Phylogeny of Decapoda (Arthropoda: Crustacea) Using Nuclear Protein-Coding Genes

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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 qualifications.

Signed.....

Abstract

The high diversity of decapods has attracted the interest of carcinologists but there is no consensus on decapod phylogeny in spite of the endeavors using both morphological and molecular approaches. New sources of information are necessary to elucidate the phylogenetic relationships among decapods. In the present study, I attempted to develop and apply the nuclear protein-coding gene markers on decapod phylogeny. Using only two protein-coding genes, we have successfully resolved most of the infraordinal relationships with good statistical support, indicating the superior efficiency of these markers compared to nuclear ribosomal RNA and mitochondrial genes commonly used in phylogenetic reconstruction of decapods. Apparently these two types of markers suffer from the problems of alignment ambiguities and rapid saturation, respectively. Subsequently, I tried to apply the nuclear protein-coding genes in revealing interfamilial and intergeneric evolutionary history in three selected decapod groups, the spiny lobster (family Palinuridae), the infraorder Anomura and the true crabs of the infraorder Brachyura to further evaluate the utility of these markers and reconstruct the evolutionary history the groups. Trees with robust support can

be obtained using sequences of three to five genes for the infraorders and families tested including the most speciose Brachyura. The genes are shown to be informative in elucidating interspecific phylogeny as well.

From the inferred phylogeny, we have obtained new insights on the evolution of decapods. First, the spiny lobster from the family Palinuridae is found to be paraphyletic with the polyphyletic Synaxidae nested within it. The Stridentes forms a monophyletic assemblage, indicating that the stridulating sound producing organ evolved only once in the spiny lobsters. Moreover, the spiny lobsters originated in the shallower water rocky reefs of the Southern Hemisphere and then invaded deep sea habitats and diversified.

Second, we show that hermit crabs have a single origin, but surprisingly, that almost all other major clades and body forms within the Anomura, are derived from within the hermit crabs. The crab-like form and squat lobster form have each evolved at least twice from separate symmetrical hermit crab ancestors. These remarkable cases of multiple parallelism suggest considerable phenotypic flexibility within the hermit crab ground plan, with a general tendency towards carcinization. Rather than

having a separate origin from other major clades, hermit crabs have given rise to most other major anomuran body types.

Finally, the gene tree of the true crabs, Brachyura, confirms that the basal "Podotremata" is paraphyletic, with the Raninoidea and Cyclodorippoidea more closely related to Eubrachyura than to the other podotremes. Within the monophyletic Eubrachyura, the analysis supports the reciprical monophyly of the two subsections, Heterotremata and Thoracotremata. All of the Old World freshwater crabs cluster together, representing an early diverged lineage in the Heterotremata.

In sum, I demonstrate the utility of the nuclear protein-coding gene markers in decapod phylogeny and they are informative across a wide range of taxonomic levels. I propose that nuclear protein-coding genes should constitute core markers for future phylogenetic studies of decapods, especially for higher systematics.

摘要

十足目(Order Decapoda)動物的多樣性吸引了許多分類學家的興趣。但雖然十足目系統發育經過了許多的研究，但無論係用形態還是分子的方法，都沒法達成一個大家都認同的共識。所以我們需有必要找尋新的信息來源去澄清十足目之間的親緣關係。在本研究中，我試圖發展和應用核蛋白質編碼基因分子標記於十足目系統發育研究上。只用兩個蛋白編碼基因的排序，我們成功地解決大部分的下目之間的系統發育關係具，並且得到良好的統計支持，這表示了蛋白質編碼基因比常用於十足類系統發育重建的核核糖體 RNA 和線粒體基因有著更優越的效率。顯然，這兩種類型的標記分別受到多序列比對含糊不清和快速飽和突變的問題。接著，我嘗試運用核蛋白質編碼基因去揭示三個選定的十足類組:刺龍蝦（龍蝦科 family Palinuridae）、異尾下目(Anomura)和短尾下目的螃蟹 (Brachyura) 中科和屬之間的進化史，以進一步評估這些分子標記的效用、與及去重建這些群體的演化歷史。運用三至五序列的基因測試去建立的系統發生樹都得到強大的支持，當中包括品種最多的短尾下目，而且這些基因都表現出能提供有用的信息去闡明種與種之間親緣關係。

而從是次研究中獲得的系統發生樹，讓我們對十足目動物的演化

得到新的見解的。首先，龍蝦科的刺龍蝦被發現是並系，而多系的合甲蝦科(family Synaxidae)嵌套在其中。而擁有摩擦發聲器的龍蝦形成一個單系的群，顯示摩擦發聲器官在刺龍蝦進化中只出現了一次。此外，多刺龍蝦起源於南半球的淺水岩礁，然後入侵深海棲息地和多樣化。

其次，我們證明了寄居蟹只有一個單一的起源，但有趣的是，幾乎所有其他主要的分支和異尾下目的身體形態都是由寄居蟹演變出來。擬似螃蟹和鎧甲蝦的形態各自至少兩次從不同的對稱寄居蟹祖先進化而來。這些不尋常的平行進化顯示寄居蟹的基本形態在表型上有著很高的靈活性，並且總的趨勢傾向於短尾化。寄居蟹演生出大多數其他主要的異尾類分支，而不是和其他分支各自有獨立的起源。

最後，螃蟹(短尾下目)的基因樹證實基底的"綿蟹派"(Podotremata)是並系群，蛙蟹科及圓關公蟹科和真蟹派有著比和其他綿蟹更密切的關係。在單系的真蟹派(Eubrachyura)中，分析支持兩個亞派，異孔亞派(Heterotremata)與胸孔亞派(Thoracotremata)爲單系。而所有舊世界的淡水蟹聚在一起，爲異孔亞派一個早期分歧出來的宗族。

總括而言，我展示了核蛋白質編碼基因標記於十足目系統發育研

究上的實用，他們並且能於廣泛的分類級別提供信息。因此我建議，核蛋白質編碼基因的標記應成爲未來十足目動物的系統發育研究的核心，尤其是在高系統學上。

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Chapter 1

General Introduction

1.1 Evolution and the Tree of Life

In the only single illustration of his book, *On the Origin of Species by Means of Natural Selection*, Charles Darwin (1859) was probably the first to suggest the idea that the evolution of life can be represented as a tree, with leaves corresponding to extant species and nodes to extinct ancestors. Since then, the reconstruction of a phylogeny for all groups of organisms has become a major issue in evolutionary biology. Organisms have evolved novel traits that allow them to better adapt to the environment, while many of their ancestral features might be retained at the same time. Accordingly, a phylogenetic framework of organisms is not only the basis of a meaningful and natural classification, but can also help us to explain the similarities and differences among different organisms. This provides a rigorous framework to guide research in all other biological disciplines and it is therefore an ideal model for the organization of biological knowledge.

Fossil records tell us about the evolutionary changes of organisms

over time, thus providing us with the most direct evidence and information about the tree of life. Unfortunately, fossil records are often poor and incomplete, especially for the taxonomic groups without hard structures. Analysis on the extant taxa becomes the major mean of elucidating the phylogenetic relationships among organisms.

Morphological examination is the foundation of systematics. The phylogeny of organisms could be inferred from the morphological similarities. To construct a taxonomic scheme that could reflect the evolutionary relatedness of biota is the ultimate goal of many evolutionary biologists and taxonomists. The morphologically inferred phylogeny, however, does have several limitations. There is limited number of characters available for particular creatures, restricting the amount of information that could be obtained. This is particularly true for organism that is simple in body plan (e.g. parasitic organisms or bacteria). Morphological features are also difficult to compare among distantly related organisms. Moreover, it is well documented that organism can exhibit phenotypic plasticity in response to different external environmental settings. Convergence is another commonly encountered phenomenon. Unrelated organisms can develop

similar adaptations if they live in the same habitat and face the same selective pressure (e.g. wings in bats and birds; fins in fishes and whales/dolphins). Hence, the use of different characters might point to a different phylogeny and there is no consensus on weighting/selection of characters which are more informative/important. All these limitations would introduce uncertainties and errors into phylogenetic inference.

1.2 Phylogenetic inference using molecular markers

Thanks to the rapid development of molecular techniques, in particular protein and DNA sequencing, the utility of molecular data has been playing an increasingly important role in phylogenetic reconstruction. This approach has the apparent advantage that all characters are discrete and objective, so that there would not be any bias or discrepancies among different researchers for the same taxon and the results could be easily compared among different studies. More importantly, molecular data are less affected by environmental factors and homoplasy, compared to phenotypes. Moreover, all living organisms have DNA, RNA and proteins. Therefore, we could determine the relatedness of morphologically diverged organisms

based on DNA, which is challenging for morphological cladistic analysis. The huge amount of potential information that molecular data could provide is another superior point over morphological analyses (the haploid human genome contains about three billion base pairs). Therefore, the accuracy and usefulness of the molecular phylogeny are highly appreciated and it has become the dominant mean of modern phylogenetic studies.

1.2.1 mtDNA

Mitochondrial (mt) genes have been the most commonly used markers in animal phylogenetic studies for many years (Simon et al., 1994, 2006). These markers are benefited from the ease of amplification due to relatively higher copy number than nuclear genes and the availability of many universal primers (Simon et al., 1994). The haploid and non-recombinant nature of mtDNA also presents fewer problems in phylogenetic reconstruction. The rate of nucleotide substitutions among mitochondrial genes is generally more rapid than that among genes in the nuclear genome (Moore, 1995). Accordingly, mitochondrial genes could more accurately reflect the relationships among recently diverged taxa, or

even intra-specific phylogeography (Avise, 2000).

Analysis of mtDNA has settled quite a number of disputing issues in the animal phylogeny. For example, the Alaskan king crabs (Crustacea: Decapoda: Anomura: Lithodidae), despite its crab-like appearance and strongly calcified abdomen, are close relatives of hermit crabs which utilize a gastropod shell as shelter. Both of them share an asymmetric abdomen, which suggests a close link between the two superficially dissimilar animals. Studies on adult and larval morphology, however, proposed that the two represent distinct evolutionary lineages. Hence, the origin of king crabs is contentious. Using sequence of the mt 16S rRNA gene, Cunningham et al. (1992) provided strong evidence for the phylogenetic affinity between king crabs and hermit crabs. The gene tree shows that the two king crabs analyzed are nested within hermit crabs from the family Paguridae, suggesting the king crabs are not only closely related to, but even have been evolved from a hermit crab ancestor.

Knowlton et al. (1993) reported the phylogeny and divergence of trans-isthmian pairs of snapping shrimps (*Alpheus*) based on the

mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequences. They successfully demonstrated that the gene is informative in resolving phylogeny among species diverged for several millions years and confirmed the species boundary in recently diverged germinate species. Moreover, they have calibrated the divergent rate of this marker according to the approximate timing of isolation by the rise of Isthmus of Panama. This rate of divergence, known as the molecular clock in Crustacea, in particular the decapods, is subsequently widely applied to estimate the time of divergence in other taxa (e.g. Harrison and Crespi, 1999; Gouws et. al., 2006). The COI has, therefore, become a predominately employed marker in crustacean phylogenetic studies and species delimitation (e.g. Knowlton, 2000; Chu et al., 2001; Macpherson and Machordom, 2001; Ptacek et al., 2001; Lavery et al., 2004).

Just named a few examples, we would appreciate the application of mtDNA in phylogenetic inference, especially for lower taxonomic levels. Mitochondrial genes, however, are often criticized for several disadvantages. All of the mitochondrial genes are linked and inherited as a single locus. Therefore, they share a common evolutionary history and cannot provide

independent phylogeny inference. The high mutation rate of mtDNA limits its utility in phylogenetics of deep divergence. Furthermore, the highly A/T-biased mtDNA, especially the third codon position of the protein-coding genes, suffer from high level of homoplasy and thus exhibit strong negative effects in phylogenetic analysis. In this regard, mtDNA sequences are mostly limited to the phylogenetic studies at lower taxonomic levels and molecular systematists have tried to incorporate the nuclear genes which evolve at a much slower rate, in addition to the mitochondrial DNA markers, for higher level phylogeny (e.g. Ahyong and O' Meally, 2004; Porter et al., 2005; Bracken et al., 2009).

1.2.2 Nuclear ribosomal RNA

Ribosomal RNA (rRNA) is the major component of the ribosome, which synthesizes proteins for cellular function. In eukaryotes, there are four kinds of cytoplasmic rRNA (28S, 5.8S, 5S (together forming the large ribosome subunit) and 18S (small subunit)), coded by four rRNA genes. The 28S, 5.8S and 18S rRNA genes are located and encoded together, with two internal transcribed spacers in between (Pellegrini et al., 1977). The

eukaryote genome contains a lot of this gene cluster organized in tandemly repeated manner on several chromosomes. The rRNA genes have stem regions that are conserved across a wide array of taxa, even different phyla, which facilitate the design of universal primers for PCR amplification. They have therefore become one of the most commonly employed markers in molecular phylogenetic studies (Smit et al., 2007). Many early studies concerning phylogenetic relationship among different phyla were inferred by analysis of nuclear rRNA gene sequence, the 18S in most of the cases (e.g. Hedges et al., 1990; Halanych et al., 1995; Aguinaldo et al., 1997).

Analyses on the 18S rRNA gene have resolved several important issues in animal evolutionary relationships and lay the foundation of further taxonomic revision. For example, the lophophorates, comprising of the phylum Brachiopoda, Bryozoa and Phoronida, are a group of animal that exhibit unique morphological characters that make their phylogenetic placement problematic. Members of these three phyla all possess the lophophore, which is a ring of ciliated tentacles surrounding the mouth used for suspension feeding. They have been suggested to be protostomes, deuterostomes, or an independent metazoan lineage. They were originally

classified as Protostomia on the basis of the presence of lophophorates and several embryological features (Hyman, 1959). However, phylogenetic analyses of embryology (e.g. blastopore fate, coelom formation and cleavage patterns) and morphology suggest close affinity of lophophorates to the deuterostomes (Hyman, 1959). Thus, the phylogenetic position of lophophorates remains contentious. Molecular data from the complete 18S rRNA gene sequences have shown that lophophorates are allied with other protostome taxa (Halanych et al., 1995). This indicates that some developmental features which were once thought to be highly conserved (e.g. coelom formation and cleavage patterns) are more plastic in the process of animal evolution (Halanych et al., 1995). Furthermore, this study reveals that the usefulness of molecular information in settling difficult phylogenetic issues and providing new insights into animal evolution.

A study using 18S rRNA gene sequence has revealed that Arthropoda form a clade with Nematoda and other mouting animals (including Tardigrada and Onychophora etc; Aguinaldo et al., 1997). This finding suggests that moulting evolved only once in the animal kingdom and accordingly, a new clade, Ecdysozoa is proposed to refer to the animals that

would moult periodically (ecdysis). In addition to shedding light in the process in animal evolution, the finding also has significant implication to developmental genetic studies as the two commonly used model organisms, *Caenorhabditis elegans* (Nematoda) and *Drosophila melanogaster* (Arthropoda) are much more closely related than researchers once believed. Hence, many developmental characters the two animals share in common might be specific to the Ecdysozoa, instead of represent a universal pattern that was once implied.

In sum, it is no doubt that nuclear rRNA genes have resolved many controversial issues and provided us with much new insight into animal evolution. The nuclear rRNA genes, however, suffer from alignment ambiguities. This poses problem in phylogenetic inference, particularly in nodes with deep divergence (i.e. infraordinal relationships). In the worst case, it sometimes could result in misleading phylogenetic tree. One famous example is about the phylogenetic position of birds. Morphology, fossil evidences and some molecular data suggest close affinity of bird to crocodilians (Ostrom, 1976; Hedges et al., 1990). Analysis of the 18S rRNA gene sequence, however, supports a bird-mammal clade (Hedges et al., 1990),

which contradicts all the other lines of evidence. This hypothesis, accordingly, is subjected to many re-examinations on the original and new datasets. It is found that the "unexpected" bird-mammal grouping is caused by errors in sequence alignment (Xia et al., 2003). The bird is recovered to the more closely related to crocodiles instead of mammals after re-aligning the 18S sequence with the aid of secondary structure model of the molecule (Xia et al., 2003). As a result, the topology inferred from the rRNA sequence should be interpreted with caution and critical examination when it is incongruent with other lines of evidence. Moreover, there is a limitation in the number of loci, and thus length of sequences, available for the rRNA genes, thus restricting the amount of information that could be provided by these markers. The resolution in most of the rRNA gene trees in the aforementioned studies remains largely unresolved.

1.2.3 Nuclear protein-coding genes

Nuclear protein-coding genes could serve as an excellent new source of information for molecular phylogeny. These genes have clear advantages of being easy to align. Moreover, many potential candidates are

present in the genome with diverse evolutionary rates that are suitable to address phylogeny at different taxonomic levels. Comparison of the information provided by mitochondrial and nuclear protein-coding gene markers has shown that the latter genes consistently perform better than mtDNA (Baker et al., 2001; Springer et al., 2001). This is because the nuclear protein-coding genes exhibit significantly lower level of homoplasy (especially at higher taxonomic levels), and the information provided by individual markers are less incongruent among themselves and with the combined dataset (Baker et al., 2001). In addition to the rapid saturation caused by the high substitution frequency observed in mtDNA, the heterogenous patterns of among-site rate variation (i.e. mutations are concentrated on several regions) and highly asymmetrical transformation rate matrices in mtDNA further make the mtDNA perform worse than the nuclear protein-coding genes (Lin and Danforth, 2004). On the other hand, little difference in nucleotide substitution patterns are observed between nuclear rRNA genes compared to the nuclear protein-coding genes (Danforth et al., 2005). Hence, the apparent advantages of choosing protein-coding genes over rRNA genes appear to be the simplicity in alignment and the number of available markers, an issue that the rRNA markers are proven to be

insufficient to address.

The deep-level phylogenetic relationships among animal phyla are one of the most interesting questions that evolutionary biologists concern. The analyses of 18S rRNA gene sequences have revealed several new clades and provided much new insights into the issues (see section 1.2.2 above). However, the resolutions regarding the nodes among these clades are mostly unsatisfactory. Some clades are highly diverged from the others, leading to the concerns of long-branch attraction (Felsenstein, 1988; Bergsten, 2005). Hence, the validity of the clades remains contentious, especially for those contradicting greatly to morphologically inferred phylogeny (e.g. Ecdysozoa, reviewed in Jenner and Scholtz, 2005). Anderson et al. (2004) have reported sets of primers for amplifying the nuclear protein-coding gene marker, sodium-potassium ATPase α-subunit (NaK) gene for the metazoan molecular phylogenetic studies. The NaK gene appears to be able to provide new information for the purpose and the resulting gene tree recovers the monophyletic Ecdysozoa, Lophotrochozoa, Vertebrata, Mollusca etc., which corroborates the results from rRNA genes (Halanych et al., 1995; Aguinaldo et al., 1997) and the myosin heavy chain II (Ruiz-Trillo et al., 2002).

Another contentious issue raised by early molecular studies using 18S rRNA gene is the phylogenetic position and origin of Hexapoda. Hexapoda, comprising of insects and three other small classes (Collembola, Protura and Diplura), is the largest group in the animal kingdom. Based on the similarity in morphology, it was long believed that Hexapoda is closely related to the Myriapoda (the centipedes and millipedes). Surprisingly, early molecular studies using 18S gene suggest that Hexapoda allies with the Crustacea which are largely marine, instead of the terrestrial Myriapoda (Field et al., 1988). This hypothesis would have significant evolutionary implication that the insects might have been evolved from aquatic crustacean ancestors that invaded the terrestrial habitat. The Hexapoda + Crustacea clade is challenged by the fact that many of taxa analyzed show highly diverged sequences, leading to the concern of "long-branch attraction". Furthermore, the clade is only recovered with weak nodal support in subsequent study based on expanded taxonomic sampling and the topologies vary greatly with the analytical methods and taxa included (Spears and Abele, 1998). Hence, the reliability of the 18S inferred topology remains puzzling. Using sequence data from two nuclear protein-coding genes (elongation

factor 1-α and RNA polymerase II), Shultz and Regier (2000) have provided strong evidence for the Hexapoda + Crustacea (Pancrustacea) clade. Soon after that, they have reported the result of the analysis of another nuclear protein-coding gene, elongation factor 2, on arthropod phylogeny (Regier and Shultz, 2001) which offer further support to the Pancrustacea and falsify the traditional grouping of Hexapoda + Myriapoda. From the results of these studies, we would recognize the higher resolving power provided by the nuclear protein-coding genes over rRNA genes. The availability of large number of candidate markers allows researchers to verify any phylogenetic hypotheses proposed with additional markers and achieve well-supported, robust phylogeny.

Despite the apparently high potential utility of protein-coding gene markers, several limitations have restricted the development and application of these markers. First, the protein-coding genes have a much lower number of copies in the genome, compared to highly abundant nuclear rRNA and mitochondrial genes, and therefore would be more difficult to amplify through PCR. The degenerate third codon positions further challenge the design of PCR primers and long stretches of introns might be present,

making amplification difficult or even impossible. Furthermore, paralogs might be present resulting in problems in phylogenetic analyses. Thus, though these genes appear to be informative, their application in phylogenetic study has been limited chiefly to the groups of high diversity or public interest, such as vertebrates and insects (e.g. Wiegmann et al., 2000; Leyes et al., 2002; Meyer, 2003; Brinkmann et al., 2004; Danforth et al., 2004) for which we have relatively more knowledge on their genomes.

With the recent advances in molecular techniques that could generate a large number of protein-coding gene sequence from non-model organisms (e.g. EST) and accumulation of large amount of genome sequence data, scientists can search for new molecular markers or apply the existing ones to their target organisms much easier than before. New protein-coding gene markers have also been successfully developed and employed for other arthropods (e.g. Myriapoda, Regier et al., 2005; spider, Ayoub et al., 2007; Mysida, Audzijonyte et al., 2008), fungi (Liu et al., 1999) etc., and proved to be informative or superior over the nuclear rRNA and mitochondrial genes in resolving power (Lu et al., 1999; Audzijonyte et al. 2008). Thus, the development and application these markers in molecular systematic study

would be a new strategy in addressing the controversial issues in animal phylogeny and the markers will play an increasingly dominant role.

1.3 Diversity, taxonomy and phylogeny of decapod crustaceans

With an estimated 15,000 described species, the Decapoda is one of the most species-rich and diverse extant group of crustaceans (De Grave et al., 2009). Decapods are also one of the most popular invertebrate groups among the general public as they contain many of the economically important species. Shrimps, lobsters and crabs, contribute to a large proportion to the global fisheries catch and seafood consumption, while many caridean shrimps and hermit crabs are favorite aquarium pets. Accordingly, the evolutionary relationships and systematics of decapods have attracted much attention. The extraordinary morphological diversity, however, poses challenges to their phylogenetic study, and many taxonomic schemes and phylogenetic hypotheses have been proposed for Decapoda (Schram, 2001). Despite attempts to test the different hypothesis concerning decapod evolution (e.g. Scholtz and Richter, 1995; Dixon et al., 2003; Schram and Dixon 2004; Ahyong and O'Meally, 2004), consensus is yet to

be reached.

There is little controversy over the monophyly of the two decapod suborders, Dendrobranchiata (penaeid and sergestid shrimps) and Pleocyemata (all the other decapods) (Burkenroad, 1963, 1981; Martin and Davis, 2001). The previous separation of Natantia (swimming group) and Reptantia (crawling group) based on the mode of locomotion (Boas, 1880) has long been rejected. Natantia is recognised as paraphyletic while Reptantia is universally regarded as monophyletic (Abele and Felgenhauer, 1986; Dixon et al., 2003; Ahyong and O'Meally, 2004). However, the relationships of several natant lineages relative to the Reptantia have been disputed (Burkenroad, 1963, 1981; Abele and Felgenhauer, 1986; Christoffersen, 1988; Abele, 1991; Fig. 1A-C), particularly in the relative positions of the Caridea and Stenopodidea. The composition and internal relationships of the highly diverse Reptantia is even more contentious (e.g. Dixon et al., 2003; Scholtz and Richter, 1995; Schram and Dixon, 2004; Fig. 1D-F). Morphological cladistic analyses are impeded by the sheer diversity of forms and limited available character sets (e.g. Tshudy and Sorhannus, 2000; Schram, 2001; Dixon et al., 2003; Schram and Dixon, 2004).

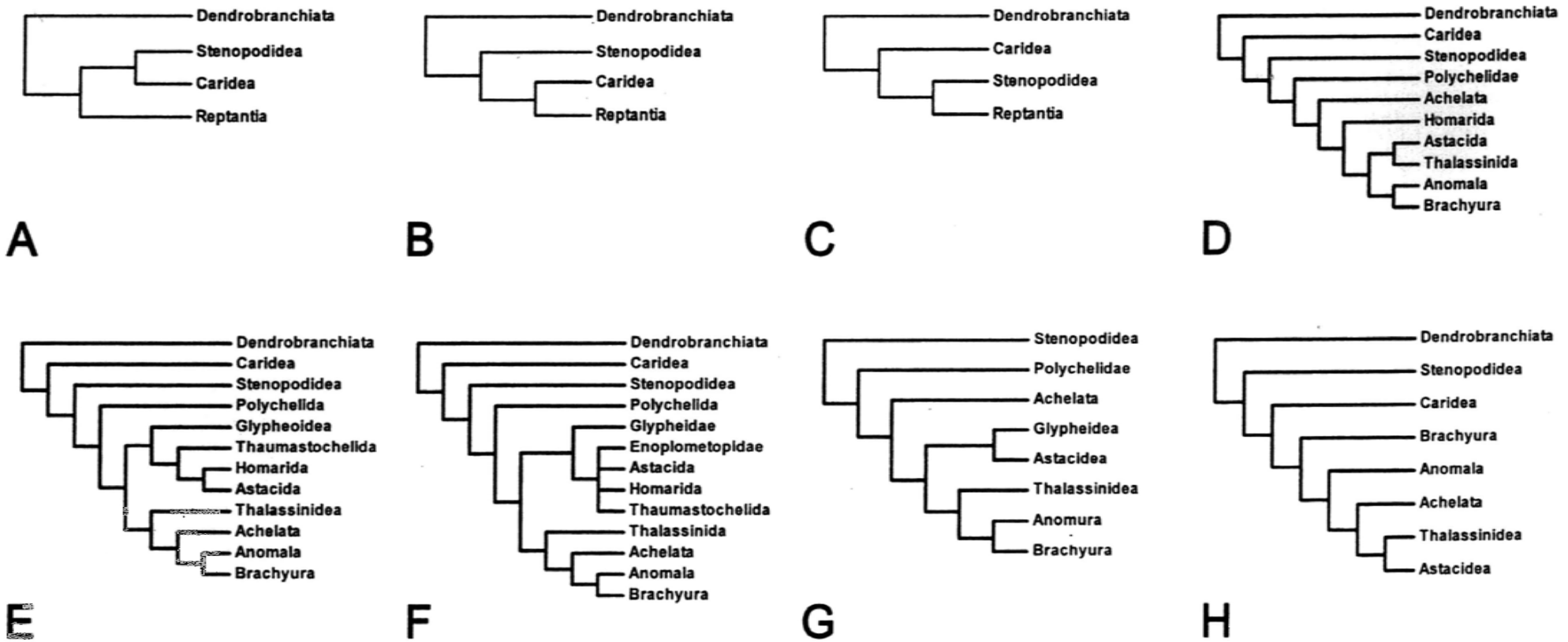

Figure 1.1 Hypotheses of phylogenetic relationships among Decapoda lineages. A-C: the relationships of Reptantia relative to natant lineages based on morphology, D-F: relationships among reptant lineages based on morphological cladistic analyses; G-H: phylogenetic hypotheses of decapod infraorders based on molecular data. (A) Burkenroad (1963, 1981); (B) Christoffersen (1988); (C) Abele and Felgenhauer (1986); (D) Scholtz and Richter (1995); (E) Dixon et al. (2003); (F) Schram and Dixon (2004); (G) Ahyong and O'Meally (2004); and (H) Porter et al. (2005).

Molecular data provide an alternative means to address these issues, and in particular, offer a much larger potential pool of characters data than presently available from morphology. Yet comprehensive molecular phylogenetic studies of high level relationships were not available until recently (Ahyong and O'Meally, 2004; Porter et al., 2005). The two studies, however, yielded significantly contrasting results (Fig. 1G and H), although based on similar data (mitochondrial 16S, nuclear 18S and 28S, in addition to histone 3 in Porter et al., 2005). Ahyong and O'Meally's (2004) topologies support Polychelidae as sister to the remaining Reptantia and Meiura (= Anomura + Brachyura) (Fig. 1G), as recovered by previous morphological analyses (Scholtz and Richter, 1995; Schram, 2001, Dixon et al., 2003, Schram & Dixon, 2004). In contrast, Porter et al. (2005) recovered Brachyura and Anomura (as Anomala) as basal or near basal reptant clades (Fig. 1H), such that the topologies were essentially a reversal of those recovered by Ahyong & O'Meally (2004). Admittedly, topologies of Porter et al. (2005) excluded Polychelidae, and all infraordinal relationships received low nodal support, meaning conflict was more apparent than actual. Therefore, it is no doubt that the development new molecular markers are prompted to resolve the controversies.

In the present thesis research study, I attempted to develop new nuclear protein-coding gene markers for phylogenetic study of decapods. I first tested the utility of these markers on resolving infraordinal relationships (Chapter 2). Subsequently, I applied these markers on phylogenetic study of different decapod groups to resolve a number of phylogenetic controversies. I

have focused my study on three groups, aiming at three different taxonomic levels to elucidate the resolving "spectrum" of the markers. I have investigated the inter-generic relationship of the spiny lobsters from the family Palinuridae (Chapter 3), the familial relationships of the infraorder Anomura (Chapter 4), and finally the phylogeny of different sections and superfamilies of the true crabs, Brachyura (Chapter 5).

Chapter 2

Phylogeny of Decapoda using two nuclear protein-coding genes:

Origin and evolution of the Reptantia

2.1 Introduction

Most molecular phylogenetic studies of Decapoda have relied heavily on mitochondrial DNA and nuclear ribosomal DNA markers. The former, however, exhibit rapid substitution saturation that limits their utility in resolving deep nodes, whereas the latter suffer from alignment ambiguities (see Chapter 1 for more detail). These disadvantages can complicate analysis and hamper accurate recovery of phylogenetic signal. Consequently, nuclear protein-coding genes could serve as an excellent new source of information. These genes are easy to align, with many potential candidates in the genome. They have been commonly used in phylogenetic studies of vertebrates (e.g. Meyer, 2003; Brinkmann et al., 2004) and insects (e.g. Wiegmann et al., 2000; Leyes et al., 2002; Danforth et al., 2004), and are informative across a wide range of taxonomic levels (Rokas et al., 2002). To date, however, their application in decapod phylogenetics has been limited (e.g. histone H3, Porter et al., 2005; glyceraldehydes-3-phosphate dehydrogenase, Buhay et al., 2007), because of the lack of genomic information for decapods that hampers the development of nuclear markers. In the present study, we applied two nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (PEPCK) and sodium-potassium ATPase α-subunit (NaK), for decapod phylogenetics.

PEPCK and sodium-potassium ATPase play fundamental roles in diverse life forms and are well conserved throughout evolution. PEPCK catalyzes the first step of gluconeogenesis, interconverting oxaloacetate and phosphoenolpyruvate in organisms ranging from bacteria to human. In addition, the enzyme may also be involved in the citric acid cycle, the activity of which parallels PEPCK abundance (Burgess et al., 2007). Low copy number in the PEPCK gene is evident in vertebrates (Yoo-Warren et al., 1983; Hod et al., 1984), whereas it may be single-copy in Lepidoptera and Diptera (Friedlander et al., 1992). Sodium-potassium ATPase is a P-type ATPase ion co-transporter found on metazoans cell membranes (Reeves and Yamanaka, 1993). The enzyme is responsible for maintaining electrochemical potential differences across membranes, and is essential for cell signaling and secondary transport. Sodium-potassium ATPase is a heterodimer composed of α- and β-subunits. The α-subunit is catalytically active with a highly conserved polypeptide chain (the nucleotide sequence of which is used in this study as a phylogenetic marker), while the function of the glycosylated β-subunit remains unknown (Reeves and Yamanaka, 1993; Emery et al., 1998). Although in vertebrates, the α- and β-subunits have evolved into multiple copies (Emery et al., 1998), sodium-potassium ATPase remains a single copy gene in invertebrates (Reeves and Yamanaka, 1993). The two genes have been successfully used to resolve deep-level phylogenetic relationships among insects (e.g. Friedlander et al., 1996; Leyes et al., 2002) and even among bilaterian metazoans (Anderson et al., 2004). Thus, we anticipate they are applicable in decapod phylogenetics.

We attempted to reconstruct the phylogeny of major infraorders within Decapoda, with emphasis on the Pleocyemata based on PEPCK and NaK sequences, as the relationships within Dendrobranchiata are less complicated owing to the smaller number of taxa (only two superfamilies with seven families; Pérez-Farfante and Kensley, 1997). Our primary goal was to study infraordinal relationships and test the phylogenetic positions of several controversial taxa. This should provide important insights into the origin and evolution of the extraordinarily diverse Decapoda, especially the morphologically diverse Reptantia. Moreover, we evaluated the potential utility of these two protein-coding genes for future phylogenetic studies of decapods.

2.2 Materials and methods

2.2.1 Taxon sampling

Representatives from all infraorders of Pleocyemata, except Glypheidea were included in the ingroup. Current studies ally the glypheoids to the astacideans (Schram and Ahyong, 2002), and either include them within Astacidea (Martin and Davis, 2001) or as a separate infraorder Glypheidea (Ahyong and O'Meally, 2004). The glypheideans were a significant radiation of largely extinct lobsters of Triassic origin (Glaessner, 1969) of which only two species are extant (Richer de Forges, 2006). Unfortunately, efforts to sequence *Neoglyphea inopinata*, one of two extant glypheoids, were unsuccessful probably due to the age of the sample which

was collected more than 30 years ago. Whereas many taxa are uncontroversial in their taxonomic positions, some have attracted controversy and we have made special effort to include them. These include the symmetrical hermit crabs (Pylochelidae) which have recently suggested to be allied to the galatheoids, the deepwater lobsters, Thaumastochelidae, which might be nested within Nephropidae, and the reef lobsters, Enoplometopidae, which have been variously placed in Astacidea or Thalassinidea (Ahyong and O'Meally, 2004). There is little dispute that Dendrobranchiata is the sister group to Pleocyemata (reviewed in Martin and Davis, 2001), so analyses were rooted to five dendrobranchiates (Table 2.1). Rooting the tree with dendrobranchiates should enable recovery of the positions of basal pleocyematan clades that are outside of the Reptantia, namely Caridea and Stenopodidea. We generally followed the classification scheme of Martin and Davis (2001), though the infraorder name Achelata was used instead of Palinura, with the exclusion of Polychelidae which is placed in its own infraorder, Polychelida, following Scholtz and Richter (1995), Dixon et al. (2003), and Ahyong and O'Meally (2004).

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

					Gene	
ıperfamily	Family	Species	Sampling location	Voucher ID	NaK	PEPKC
endrobranchiata						
rgestoidea	Sergestidae	*Sergia maxima*	Taiwan	NTOU M00702	EU427145	EU427214
naeoidea	Aristeidae	*Aristeus virilis*	Taiwan	NTOUM00703	EU427143	EU427212
naeoidea	Penaeidae	*Penaeus monodon*	Fish market, Hong Kong	MSLKHC-Pemon	EU427144	EU427213
naeoidea	Sicyoniidae	*Sicyonia lancifer*	Dasi, Taiwan	NTOU M00704	EU427146	EU427215
naeoidea	Solenoceridae	*Solenocera melantho*	Taiwan	NTOU M00705	EU427147	EU427216
eocyemata						
ıridea						
angonoidea	Glyphocrangonidae	*Glyphocrangon perplexa*	Dasi, Taiwan	NTOU M00706	EU427173	EU427242
laemonoidea	Gnathophyllidae	*Gnathophyllum americanum*	Aquarium shop, Hong Kong	MSLKHC-Gname	EU427179	EU427248
laemonoidea	Hymenoceridae	*Hymenocera picta*	Aquarium shop, Hong Kong	MSLKHC-Hypic	EU427175	EU427244
laemonoidea	Palaemonidae	*Macrobrachium rosenbergii*	Fish market, Hong Kong	MSLKHC-Maros	EU427176	EU427245
ndaloidea	Pandalidae	*Heterocarpus gibbosus*	Dasi, Taiwan	NTOU M00707	EU427174	EU427243
ndaloidea	Pandalidae	*Plesionika hsuehyui*	Dasi, Taiwan	NTOU M00708	EU427180	EU427249
olophoroidea	Oplophoridae	*Acanthephyra eximea*	Dasi, Taiwan	NTOU M00709	EU427181	EU427250
ematocaricinoidea	Rhynchocinetidae	*Rhynchocinetes durbanensis*	Aquarium shop, Hong Kong	MSLKHC-Rhdur	EU427177	EU427246
enopodidea						
	Stenopodidae	*Stenopus hispidus*	Aquarium shop, Hong Kong	MSLKHC-Sthis	EU427178	EU427247
eptantia						
ıomura						
ılatheoidea	Galatheidae	*Munida albiapicula*	Dasi, Taiwan	NTOU A00837	EU427119	EU427188
ılatheoidea	Galatheidae	*Paramunida scabra*	Dasi, Taiwan	NTOU A00838	EU427122	EU427191
ılatheoidea	Porcellanidae	*Petrolisthes japonicus*	Hainan, China	MSLKHC-Ptjap	EU427123	EU427192
ppoidea	Albuneidae	*Albunea holthuisi*	Matsu, Taiwan	NTOU A00839	EU427113	EU427182
omisoidea	Lomisidae	*Lomis hirta*	South Australia	NTOU A00840	EU427118	EU427187

ıguroidea	Coenobitidae	*Coenobita violascens*	Kenting, Taiwan	NTOU A00841	EU427115	EU427184
ıguroidea	Coenobitidae	*Coenobita rugosus*	Kenting, Taiwan	NTOU A00842	EU427116	EU427185
ıguroidea	Diogenidae	*Dardanus impressus*	Dasi, Taiwan	NTOU A00843	EU427117	EU427186
ıguroidea	Diogenidae	*Paguristes seminudus*	Dasi, Taiwan	NTOU A00844	EU427121	EU427190
ıguroidea	Lithodidae	*Neolithodes nipponensis*	Taiwan	NTOU A00845	EU427120	EU427189
ıguroidea	Paguridae	*Pagurodofleinia doederleini*	Dasi, Taiwan	NTOU A00846	EU427114	EU427183
ıguroidea	Paguridae	*Propagurus obtusifrons*	Dasi, Taiwan	NTOU A00847	EU427124	EU427193
ıguroidea	Pylochelidae	*Pylocheles macrops*	Taiwan	NTOU A00848	EU427125	EU427194
ıstacidea						
ıstacoidea	Cambaridae	*Procambarus clarkii*	Aquarium shop, Hong Kong	NTOU M00726	EU427153	EU427222
ıoplometopoidea	Enoplometopidae	*Enoplometopus debelius*	Aquarium shop, Hong Kong	MSLKHC-Endeb	EU427149	EU427218
ɛphropoidea	Nephropidae	*Homarus gammarus*	Fish market, Paris, France	NTOU M00711	EU427150	EU427219
ɛphropoidea	Nephropidae	*Nephropides caribaeus*	Guadeloupe	MNHN-As642	EU427151	EU427220
ɛphropoidea	Nephropidae	*Nephropsis stewarti*	Dasi, Taiwan	NTOU M00505	EU427152	EU427221
ɛphropoidea	Thaumastochelidae	*Thaumastocheles dochmiodon*	Dasi, Taiwan	NTOU M00150	EU427154	EU427223
ırastacoidea	Parastacidae	*Cherax quadricarinatus*	Aquarium shop, Hong Kong	NTOU M00710	EU427148	EU427217
rachyura						
ılappoidea	Calappidae	*Calappa philargius*	Fish market, Hong Kong	MSLKHC-Caphi	EU427126	EU427195
ıncroidea	Corystidae	*Jonas distincta*	Dasi, Taiwan	NTOU B00002	EU427133	EU427202
ıncroidea	Cancridae	*Cancer japonica*	Dasi, Taiwan	NTOU B00003	EU427127	EU427196
orippoidea	Dorippidae	*Paradorippe granulata*	Dasi, Taiwan	NTOU B00004	EU427137	EU427206
romioidea	Dromiidae	*Conchoecetes artificiosus*	Dasi, Taiwan	NTOU B00005	EU427129	EU427198
romioidea	Dromiidae	*Lauridromia dehaani*	Longdong, Taiwan	NTOU B00006	EU427134	EU427203
rapsoidea	Varunidae	*Eriocheir japonica*	Hong Kong	MSLKHC-EjapHK	EU427132	EU427201
omoloidea	Latreillidae	*Eplumula phalangium*	Taiwan	NTOU B00007	EU427131	EU427200
ɛucosioidea	Leucosiidae	*Tokoyo eburnea*	Dasi, Taiwan	NTOU B00008	EU427141	EU427210
ajoidea	Epialtidae	*Pugettia nipponensis*	Taiwan	NTOU B00009	EU427139	EU427208
ajoidea	Inachidae	*Platymaia remifera*	Dasi, Taiwan	NTOU B00010	EU427138	EU427207
cypodoidea	Mictyridae	*Mictyris longicarpus*	Hong Kong	MSLKHC-Milon	EU427135	EU427204
cypodoidea	Ocypodidae	*Uca crassipes*	Hong Kong	MSLKHC-Uccra	EU427142	EU427211
ırthenopoidea	Parthenopidae	*Cryptopodia fornicata*	Hong Kong	MSLKHC-Crfor	EU427130	EU427199
ɔrtunoidea	Portunidae	*Ovalipes punctatus*	Dasi, Taiwan	NTOU B00011	EU427136	EU427205

aninoidea	Raninidae	*Ranina ranina*	Kengfang, Taipei, Taiwan	NTOU B00012	EU427140	EU427209
anthoidea	Goneplacidae	*Carcinoplax longimana*	Dasi, Taiwan	NTOU B00013	EU427128	EU427197
olychelida						
yonoidea	Polychelidae	*Polycheles amemiyai*	Dasi, Taiwan	NTOU M00719	EU427165	EU427234
chelata						
ılinuroidea	Palinuridae	*Jasus edwardsii*	Fish market, Hong Kong	Unvounchered	EU427157	EU427226
ılinuroidea	Palinuridae	*Justitia japonica*	Taiwan	NTOU M00712	EU427158	EU427227
ılinuroidea	Palinuridae	*Linuparus trigonus*	New Caledonia	NTOU M00713	EU427159	EU427228
ılinuroidea	Palinuridae	*Palinurus elephas*	Fish market, Hong Kong	Unvounchered	EU427160	EU427229
ılinuroidea	Palinuridae	*Panulirus longipes*	Fish market, Hong Kong	Unvounchered	EU427161	EU427230
ılinuroidea	Palinuridae	*Panulirus polyphagus*	Fish market, Hong Kong	Unvounchered	EU427162	EU427231
ılinuroidea	Palinuridae	*Panulirus versicolor*	Fish market, Hong Kong	MSLKHC-Paver	EU427163	EU427232
ılinuroidea	Palinuridae	*Projasus bahamondei*	Fish market, Keelung, Taiwan	NTOU M00714	EU427166	EU427235
ılinuroidea	Palinuridae	*Puerulus angulatus*	Taiwan	NTOU M00715	EU427164	EU427233
ılinuroidea	Scyllaridae	*Eduarctus martensii*	Dasi, Taiwan	NTOU M00716	EU427155	EU427224
ılinuroidea	Scyllaridae	*Ibacus novemdentatus*	Taiwan	NTOU M00717	EU427156	EU427225
halassinidea						
xioidea	Axiidae	*Calaxius manningi*	Taiwan	NTOUA0053	EU427169	EU427238
xioidea	Axiidae	*Calocarides chani*	Taiwan	NTOUA00423	EU427170	EU427239
xioidea	Calocarididae	*Calastacus crosnieri*	Taiwan	NTOUA00212	EU427168	EU427237
xioidea	Eiconaxiidae	*Eiconaxius indicus*	Pratas Is., South China Sea	NTOUA00829	EU427171	EU427240
allianasoidea	Upogebiidae	*Austinogebia edulis*	Hong Kong	MSLKHC-AeduHK	EU427167	EU427236
nalassinoidea	Thalassinidae	*Thalassina anomala*	Singapore	ZRC1998.2263	EU427172	EU427241

2.2.2 Primer design, DNA extraction, PCR and sequencing

Initially, we applied the primers reported in other studies (Friedlander et al., 1996 for PEPCK; Anderson et al., 2004, and Danforth et al., 2004 for NaK) with modifications that reduce the level of degeneracy for a higher efficiency of PCR amplification. We successfully obtained sequences of the two target gene segments from some but not all of our decapod samples (Table 2.1). Subsequently we designed new Decapoda specific primers based on these sequences in combination with sequences of other arthropod species available in GenBank (Table 2.2). The new primer sets resulted in PCR amplicons of 590-689 bp for PEPCK and 683-902 bp for NaK.

Total genomic DNA was extracted from pleopod or pereiopod of the target species using the commercial QIAamp Tissue Kit (QIAGEN). The amplifications were conducted in a reaction mix containing 1-5 μL of template DNA, 1X PCR reaction buffer, 3 mM $MgCl_2$, 200 nM of each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (Amersham) and ddH_2O to a total volume of 50 μl. The PCR profile PEPCK was as follows: 3 min at 94°C for initial denaturation, then 35 cycles of 30 s at 94°C, 30 s at 60°C, 1

Table 2.2
Primer sequences used for PCR amplification

Primer	Sequence (5' to 3')	Source
PEPCK		
PEPCK for	GTA GGT GAC GAC ATT GCY TGG ATG AA	Modified from 19.5df of Friedlander et al. 1996
PEPCK for2	GCA AGA CCA ACC TGG CCA TGA TGA C	This study
PEPCK rev	GAA CCA GTT GAC GTG GAA GAT C	Modified from 22.5drc of Friedlander et al. 1996
PEPCK rev3	CGG GYC TCC ATG CTS AGC CAR TG	This study
NaK		
NaK for-a	GTG TTC CTC ATT GGT ATC ATT GT	This study
NaK for-b	ATG ACA GTT GCT CAT ATG TGG TT	Modified from fATPa of Anderson et al. 2004
NaK rev	ACC TTG ATA CCA GCA GAT CGG CAC TTG GC	Modified from NaKrev2 of Danforth et al. 2004
NaK rev2	ATA GGG TGA TCT CCA GTR ACC AT	This study

min 30 s at 72°C with a final extension for 10 min at 72°C. The same profile was employed for NaK with an annealing temperature of 55-60°C depending on individual samples. The PCR products were then purified using the QIAquick gel purification kit (QIAGEN) according to manufacturer's instructions. Sequencing reactions were carried out using the same sets of primers using an Applied Biosystems (ABI) 3100 automated sequencer using the ABI Big-dye Ready-Reaction mix kit, following the standard cycle sequencing protocol.

2.2.3 Phylogenetic analyses

Sequences were aligned using CLUSTAL W (Thompson et al., 1994) using default parameters, manually adjusted and confirmed by translating into amino acid sequences. Departures from base compositional homogeneity across taxa were evaluated by χ^2 test using PAUP*4.0b10 (Swofford, 2002) for the two genes as a whole and for individual codon positions. As a test of partition combinability, the incongruence length-difference test (ILD) (Farris et al., 1994) has been criticised (Gatesy et al., 1999; Yoder et al., 2001), so we followed the method suggested by Wiens (1998), which identifies any strongly supported conflicting nodes between a Bayesian phylogeny generated from individual markers. The support is considered to be strong where Bayesian posterior probability ≥ 0.95.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were conducted in PAUP*, and Bayesian inference (BI) was conducted using

MrBayes v.3.12 (Ronquist and Huelsenbeck, 2003). The best-fit model of nucleotide substitution for each dataset analyzed under ML was determined by Modeltest 3.7 (Posada and Crandall, 1998). MP analysis was carried out by heuristic search and tree-bisection-reconnection with 1000 random addition sequence replicates. Bootstrap (BP) support was evaluated using 1000 pseudoreplicates, each based on 100 random addition sequence replicates. ML topologies were generated using a heuristic search with 100 random addition sequence replicates, with nodal support estimated from 100 bootstrap pseudoreplicates, each with one random addition sequence replicate. For the Bayesian analysis, 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 the analysis. Chains were sampled every 500 generations and the first 40% of trees were discarded as burn-in. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PP). Four replicates of these Bayesian runs were conducted to ensure convergence was repeatable.

Alternative a priori 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.

2.3 Results

2.3.1. Sequence characteristics

A total of 64 ingroup and 5 outgroup species were sequenced for the two protein-coding genes (Table 2.1). The PCR products generated using different primer sets varied in length and only partially overlapped. We tried to remove most of the non-overlapping regions in order to minimize the amount of missing data. The final aligned sequences consisted of 570 bp of PEPCK and 534 bp of NaK. No introns or indels were present. Sequence ambiguities (i.e. double peaks in the chromatograms) were observed, probably due to heterozygosity of the individuals from which the sequence was derived. These sites were coded as ambiguous using the IUB symbols, i.e. R, Y, S, W, K, or M and they were only present at several sites within a single sequence that would not exhibit significant effect in phylogenetic inference. Base composition of PEPCK sequences were slightly G/C biased (57%), whilst NaK was slightly A/T biased (51.5%). There was significant base heterogeneity among taxa at the third codon position of the two genes, but not at the first and second codon positions (Table 2.3).

2.3.2. Phylogenetic inference

The trees derived from separate analysis of the individual genes

Table 2.3
Summary of parsimony results

Gene	No. of sites	No. of variable sites	No. of parsimony informative sites	% A/T	Chi-square
PEPCK					
nt1	190	70	54	42.2	$p = 1.000$
nt2	190	45	30	50.8	$p = 1.000$
nt3	190	177	174	33.1	$p < 0.001$
All sites	570	292	258	43.0	$p < 0.001$
Amino acid	190	72	52		
NaK					
nt1	178	72	52	44.1	$p = 1.000$
nt2	178	43	21	62.7	$p = 1.000$
nt3	178	172	171	47.8	$p < 0.001$
All sites	534	287	244	51.5	$p < 0.001$
Amino acid	178	74	38		
Overall: nucleotide	1104	579	502	47.2	$p < 0.001$
Amino acid	368	146	90		

exhibited little conflict in topology. Therefore we concatenated the sequences from the two genes resulting in a dataset with 1114 bp. Of the sites, 579 (52%) are variable and 502 (45%) were parsimony informative (Table 2.3). The third codon position contributed to most of the phylogenetic signal, accounting for 69% (345) of parsimony-informative sites. The first and second codon positions account for only 21% and 10% of parsimony-informative sites respectively (Table 2.3).

The best-fit model selected using Modeltest was GTR+I+G (base frequencies = 0.2928, 0.2555, 0.1983; Rmat = 1.2648, 3.7699, 1.4709, 1.1466, 5.2397; gamma shape parameter = 1.2005; proportion of invariable sites = 0.4528) for ML analysis; the same model was also used for BI. Topologies derived from MP, ML and BI analyses were largely congruent with some clades consistently showing high support values. Yet the arrangements of several internal nodes varied according to the analysis type, with low statistical support for these conflicting nodes. 68 most parsimonious trees were found by MP analysis but the infraordinal relationships recovered in the strict consensus tree were congruent with those recovered under ML and BI but the nodal supports of the MP tree were generally lower. Only support values of ML and BI are shown on the ML tree (Fig. 2.1).

The Reptantia, and all but one of the infraorders, are strongly supported as monophyletic under both ML and BI (Fig. 2.1). Thalassinidea, however, is polyphyletic and the a priori hypothesis of thalassinidean monophyly is rejected by the KH and SH tests ($P < 0.001$ for both tests). The nodal support

for most of the infraordinal and inter-familial relationships is high. Unless otherwise stated, all the relationships discussed below are strongly supported by both ML (BP $\geq$ 70) and BI (PP $\geq$ 0.95) analyses. Stenopodidea and Caridea form a clade as sister to Reptantia. The alternative hypotheses of Stenopodidea + Reptantia or Caridea + Reptantia are significantly worse than the inferred result (KH P = 0.007; SH P = 0.006).

The Reptantia comprises two major clades. The first major clade consists of Astacidea, Achelata, Polychelida, and three thalassinidean families (Axiidae, Calocarididae and Eiconaxiidae). The second major clade includes Anomura, Brachyura and two thalassindean families (Thalassinidea and Upogebiidae). Notably then, thalassinidean clades are recovered as sister to both major reptant clades. In the first major clade, the three thalassinidean families form a monophyletic group, though with a paraphyletic Axiidae. All lobsters and crayfish form a clade with Astacidea as sister to Achelata + Polychelida. Within Astacidea, the two superfamilies of freshwater crayfish (Astacoidea and Parastacoidea) form a monophyletic group, as do the marine clawed lobsters (Enoplometopidae, Nephropidae and Thaumastochelidae). Enoplometopidae is sister to Nephropidae, but the latter is paraphyletic with Thaumastochelidae nested with it. Polychelida is more closely related to Achelata but with low support (ML BP = 47, BI PP = 0.9). Additionally, the possible association of Polychelida with Astacidea cannot be rejected (KH P = 0.722, SH P = 0.366). However, the a priori hypothesis of Polychelidae as sister to the remaining reptants is not supported (KH P = 0.016, SH P = 0.012). Achelata, comprising Palinuridae and Scyllaridae, forms a strongly

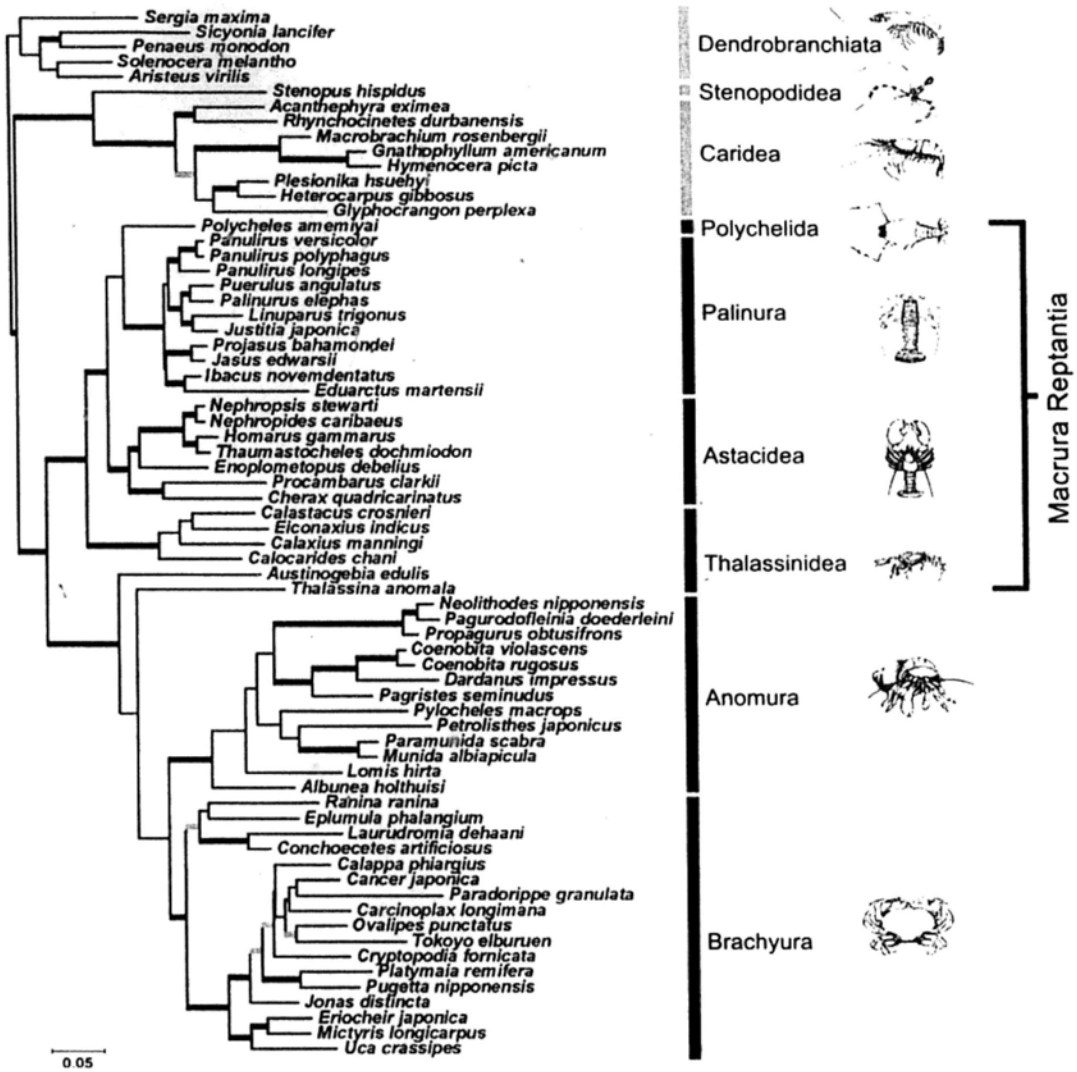

Fig. 2.1 Maximum likelihood tree from combined PEPCK and NaK analysis under the best-fitting model GTR+I+G. The branches strongly supported by both ML (BP≥70) and BI (PP≥0.95) are indicated by thick black lines; branches only strongly supported by one of the analyses are indicated with thick grey line. The infraorder classification of the species mainly based on Martin and Davis (2001) are indicated on the right-hand side with Reptantia indicated in black bars and Natantia in grey bars.

supported clade. The reciprocal monophyly of Scyllaridae and Palinuridae, however, is ambiguous. A scyllarid clade and two palinurid clades are distinct, but their interrelationships are unresolved.

In the second major clade, Upogebiidae and Thalassinidae do not group together, though their internode is poorly supported (ML BP = 47, BI PP = 0.68). Anomura and Brachyura form Meiura, with intermediate support (ML BP = 62, BI PP = 0.81). The hypothesis that Brachyura and Anomura are basal or near basal reptant clades is rejected by the KH and SH tests ($P <$ 0.001 for both analyses). Brachyura comprises three clades corresponding to Podotremata (*Conchoecetes*, *Lauridromia*, *Eplumula* and *Ranina*), Heterotremata (*Jonas*, *Calappa*, *Cancer*, *Paradorippe*, *Tokoyo*, *Pugettia*, *Platymaia*, *Cryptopodia*, *Ovalipes* and *Carcinoplax*) and Thoracotremata (*Eriocheir*, *Mictyris* and *Uca*), with podotremes as sister to the remaining. This contradicts the result of a recent study which indicates paraphyly of Podotremata (Ahyong et al., 2007). However, the monophyly of Podotremata is only strongly supported by BI analysis (BI PP = 0.97) while the bootstrap support was intermediate under ML analysis (BP = 68). Moreover, the topology based on sequence of NaK alone does indicate the paraphyly of Podotremata, with *Ranina* more closely related to Heterotremata + Thoracotremata clade than to other species from Podotremata, though the group is monophyletic based on PEPCK gene tree.

2.4 Discussion

2.4.1 Utility of nuclear protein-coding genes in decapod phylogenetics

Our study demonstrates the utility of nuclear protein-coding genes in reconstruction of decapod phylogeny. Most of the infraordinal/familial relationships are robust and concordant between different methods of phylogenetic inference. We did not detect evidence for the presence of paralogs in the sequences and both gene segments lack introns, facilitating PCR amplification from genomic DNA. Thus, they show obvious promise in phylogenetic reconstruction and appear to be excellent new markers for future multi-locus studies. Moreover, the two genes appear to be informative across a wide range of taxonomic levels. In the present study, we focused mainly on infraordinal and familial relationships, but nevertheless found high statistical support for generic relationships within families for which there is more extensive taxon sampling (e.g. Palinuridae, Nephropidae). The three spiny species of *Panulirus* analyzed exhibit up to 6% and 3.5% sequence divergence in PEPCK and NaK respectively, suggesting potential for resolving species level relationships as well. We propose that nuclear protein-coding genes, like the two genes we have used here, should become core markers for future phylogenetic studies of decapods, especially for reconstructing deep nodes. More markers should also be explored for their phylogenetic potential. In combination with the available mtDNA and nuclear ribosomal DNA markers, the use of the protein-coding genes could lead us closer to the goal of reconstructing the tree of life of Decapoda.

2.4.2 *Implications to higher classification of Decapoda*

Monophyly of Thalassinidea has been controversial for decades (e.g. Gurney, 1938; de Saint Laurent, 1973; Poore, 1994; Schram, 2001). Cladistic analyses of somatic morphology suggest Thalassinidea is monophyletic (e.g. Poore, 1994; Scholtz and Richter, 1995; Dixon et al., 2003), though analyses of spermatozoal ultrastructure suggest polyphyly (Tudge, 1997). Recent molecular studies of interfamilial relationships in Thalassinidea showed low support for the monophyly of the infraorder (Tudge and Cunningham, 2002; Ahyong & O'Meally, 2004; Tsang et al., 2008a). Porter et al. (2005) recovered a monophyletic thalassinidean group, but on the basis of terminals restricted to a single family, Callianassidae. In the present study, we found strong support for thalassinidean polyphyly. This corroborates the results of a previous study based on mitochondrial gene rearrangements and sequences from both mitochondrial and nuclear ribosomal genes (Morrison et al., 2002). We recovered two distinct lineages in Thalassinidea that correspond to the two strongly supported clades obtained by previous molecular studies (Tudge and Cunningham, 2002; Ahyong and O'Meally, 2004; Tsang et al. 2008a) though our analysis includes fewer taxa than the aforementioned studies. The two thalassinidean groups correspond to the "Homarine Group" (Axiidae + Callianassidae) and "Anomuran Group" (Upogebiidae + Laomediidae) as proposed by Gurney (1938) based on larval morphology (but see Burkenroad, 1981). The division of Thalassinidea into the two major groups is also supported by external somatic morphology and foregut ossicles (de Saint

Laurent, 1973; Sakai 2005; Tsang et al. 2008a). Notably, members of the "Homarine Group", which are most closely related to the lobsters, have chelate first and second pereiopods. Members of the "Anomuran Group", which are closest to the Meiura, have chelate or subchelate first pereiopods and subchelate or simple second pereiopods. Although the Upogebiidae + Thalassinidae clade is paraphyletic in our gene tree, the nodal support is low. This might be an artefact of low taxon sampling, because the two families form a well-supported clade in more comprehensive molecular analyses (Tudge and Cunningham, 2002; Ahyong and O'Meally, 2004; Tsang et al., 2008a). Thus, we believe that Thalassinidea comprises two, major, disparately placed clades that correspond to the two superfamilies, Thalassinidea and Callianassoidea, proposed by Tsang et al. (2008a). The presence of thalassinideans at the base of both major reptant clades recovered here, suggests that the stem reptantian was most probably thalassinidean-like (see section 4.3). Moreover, these two clades may each warrant infraorder status if thalassindean polyphyly is corroborated by further analyses.

According to our results, all lobsters and crayfish (Astacidea, Achelata, Polychelida), form a strongly supported monophyletic group, and together with a clade of thalassinideans, corresponds essentially to the old suborder Macrura Reptantia (Bouvier, 1917). This contrasts with the results of previous morphological and molecular analyses that indicate Polychelida to be the basal reptantian, with Astacidea and Achelata sensu lato being paraphyletic or polyphyletic (e.g. Scholtz and Richter, 1995; Schram, 2001; Dixon et al., 2003; Ahyong and O'Meally, 2004; Schram and Dixon, 2004;

Fig. 1D-H). These authors thus proposed the usage of Achelata for Palinuridae + Scyllaridae. Our results find Polychelida to be more closely related to Achelata despite the relatively low statistical support. On this basis, some might argue for the reunion of these groups into the single infraorder Palinura as reflected in Martin and Davis' (2001) classification. The association of Polychelida with Achelata (i.e., Palinuridae + Scyllaridae), however, is relatively weak, they are genetically quite divergent, and morphologically disparate. Indeed, there are no morphological characters uniquely shared by polychelidans and palinurans, the most obvious difference being the chelate versus non-chelate pereiopods. As remarked by Burkenroad (1981: 264), "it is not easy to define Borradaile's "Palinura" with precision except by their possession of a peculiar button fastening the carapace to the last thoracic somite". However, Scholtz and Richter (1995) demonstrated that even the 'button fastening' device supposedly uniting palinurans and polychelidans is not homologous, having a similar function, but being derived from different structures, namely the thorax and abdomen, respectively. Thus, the recognition of two separate infraorders, Polychelida and Achelata is preferable, not only because both clades are highly distinct morphologically, but because a Polychelida + Achelata taxon would lack synapomorphies.

Our results suggest a very different pattern of cladogenesis amongst the macrurous reptantians than indicated by previous phylogenetic studies (Scholtz and Richter, 1995; Dixon et al., 2003; Ahyong and O'Meally, 2004; Porter et al., 2005). Also, it should be stated that it is difficult to diagnose a

Macrura Reptantia clade. The major characters traditionally used, such as the long, well-developed tail, and reduced first abdominal pleuron, are plesiomorphies (Scholtz & Richter, 1995), though further morphological study may reveal unrecognized synapomorphies. It is noteworthy, however, that when reptant relationships are interpreted as an unrooted network, present results and those of Ahyong and O'Meally (2004) are congruent except for the monophyly of the thalassinideans (which received low support in the latter study). Thus, the chief topological differences between present results and Ahyong and O'Meally (2004) are in the position of reptant root. Several factors can affect the placement of the root, including long-branch effects and 'random outgroup effects' (Wheeler, 1990; Maddison et al., 1992). The accuracy of the position of the reptant root will have to be further evaluated when additional loci and taxa can be added to existing datasets.

Within Achelata, interrelationships of Scyllaridae and Palinuridae remain unclear according to our results. Under ML, Scyllaridae is nested within a paraphyletic Palinuridae. Likewise, Porter et al. (2005) reported a similar result but with low statistical support. The position of Scyllaridae relative to palinurans is unresolved in BI analysis. Thus, our results raise important questions about the status of Palinuridae in relation to Scyllaridae, questions that are currently the subject of more detailed investigation in our laboratory.

Thaumastochelidae is nested within Nephropidae in contrast to previous studies finding the two families to be reciprocally monophyletic

(e.g., Tshudy and Sorhannus, 2000; Dixon et al., 2003; Ahyong and O'Meally, 2004; Ahyong, 2006; Fig. 1E-F). Our result, however, is concordant with analyses using 12S and 16S rDNA based on more extensive taxon sampling from Nephropidae (Tshudy et al., 2005; Chu et al., 2006). Thus, the family status of Thaumastochelidae requires re-evaluation. The reef lobsters, Enoplometopoidea, with the single extant family Enoplometopidae, have been somewhat controversial in being variously included in the Astacidea or Thalassinidea (for discussion see Ahyong & O'Meally, 2004). Our data show that Enoplometopidae is sister to Nephropidae + Thaumastochelidae, corroborating previous morphological and molecular analyses (Scholtz & Richter, 1995; Ahyong & O'Meally, 2004; Ahyong, 2006). Thus, inclusion of Enoplometopoidea within Astacidea is supported by the present data.

It is beyond the scope of the present study to reconstruct a detailed phylogeny within Brachyura or Anomura, but their sister relationship corroborates the Meuira concept (Scholtz and Richter, 1995; Schram, 2001; Dixon et al., 2003; Ahyong & O'Meally, 2004; Miller and Austin, 2006). Several aspects of our topologies, however, are noteworthy. First, our results recover three clades corresponding to Podotremata, Heterotremata and Thoracotremata, with Podotremata as basal. The finding of a monophyletic Podotremata based on the combined gene tree contrasts with a recent 18S rDNA analysis indicating podotreme paraphyly (Ahyong et al., 2007). The paraphyly of this group, however, is supported by the tree based on NaK alone. Moreover, our taxonomic sampling of podotremes is very limited and

wider sampling will most likely recover a paraphyletic Podotremata, particularly when representatives of the Cyclodorippoidea (sister to Eubrachyura fide Ahyong et al., 2007), are included. Secondly, the symmetrical hermit crab *Pylocheles macrops* (Pylochelidae) is recovered as sister to the galatheoids rather than other hermit crabs, also observed by Ahyong and O'Meally (2004). This result is consistent with the finding that pylochelid sperm morphology differs considerably from that of other hermit crabs (Tudge, 2001). Apart from the position of *Pylocheles macrops*, the remaining hermit crabs are distributed in two major clades corresponding to the paguroids and coenobitoids. Within the paguroid group, Lithodidae (king crabs) is nested within Paguridae, corroborating most previous studies (e.g., Cunningham et al., 1992; Richter & Scholtz, 1994; Morrison et al., 2002) challenging the monophyly of Paguridae as well as the distinct superfamily status for king crabs proposed by McLaughlin et al. (2007). Interestingly, relationships within the coenobitoid group somewhat parallel that of the paguroids in that the coenobitid species are nested within the Diogenidae, challenging monophyly of Diogenidae. Both coenobitoid and paguroid clades have consistently received molecular and morphological support (e.g., Ahyong and O'Meally, 2004; McLaughlin et al., 2007), but molecular support for the sister relationship between these clades is consistently weak or lacking (Ahyong and O'Meally, 2004; this study). The inability of our analysis to robustly resolve the positions of several of the major clades within Anomura suggests higher taxon sampling and additional molecular loci may be required to address this issue. Clearly, the controversial issue of paguroid monophyly is an obvious point of exploration for future studies.

2.4.3. *Origin and evolution of Reptantia*

The sister group of Reptantia has been a debated issue with several hypotheses proposed (Fig. 1A-C). Analysis of 18S rDNA (Abele, 1991) and recent morphological studies derive Stenopodidea as sister to Reptantia (e.g. Scholtz and Richter, 1995; Schram, 2001; Dixon et al., 2003; Schram and Dixon, 2004; Fig. 1D-F). However, we found a close association between Caridea and Stenopodidea forming a clade that is the sister clade of Reptantia, supporting Burkenroad (1963, 1981) (Fig. 1A). This suggests that apomorphic characters used to align Stenopodidea and Reptantia may be convergent. Our finding, if corroborated by further analyses, could have important implications in the evolution of character systems in decapods.

Within the Reptantia, polychelidans, with a fossil record dating back to the Jurassic, have been proposed as the basal reptantian clade based on morphological and molecular analyses (e.g. Scholtz & Richter, 1995; Dixon et al., 2003; Ahyong and O'Meally, 2004; Schram and Dixon, 2004; Fig. 1D-G). Present results suggest a different ancestry. If basal groups in both major clades of reptants are thalassinideans, the most parsimonious inference is that the stem reptantians were thalassinidean-like. In favour of this hypothesis are Carboniferous trace fossils, believed to be astacidean burrows (Hasiotis, 1999) which, however, are equally interpreted as produced by thalassinideans (Dixon et al., 2003). Of even greater potential interest is the fact that the oldest known fossil decapod, the Devonian *Palaeopalaemon*

newberryi, bears the habitus of a macrurous lobster, along with carapace lineae as in thalassinideans (Schram et al., 1978). Thus, stratigraphic data are readily compatible with present topologies. The significance of this fact should not be overweighted, however, because pre-Jurassic decapod fossils are rare and the oldest fossil dendrobranchiates are of Triassic age, considerably younger than *Palaeopalaemon*. As cautioned by Schram (2001: 9), "a tremendous number of discoveries in the fossil record await us".

Scholtz and Richter (1995) identified the Fractosternalia, comprised of several reptant groups possessing a combination of articulating seventh and eighth thoracic somites and the presence of a tripartite secula. The fractostern characters provide additional somatic flexibility and may be advantageous to burrowing forms (Dixon et al., 2003). Topologies of Scholtz and Richter (1995), Schram (2001) and Ahyong and O'Meally (2004) indicated that fractostern characters were a derived feature of 'higher' reptants (but secondarily lost in nephropoids and brachyurans). Similarly, the carapace lineae (i.e., linea thalassinica, linea anomurica and linea dromica, as they are referred to in thalassinideans, anomurans and brachyurans, respectively) were regarded as a derived feature, uniting Thalassinidea, Anomura and Brachyura in the clade Lineata Ahyong and O'Meally, 2004. Polarization of fractostern characters according to present results suggests the opposite scenario. All thalassinideans are fractosterns, suggesting that the fractosternate condition was present in reptant stem-lineage, and lost in Polychelida, Achelata, Nephropoidea and Brachyura. Likewise, the presence of carapace lineae would be inferred to be a reptant stem character that was

subsequently lost in Polychelida, Achelata, Astacidea and some Thalassinidea.

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Chapter 3

Molecular evidence for the Southern Hemisphere origin and deep sea diversification of spiny lobsters (Crustacea: Decapoda: Palinuridae)

3.1 Introduction

Spiny lobsters (Decapoda: Palinuridae) are one of the most commercially important types of marine animal (Phillips, 2006). Their biology, ecology and population genetics have therefore been the subject of intensive research for aquaculture and fishery management purposes. The classification of spiny lobsters is relatively stable except for a few taxa (see Davie, 1990; Booth et al., 2002; George, 2006b). However, the phylogeny and evolutionary origin of the family and its allies are more contentious (e.g. George and Main, 1967; Davie, 1990; Patek and Oakley, 2003; George, 2006b; Patek et al., 2006).

Two major lineages, Stridentes and Silentes, have long been recognized in the Palinuridae, based on the presence or absence of the stridulating organ at the antennae (Parker, 1884; George and Main, 1967). George and Main (1967) first proposed a phylogeny of the extant palinurid genera known at that time based on non-cladistic analysis of morphological characters (Fig. 3.1A). They concluded that there had been an early divergence into Silentes and Stridentes within spiny lobsters. They further

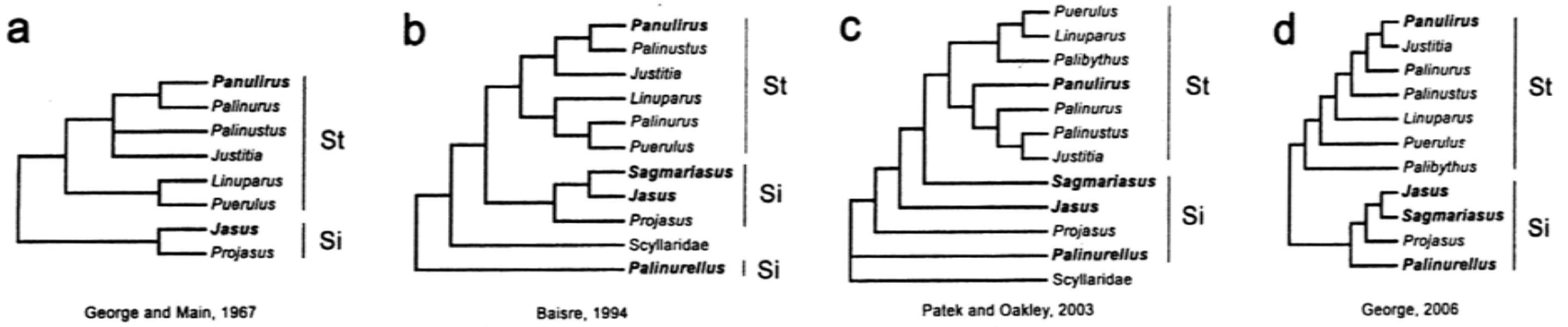

Fig. 3.1 Hypotheses of phylogenetic relationships among Palinuridae genera. (A) The evolutionary hypothesis of George and Main (1967) based on non-cladistic morphological analysis. (B) The phylogeny of Baisre (1994) based on larval and adult morphology. (C) The morphological phylogeny of Patek and Oakley (2003). (D) The phylogenetic hypothesis of George (2006b) based on review of existing information from species distribution, biology, morphology and molecular data. The Stridentes and Silentes are indicated by "St" and "Si", respectively, at the right. The genera usually found in shallow water region (< 100 m) are in bold.

hypothesized that the ancestral stock of the two lineages lived in deep-water, high-latitude areas, and subsequently invaded and diversified in shallower warm water habitats in lower latitudes. The shallow water genera *Panulirus* and *Jasus* (with most species distributed between 0 - 200 m) are considered to be more derived and specialized, as their adaptations include an elevated eye position and an enlarged supra-orbital process which enhance vision. George (2005) further suggested that the presumably more primitive deep water genera like *Puerulus* and *Linuparus* (generally found in depths > 200 m), usually have a longer larval incubation time and spawn year-round in contrast to the recently evolved *Panulirus*. This trend of biological modifications from deep to shallower water is observed in both Stridentes and Silentes, and has therefore been assumed to be the dominant process in the early diversification of the Palinuridae.

Davie (1990) challenged this hypothesis after discovering the stridulating lobster, *Palibythus magnificus*. This species closely resembles the coral lobster *Palinurellus* of the family Synaxidae in morphological terms, but possesses a fully developed stridulating organ which is absent in *Palinurellus* (Davie, 1990). The author argued that the independent origins of such a highly developed organ are unlikely, and suggested that the Synaxidae is synonymous with the Palinuridae. *Palibythus* and *Palinurellus* retain many primitive features that probably represent an early offshoot of the Stridentes and Silentes respectively. This suggests that, contrary to the view held by George and Main (1967), the ancestral form of the family initially inhabited shallower waters and then retreated into the deeper region,

not the other way round.

To decide between the two alternative hypotheses of lobster evolution, a robust phylogeny is a prerequisite. Baisre (1994) attempted to reconstruct the phylogeny of Palinuridae and Scyllaridae based on larval and adult morphology using non-cladistic clustering analyses. His topology recovered the reciprocal monophyly of Stridentes and Silentes, and the generic relationships inferred are largely congruent to those proposed by George and Main (1967), with deep-water genera being basal in the group (Fig. 3.1B). *Palinurellus* (as Synaxidae) is the most primitive genus in his tree. However, *Palibythus* was not included in his analyses and tree topologies varied with data and species included so that his result remains inconclusive. In the first cladistic analysis on morphological characters of the spiny lobsters, *Palibythus* is nested within Palinuridae while *Palinurellus* fell outside of the Palinuridae (Patek and Oakley, 2003; Fig.3.1C). This provides some support for the placement of *Palinurellus* in the Synaxidae, which should not include *Palibythus*. The generic relationships, however, are poorly resolved, as in other morphological studies. Apparently molecular data serve as an alternative solution to the problem.

Most of the molecular phylogenetic studies on spiny lobsters to date have focused on species-level relationships within a genus (e.g. Ovenden et al., 1997; Ptacek et al., 2001; Groeneveld et al., 2007), and a comprehensive study on the family as a whole remains lacking. Patek and Oakley (2003) presented the first attempt to reconstruct the molecular phylogeny of

palinurid genera. However, the phylogenetic trees constructed from mitochondrial 16S rRNA, nuclear 18S and 28S rRNA genes were inconsistent, while analyses based on the concatenated sequence did not increase the resolution. The inclusion of mitochondrial COI and nuclear histone 3 gene sequences by Palero et al. (2009) in addition to the three markers previously used does not provide enough resolution to the generic relationships. Thus, most of the taxonomic uncertainties and evolutionary hypotheses of the spiny lobsters could not be settled unambiguously. On the other hand, although previous studies have provided little information concerning generic relationships, the molecular data consistently show that *Palibythus magnificus* is nested within the Palinuridae and is the sister group of *Panulirus* (Patek and Oakley, 2003; Palero et al., 2009). This is concordant with the result of the morphological analyses, challenging the placement of *Palibythus* in the Synaxidae. George (2006b) recently argued that the available data from adult and larval morphology, ecology, geographical distribution, fossil records, plate tectonics, ocean currents, molecular and cladistic analysis generally support George and Main's (1967) hypothesis of palinurid evolution, and further suggested that spiny lobsters arose in the Atlantic-European region of the Tethys Sea, probably during the early Mesozoic. George (2006b) concluded that the stridulating organ is important in the evolution of spiny lobsters and therefore the family Synaxidae should be synonymized with Palinuridae. A refined scenario of deep-sea to shallow water evolutionary trends in spiny lobsters was also suggested by George (2006b) but in a non-cladistic way (Fig. 3.1D).

Recent studies of decapod phylogeny using sequences of nuclear protein-coding genes, although limited in number, have strikingly demonstrated their potential utility in resolving generic and familial relationships (Tsang et al., 2008b; Mahon and Neigel, 2008; Chu et al., 2009). Using sequences from three nuclear protein-coding genes and the most comprehensive sampling to date, we attempted to elucidate the phylogenetic relationships between different genera of the Palinuridae and its allies. We tested the hypothesis of the high-latitude, deep-sea origin of the family and the separation of the family with the evolution of the stridulating organ. In the light of the inferred phylogeny, we propose important revisions in the taxonomy of the Palinuridae and Synaxidae.

3.2 Materials and methods

3.2.1 Taxon sampling

Representatives from all genera of Palinuridae were included in this study (Table 3.1). The validity of the notion of a Synaxidae family consisting of two genera, *Palinurellus* and *Palibythus* (e.g. Davie, 1990; Holthuis, 1991; Martin and Davis, 2001; Patek and Oakley, 2003; Patek et al., 2006), has been questioned repeatedly, and we have taken into account the fragility of this classification in the present study. We follow the classification scheme of Holthuis (1991), keeping Synaxidae as a separate family from Palinuridae before evaluating its taxonomic status based on the results. The taxon *Sagmariasus*, originally proposed by Holthuis (1991) as a subgenus of *Jasus*,

Table 3.1 Classification, sampling locations and voucher ID of the species and GenBank accession numbers of the gene sequences of the present study.

Species	Sampling location	Vouncher ID	Gene		
			NaK	PEPCK	H3
Ingroup					
Achelata					
Palinuridae					
Jasus edwardsii	Fish market, Hong Kong	Unvouchered	EU427157	EU427226	FJ558545
Jasus tristani	Tristan da Cunha	MNHN Pa679	FJ558583	FJ558570	FJ558547
Justitia japonica	Taiwan	NTOU M00712	EU427158	EU427227	FJ558548
Justitia longimanus	Iles Australes	MNHN Pa1399	FJ558582	FJ558569	FJ558546
Justitia vericeli	Vanuatu	MNHN Pa1852	FJ558584	FJ558570	FJ558548
Linuparus sordidus	Taiwan	NTOU M00728	FJ558584	FJ558572	FJ558550
Linuparus trigonus	New Caledonia	NTOU M00713	EU427159	EU427228	FJ558551
Palinurus elephas	Fish market, Paris, France	NTOU M00727	EU427160	EU427229	FJ558552
Panulirus longipes	Fish market, Hong Kong	Unvouchered	EU427161	EU427230	FJ558556
Panulirus polyphagus	Fish market, Hong Kong	Unvouchered	EU427162	EU427231	FJ558558
Panulirus ornatus	Fish market, Hong Kong	MSLKHC-Paorn	FJ558589	FJ558576	FJ558557
Panulirus stimpsoni	Fish market, Hong Kong	MSLKHC-Pasti	FJ558588	FJ558575	FJ558555
Panulirus versicolor	Fish market, Hong Kong	MSLKHC-Paver	EU427163	EU427232	FJ558559
Palinustus holthuisi	Taiwan, holotype	NTOU M00730	FJ558586	FJ558573	FJ558553
Palinustus unicornutus	New Caledonia	NTOU M00729	FJ558587	FJ558574	FJ558554
Projasus bahamondei	Fish market, Keelung, Taiwan	NTOU M00714	EU427166	EU427235	FJ558562
Puerulus angulatus	Taiwan	NTOU M00715	EU427164	EU427233	FJ558563
Puerulus velutinus	Vanuatu	NTOU M00731	FJ558591	FJ558578	FJ558564
Sagmariasus verreauxi	New Zealand	Unvouchered	FJ558593	FJ558580	FJ558566

Scyllaridae					
Eduarctus martensii	Taiwan	NTOU M00716	EU427155	EU427224	FJ558543
Ibacus novemdentatus	Taiwan	NTOU M00717	EU427156	EU427225	FJ558544
Scammarctus batei	Philippines	NTOU M00732	FJ558593	FJ558581	FJ558567
Synaxidae					
Palinurellus wieneckii	Aquarium shop, Singapore	ZRC2000.743	FJ558592	FJ558579	FJ558565
Palibythus magnificus	Coral Sea	NTOU M00733	FJ558590	FJ558577	FJ558560
Outgroup					
Polychelida					
Polychelidae					
Polycheles amemiyai	Taiwan	NTOU M00719	EU427165	EU427234	FJ558561
Astacidea					
Enoplometopidae					
Enoplometopus debelius	Aquarium shop, Hong Kong	MSLKHC-Endeb	EU427149	EU427218	FJ558568
Nephropidae					
Homarus gammarus	Fish market, Paris, France	NTOU M00711	EU427150	EU427219	DQ079676

has been elevated into a generic rank by some authors (e.g., Booth et al., 2002; George, 2006b). This taxon, containing only one species, is included in the present analysis as a genus to test if this status is valid. Three species from Scyllaridae (*Eduarctus martensii*, *Ibacus novemdentatus* and *Scammarctus batei*) were investigated for comparison as well. Föster (1973) argued that Palinuridae is not monophyletic, with Scyllaridae nested within it

We therefore also included Polychelida and Astacidea as outgroups which were revealed to be the sister taxa to Achelata (Ahyong and O'Meally, 2004; Porter et al., 2005; Tsang et al., 2008b). The species used were *Polycheles amemiyai* and two astacid species, *Homarus gammarus* and *Enoplometopus debelius*.

3.2.2 DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the pleopod or pereiopod of the target species using the commercial QIAamp DNA Mini Kit (QIAGEN). Primers for amplifying the nuclear phosphoenolpyruvate carboxykinase (PEPCK), sodium-potassium ATPase α-subunit (NaK) and histone 3 (H3) genes were based on Tsang et al. (2008b) for the former two genes and Colgar et al. (1998) for the last one. The amplifications were conducted in a reaction mix containing 1-5 μl of template DNA, 1X PCR reaction buffer, 3 mM $MgCl_2$, 200 nM of each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (Amersham) and ddH_2O to a total volume of 50 μl. The PCR profiles were as follows: 3 min at 94°C for initial denaturation, followed by

35 cycles of denaturation at 94°C for 30 s, annealing at 50-60°C depending on the primers for 30 s, elongation at 72°C for 0.5-1.5 min depending on the length of the target gene region, and a final extension at 72°C for 10 min. The PCR products were then purified using the QIAquick gel purification kit (QIAGEN), in accordance with the manufacturer's instructions. Sequencing reactions were carried out using the same sets of primers and the ABI Big-dye Ready-Reaction mix kit, following the standard cycle sequencing protocol. The products were analyzed using an Applied Biosystems (ABI) 3100 automated sequencer.

3.2.3. Phylogenetic analyses

Sequences were aligned using CLUSTAL W (Thompson et al., 1994) using default parameters, manually adjusted and confirmed by translating into amino acid sequences. Departures from base compositional homogeneity across taxa were evaluated by χ^2 test using PAUP*4.0b10 (Swofford, 2002) for the genes as a whole and for individual codon positions. We evaluated the congruence among genes by investigating any strongly supported conflicting nodes between a maximum likelihood phylogeny generated from individual markers (Wiens, 1998). Support was considered to be strong where bootstrap (BP) values were ≥ 75.

The total dataset was analyzed under maximum likelihood (ML) using the online version of PhyML (Guindon and Gascuel, 2003; Guindon et al., 2005; available at: http://www.phylogeny.fr/phylo cgi/phyml) and

Bayesian inference (BI) using MrBayes v.3.12 (Ronquist and Huelsenbeck, 2003). The best-fit model of nucleotide substitution for each dataset was determined by Modeltest 3.7 (Posada and Crandall, 1998). For ML analysis, three independent runs were performed with nodal support estimated from 500 BP pseudoreplicates. For Bayesian analysis, 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 the analysis. Chains were sampled every 500 generations. Convergence of the analyses was validated by monitoring the likelihood values graphically using Tracer v1.4 (Rambaut and Drummond, 2007) and the trees prior to stationary were discarded as burn-in. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PP).

Alternative a priori 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 inferred topology and the a priori hypotheses. The tests were carried out with RELL optimization and 1000 BP pseudoreplicates.

3.3 Results

3.3.1. Sequence characteristics

We obtained 13 new gene sequences each for PEPCK and NaK, and 26 H3 sequences (Table 3.1). These were combined with the PEPCK and NaK sequences of the target species (see Tsang et al., 2008b) for analysis. The final aligned sequences consisted of 570 bp of PEPCK, 525 bp of NaK and 303 bp of H3. No introns or indels were present. Sequence ambiguities (i.e. double peaks in the chromatograms) were coded as ambiguous using the IUB symbols, i.e. R, Y, S, W, K, or M. There was no significant base heterogeneity among taxa in any of the three genes or at any codon position (Table 3.2).

3.3.2. Phylogenetic inference

There was no significant conflict among gene trees constructed from the three molecular markers used. We therefore combined the sequences from the three genes, resulting in a dataset with 1398 bp. The best-fit model selected using Modeltest was GTR+I+G for ML analysis; TIM+I+G, GTR+I+G and TVM+I+G were applied to PEPCK, NaK and H3 genes, respectively, in the BI analysis. Topologies derived from ML and BI analyses were completely congruent. As a result, the ML tree is presented with support values of ML and BI shown on the corresponding branches (Fig. 3.2) and most of the nodes receive strong support (ML BP $\geq$ 75 and BI PP $\geq$ 0.95)

Table 3.2 Summary of parsimony results

	No. of sites	No. of variable sites	No. of parsimony informative sites	%A/T	Chi square (*P*)
PEPCK					
nt1	190	24	9	45.6	1
nt2	190	12	6	51.8	1
nt3	190	137	96	33.8	1
All sites	570	173	111	43.1	1
NaK					
nt1	175	43	27	45.9	1
nt2	175	16	7	63.1	1
nt3	175	146	119	56.6	0.994
All sites	525	205	153	55.2	1
H3					
nt1	101	8	7	36.9	1
nt2	101	0	0	54.5	1
nt3	101	86	75	37.8	0.729
All sites	303	94	82	43.1	1
Overall	1398	472	346	47.9	1

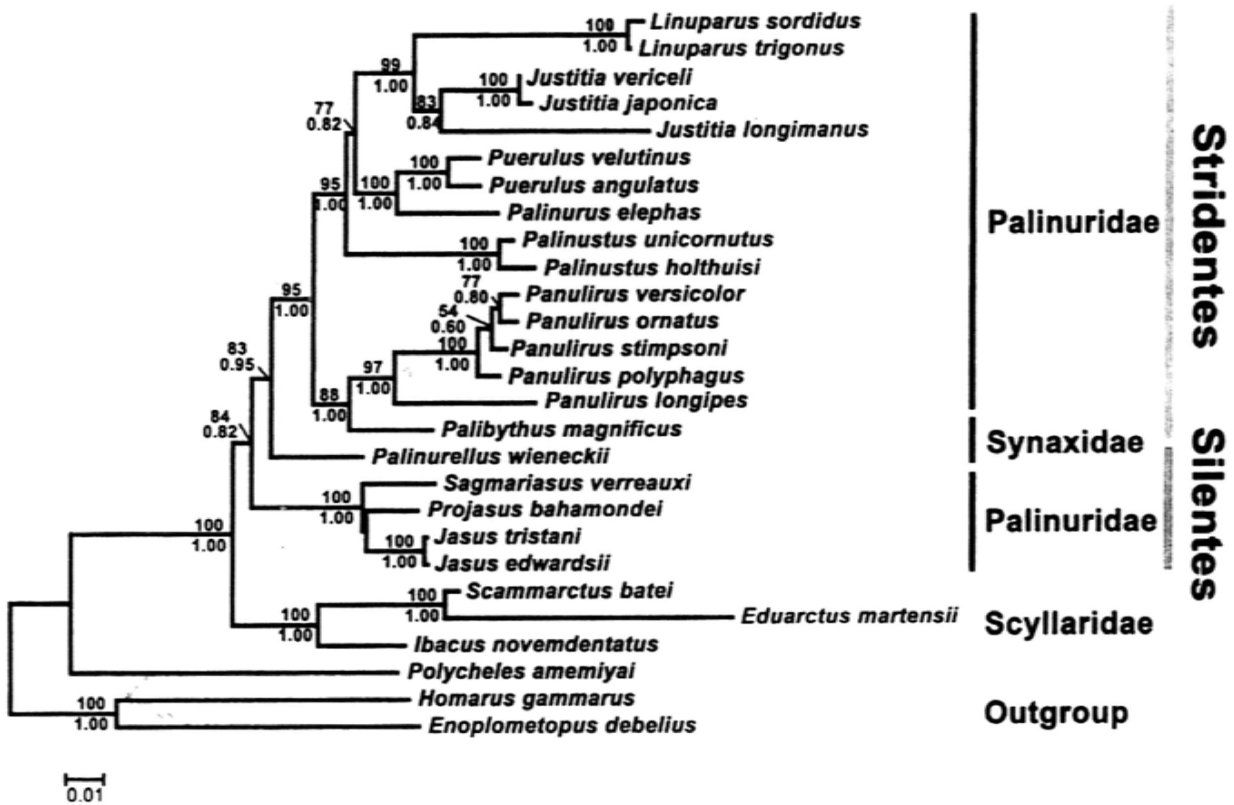

Fig. 3.2 Maximum likelihood tree inferred from the combined PEPCK, NaK and H3 analysis. ML bootstrap supports (%) are given above the corresponding branches while the Bayesian posterior probabilities are shown below the branches. The familial classification of the species based on Holthuis (1991) is indicated by black bars on the right-hand side. The two major lineages, Stridentes and Silentes, are delineated using the grey bars.

unless otherwise stated.

Achelata forms a monophyletic assemblage (Fig. 3.2), consistent with previous morphological (Scholtz and Richter, 1995; Dixon et al., 2003) and molecular studies (Ahyong and O'Meally, 2004; Porter et al., 2005; Tsang et al., 2008b). By contrast, only Scyllaridae of the three families in the infraorder was revealed to be monophyletic (Fig. 3.2), and it is not nested within Palinuridae, contrary to Föster's suggestion (1973). The two genera *Palibythus* and *Palinurellus* of Synaxidae are nested within the Palinuridae, making the latter paraphyletic. Since the genera have different affinities to the paliunirid genera, Synaxidae is polyphyletic. The a priori hypothesis for the reciprocal monophyly of the two families was disproved by the SH and KH tests (monophyletic Palinuridae: SH and KH $P < 0.01$; monophyletic Synaxidae: SH and KH $P = 0.001$). As some previous studies have placed *Palibythus* under Palinuridae instead of Synaxidae (Davie, 1990; Patek and Oakley, 2003; George, 2006b; Patek et al., 2006), we also tested the possible monophyly of Palinuridae with *Palibythus* as a member (i.e., with *Palinurellus* as the only genus in Synaxidae). This a priori hypothesis cannot be rejected by the SH ($P = 0.13$) and KH ($P = 0.239$) tests. Of the two groups of Palinuridae, Stridentes (including *Palibythus*) is revealed to be monophyletic but Silentes is paraphyletic, with *Palinurellus* more closely related to the Stridentes than to the other three Silentes genera (*Jasus*, *Projasus* and *Sagmariasus*) which form the basal group in the Palinuridae + Synaxidae clade. Yet an alternative hypothesis of a monophyletic Silentes is not significantly worse than the inferred phylogeny (SH $P = 0.192$, KH $P =$

0.107).

Within the Stridentes, the case for each of the genera being monophyletic is supported, and most of the generic relationships are well-resolved. The genera are divided into two major clades. The synaxid *Palibythus* allies with *Panulirus* and they constitute the first earlier diverged clade from the other genera. The remaining five Stridentes genera cluster into the second clade. In the second major clade, *Linuparus* is most closely related to *Justitia* while *Puerulus* groups with *Palinurus*. The sister relationship between the *Linuparus* + *Justitia* and the *Puerulus* + *Palinurus* clades is strongly supported by ML analysis (BP = 77) but only receives moderate support from BI (PP = 0.82). These four genera associate with *Palinustus* with high confidence values (ML BP = 95, BI PP = 1.00). An a priori phylogenetic hypothesis for *Puerulus* and *Linuparus* being the basal palinurids, as suggested by George and Main (1967), is strongly rejected by the SH and KH tests ($P < 0.001$ for both). On the other hand, the interrelationship among the three Silentes palinurid genera (*Jasus*, *Projasus* and *Sagmariasus*) remains unresolved, though *Sagmariasus* is shown to be genetically diverged from *Jasus*, consistent with the findings of previous studies (Ovenden et al., 1997; Booth et al., 2002, George, 2006b).

3.4 Discussion

3.4.1 Origin and evolution of Palinuridae

This study presents the most comprehensive molecular phylogeny of Palinuridae and its allies to date. The inferred topology receives strong nodal supports for most of the branches, and the results are robust across different analytical methods (maximum likelihood and Bayesian inference). This allows us to explicitly test different hypotheses concerning the evolutionary history of the spiny lobsters. Together with the information from paleobiology, species distribution and life cycle characteristics, we can synthesize a biogeographic hypothesis on the origin and diversification of the group.

The molecular dataset of Patek and Oakley (2003) shows the inclusion of Silentes in the Stridentes, indicating the possibility of acquisition and successive loss of the stridulating organ during lobster evolution. However, nodal supports in their rRNA gene trees are low and the topologies vary across genes and tree reconstruction methods, so that the result is not conclusive. Our gene tree recovers a monophyletic Stridentes, corroborating the larval and adult morphological analyses (Baisre, 1994; Patek and Oakley, 2003) which strongly support the monophyly of the group. The purpose of the stridulating organ may have been to improve the lobster's chances of escaping from predators by deterring them temporarily with a rasping sound (Mulligan and Fischer, 1977). The spiny lobsters make sound by a

stick-and-slip mechanism by rubbing the plectrum (a basal extension of each antenna) over a file located on the antennular plate (Patek, 2001). An enlarged antennular plate and the possession of plectrum evolved in the Stridentes to facilitate this action (Patek and Oakley, 2003). Most taxonomists therefore consider that it is unlikely that such a complex organ arose independently (Davie, 1990; Patek et al., 2006; George, 2006b). The present study confirms that the stridulating organ in spiny lobsters is a synapomorphy and evolved only once from a Silentes ancestor.

Palinurellus and *Palibythus* show many primitive features (e.g. small eye, broad flat rostrum, relatively narrow sternum), but *Palibythus* possesses a derived stridulating organ. Davie (1990) has therefore proposed that the two species represent an early offshoot in the Stridentes and Silentes. Our data reveal that *Palibythus* allies with *Panulirus* and that they were diverged from the other Stridentes taxa in the early stage in the radiation of the group, consistent with this hypothesis. However, *Palinurellus* was apparently derived from the other Silentes, suggesting that it is not a primitive form, as suggested by some authors (Baisre, 1994; George, 2006b). Previous molecular analyses reveal that *Palinurellus* is the sister taxon to *Jasus*, *Projasus* and *Sagmariasus*, and the Silentes is a monophyletic assemblage (Patek and Oakley, 2003; Palero et al. 2009). On the contrary, the present results show that *Palinurellus* is intermediate between the Stridentes and Silentes. This apparently is attributed to the resolution of markers employed (nuclear protein-coding genes vs. nuclear rDNA and mtDNA). The strong supports for most of the nodes in our gene tree as compared to

previous studies demonstrate the robustness of our phylogenetic hypothesis. Moreover, the ophthalmic somite in *Palinurellus* is somewhat different from those of *Jasus*/*Sagmariasus*/*Projasus*, and shows a type of structure which could be a forerunner of the stridulatory organ of *Palibythus* and other Stridentes (Davie, 1990: Fig. 5B). Thus *Palinurellus* appears to represent a transition from the Silentes to the Stridentes. Considering also the morphological similarities between *Palibythus* and *Palinurellus*, it is likely that *Palibythus* represents the most primitive extant lineage in the Stridentes.

The three Silentes genera *Jasus*, *Projasus* and *Sagmariasus* are restricted to the high latitudes (>30°) of the Southern Hemisphere. They are shown to be the basal spiny lobsters (including *Palinurellus* and *Palibythus*). This concurs with the hypothesis of high latitude origin of the group, proposed by George and Main (1967), and suggests a Southern Hemisphere origin of the extant spiny lobster genera (though George, 2006b, later suggested an Atlantic-European origin of the Palinuridae). Feldmann and Schweitzer (2006) reviewed the many extensive decapod fossil collections from the Southern Hemisphere, dating back to the Jurassic through the Eocene, and concluded that many decapod generic-level taxa originated in that hemisphere. New species formation in higher southern latitudes and subsequent colonization into lower latitudes were common (Feldmann and Zinsmeister, 1984; Zinsmeister and Feldmann, 1984; Crame, 1993; Feldmann and Schweitzer, 2006). The Tethys Sea served as an important pathway for the dispersal and exchange of marine organisms, including the decapods during the Cretaceous to early Miocene. However, the Tethys Sea

reduced in size in the Eocene due to tectonic plate movements and the formation of the cold Antarctic circumpolar current. This created a barrier to gene flow between the fauna in the Northern and Southern Hemisphere, consequently giving rise to a high level of endemism in the latter (Newman, 1991). Once the animals settled in the lower latitudes, adaptation to the warmer environment could promote divergence and speciation. In the case of the spiny lobsters, the ancestral stock from the southern high latitudes is believed to have evolved to *Palinurellus* in the tropical region, with *Panulirus* later radiating in the Indo-West Pacific (Pollock, 1992, 1993; Ptacek et al., 2001). George (1997, 2006a) suggested that *Panulirus* probably originated in the late Miocene, in association with the closure of the Tethys Sea. While this is consistent with our hypothesis that diversification in the extant lobster genera postdated the isolation between northern and southern fauna by the oceanographic and tectonic changes, our present study also shows that the Silentes is a more primitive group and *Panulirus* is basal in Stridentes.

The high southern latitudes are widely believed to have been the site of origin of many marine fauna (Crame, 1999), including many decapod crustaceans (Feldmann and Schweitzer, 2006). However, empirical data from molecular phylogenetic study remains limited. Investigations on several mollusks and echinoderms have provided some evidence in this respect (e.g. Williams et al., 2003; Lee et al., 2004; Degnan et al., 2006) but many of these studies were conducted on a relatively limited geographical scale studied or failed to clearly identify the region of origin. The recent molecular phylogenetic analysis of the clawed lobsters *Metanephrops*, combined with

the current distribution pattern of the extant species and the fossil record of the genus has provided a clear example of the Antarctica origin of some crustaceans (Chan et al. in press). The present study offers further phylogenetic evidence for the southern high latitudes as the site of origin of marine fauna.

3.4.2 Offshore shift and diversification

It has been hypothesized that radiation in the spiny lobsters occurred when the deep water ancestral stock invaded the shallow seas, with subsequent specialization and diversification (George and Main, 1967; Baisre, 1994; George, 2005, 2006b). The two most species rich genera, *Jasus* (6 species) and *Panulirus* (21 species), are believed to be the most recently diverged genera, radiated between the late-Miocene and the Pleistocene (Pollock, 1990, 1992, 1993; George, 1997, 2006a). The higher species number in the two genera (*Palinurus* now also has 6 species though *Jasus* including *Sagmariasus* would have 7 species) is attributed to transition and adaptation in the more fluctuating environments in the shallower seas. However, our inferred topology strongly opposes this point of view. *Panulirus* diverged with *Palibythus* in the early stage of Stridentes evolution instead of being the most recently derived, as long believed. Moreover, *Palibythus* is morphologically highly similar to *Palinurellus*, the sister taxon to Stridentes, suggesting that *Panulirus* + *Palibythus* most likely represent the basal lineage of Stridentes. This is further evident from the high inter-specific genetic divergence observed in *Panulirus* (differed by up to

32% and 24% in the mitochondrial COI and 16S rRNA, respectively; Ptacek et al., 2001; see also Patek and Oakley, 2003 and the present study), compared to other genera. The five genera living in deeper water habitats form another group. The two deep sea genera, considered to be the most primitive, *Puerulus* and *Linuparus* (George and Main, 1967; Baisre, 1994; George, 2006b), are relatively derived in our gene tree. *Panulirus* is a shallow water inhabitant while *Palinurellus* is found in caves of coral reef at near shore regions (Titgen and Fielding, 1986; George, 2006b). These are all inconsistent with the hypothesis of an invasion from the deep sea to shallow waters, but rather suggest a shift from onshore to offshore habitat in the deep sea. Moreover, almost all palinurids are found in coral reefs, deep-sea rocky areas or on top of sea mounts (Holthuis, 1991). Only the genus *Linuparus* has all its species generally inhabiting soft flat bottoms in the deep sea. *Puerulus*, considered to be the most primitive extant Stridentes by George (2006b), although also occurring in deep sea, has some species found in sandy mud bottoms while some are very abundant on top of sea mounts (Richer de Forges and Laboute, 1995). Species of *Palinustus*, mentioned by George (2006b) as living on soft substrates, are actually found mainly in deep parts of the reefs (Chan and Yu, 1995). *Palinurellus* lives deep within caves in shallow water reefs. Very little is known about the habitat of the rare *Palibythus*, but all the specimens so far collected have been caught by traps. Therefore, it is likely that *Palibythus* inhabits deep-sea rocky areas. Rocky reefs are common shallow water habitats while soft flat bottoms constitute a typical deep-sea environment. Thus, the present results further indicate a shallow water reef origin of the spiny lobsters, which then

dispersed into deeper reefs and eventually adapted to the typical soft deep-sea bottoms as suggested by Davie (1990).

A diverse number of fossil species of *Linuparus*, or the closely related extinct genus *Podocrates* have been discovered from the Cretaceous to Eocene in shallow water habitats worldwide (Secretan, 1964; Föster, 1973; Feldmann and Bearlin, 1988). George and Main (1967) argued that it is difficult for deep water species to leave a fossil record. Thus they suggested that the shallow-water fossils represent specialized species which have since died out, while the ancestral stock of modern species survived in deeper and cooler waters unrepresented by fossil deposits. However, Feldmann and Tshudy (1989) proposed that all *Linuparus* originated from the shallow waters around the Antarctic, and subsequently radiated into deep water, low-latitude habitats. This is supported by the present molecular analyses. Transitions from the onshore shallow sea to offshore deeper water regions are well-documented in other marine communities (Jablonski et al., 1983; Briggs, 2003). Retreats to deep water have also been observed in many decapods, including other lobster species from Eryonoidea, Prosopidae and Nephropidae (Glaessner, 1969). Thus, it appears that the invasion of deep sea environments played an important role in driving early diversifications in diverse groups of decapods. If our shallow to deep waters and Silentes to Stridentes evolutionary scenarios are correct, the extant palinurids would have a much longer history than previously thought. As mentioned above, the most derived lineage *Linuparus* (as shown in the present study) has many fossils dating back to the Cretaceous. Most of the other genera would have

even earlier origins. Thus, mutational saturation of mitochondrial genes and alignment ambiguities of nuclear ribosomal genes might have led to poor resolution in previous molecular studies on the genera (e.g. *Panulirus*, Ptacek et al., 2001), or the family as a whole (Patek and Oakley, 2003; Palero et al., 2009). The protein-coding genes used in the present study are powerful tools in resolving the phylogeny at various taxonomic levels of decapods (Chu et al., 2009).

Nevertheless, the relationship among *Jasus*, *Projasus* and *Sagmariasus* remains unresolved using the three protein-coding genes, so that we cannot determine whether *Jasus* is the most recent genus within the Silentes. Yet the genetic divergences among six of the *Jasus* species are relatively low (< 10% in COI and < 3.5% in 16S, Ovenden et al., 1997). This provides some evidence for recent radiation in the genus. Nevertheless, the evolutionary trend in the Silentes cannot be determined purely on the basis of the results of the present study.

3.4.3 Taxonomic implications

The validity of the family Synaxidae has long been contentious (reviewed in Patek et al., 2006, George, 2006b). Davie (1990) argued that Synaxidae is synonymous to Palinuridae and Patek and Oakley (2003) and Palero et al. (2009) provided some support for his view. Both molecular and morphological evidence consistently indicate the close association of *Palibythus magnificus* with the Stridentes palinurids (Patek and Oakley, 2003;

George, 2006b). We unambiguously confirm that *Palibythus magnificus* belongs to the Palinuridae instead of Synaxidae. The position of *Palinurellus* has been more controversial (Davie, 1990; Baisre, 1994; Patek and Oakley, 2003; George, 2006b). The present study clearly demonstrates that *Palinurellus* is nested within Palinuridae, as in the case of *Palibythus*. Thus, our results agree with almost all the hypotheses proposed by Davie (1990) including: Synaxidae is not a valid family; *Palinurellus* is a primitive palinurid that led to the Stridentes genera; and the evolution within the Palinuridae involves invasion from shallow to deeper waters. Nevertheless, the present molecular data does not support a close relationship between *Palibythus* and *Palinustus*, as suggested by Davie (1990).

Baisre (1994) suggested a formal recognition of the Silentes and Stridentes at the subfamily level. The present study supports the view of George (2006b) that only Stridentes is a monophyletic group while the Silentes is paraphyletic. Thus, Synaxidae should be synonymized with Palinuridae and a subfamily for the Silentes is unwarranted. As a result, Achelata would only consist of two families, Palinuridae and Scyllaridae.

Other than the generally used classification scheme of Palinuridae in Holthuis (1991), a subgenus *Nupalirus* has been proposed in *Justitia* (Kubo, 1955; George and Main, 1967; Baisre, 1994; George, 2006b) and it has also been proposed to raise the subgenus *Sagmariasus* to a generic rank (Booth et al., 2002; George, 2006b). Although our results could not clearly resolve if *Sagmariasus* and *Jasus* are poly- or paraphyletic, *Sagmariasus* is clearly a

distinct lineage from *Jasus* and therefore its generic status is well supported. *Nupalirus* has been proposed for the three closely related species *Justitia japonica*, *J. chani* and *J. vericeli*, with *Justitia* restricted to *J. longimanus* (= *J. mauritiana*) (George and Main, 1967; Baisre, 1994; George, 2006b). Our result shows that *J. longimanus*, *J. japonica* and *J. vericeli* constitute a monophyletic group. However, this association is only strongly supported by ML analysis: the posterior probability of Bayesian inference is only moderate. Moreover, the two groups (*i.e. J. longimanus* and *J. japonica*/*J. vericeli*) exhibit high genetic divergence comparable to the magnitude of the comparisons between the other lobster genera (Fig. 2). Thus, we agree with George (2006b) that the two groups deserve at least the rank of subgenera. In view of the very unique trait in males of an extremely long and subchelate first pereiopod in *J. longimanus*, a separate genus seems to be more suitable for this species.

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Chapter 4

Hermit to king, or hermit to all: Multiple transitions to crab-like forms from hermit crab ancestors

4.1 Introduction

The 17,600+ species of decapod crustaceans are presently distributed in 10 infraordinal clades (De Grave et al., 2009). Of these Anomura presents the greatest degree of morphological disparity, with the symmetrical and asymmetrical hermit crabs, deepwater and freshwater squat lobsters, king and porcelain crabs, and fossorial mole crabs (McLaughlin et al., 2007; Ahyong et al., 2009; Fig. 4.1). Possibly, the most familiar are the hermit crabs, Paguroidea, with more than 1,000 species. They occur in shallow waters, the deep sea, and even on land. Hermit crabs are so named because they use a gastropod shell or other hollow objects to protect their pleons, which in most species are asymmetrically coiled to fit dextral gastropod shells (Fig. 4.1a-d). Conversely, some putatively more 'primitive' hermit crabs (family Pylochelidae) have more highly calcified and more symmetrical pleons (Fig. 4.1f). They usually live in pieces of hollow wood or straight worm tubes instead of coiled gastropod shells. The squat lobsters (Fig. 4.1j-m), include the freshwater squat lobsters (Aeglidae), yeti crab (Kiwaidae) from hydrothermal vents, coral associated Chirostylidae, and generally free-living Galatheidae. Squat lobsters share an elongated pleon

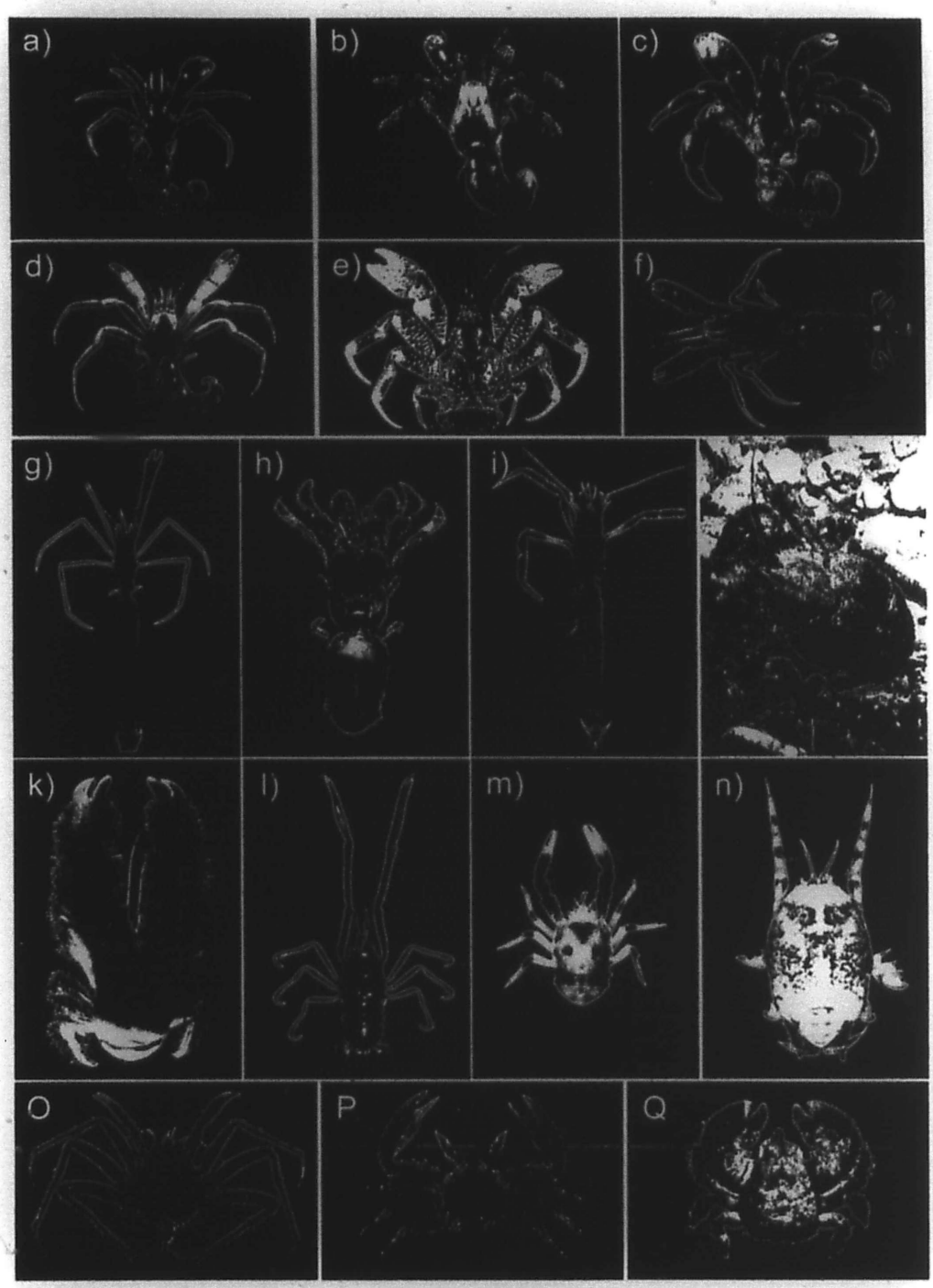

Figure 4.1. Body forms and morphological diversity of Anomura. (a) *Bathypaguropsis kuroshioensis* (Paguridae). (b) *Calcinus elegans* (Diogenidae). (c) *Coenobita rugosus* (Coenobitidae). (d) *Sympagurus burkenroadi* (Parapaguridae). (e) *Birgus latro* (Coenobitidae). (f) *Xylocheles macrops* (Pylochelidae: Pylochelinae). (g) *Xylopagurus philippinensis* (Paguridae). (h) *Cancellis panglaoensis* (Diogenidae). (i) *Tsunogaipagurus chuni* (Parapaguridae). (j) *Aegla neuquensis* (Aeglidae). (k) *Kiwa hirsuta* (Kiwaidae). (l) *Uroptychus orientalis* (Chirostylidae). (m) *Galathea rubromaculata* (Galatheidae). (n) *Hippa marmorata* (Hippidae). (o) *Neolithodes nipponensis* (Lithodidae). (p) *Petrolisthes coccineus* (Porcellanidae). (q) *Lomis hirta* (Lomisidae).

that is held partially folded under the body – they have a somewhat lobster-like body form, hence their common name. Some anomurans are distinctly crab-like; they have a broadened carapace and sternum, and a reduced pleon that is fully folded beneath the body as in true crabs (Brachyura): these are the king crabs (Lithodidae) (Fig. 4.1o), porcelain crabs (Porcellanidae) (Fig. 4.1p) and hairy stone crabs (Lomisidae) (Fig. 4.1q). The fossorial mole crabs (Hippoidea) (Fig. 1n) have the pleon partially folded under the body and with a general appearance very similar to some primitive brachyuran crabs (e.g. the frog crabs Raninoida).

Not surprisingly, the evolution and phylogeny of the anomurans have been surrounded by controversies (Ahyong and O'Meally, 2004; McLaughlin et al., 2004, 2007; Ahyong et al., 2009). The hermit crabs are unusual in using portable, hollow domiciles to protect the pleon (unique in Decapoda), and in the presence of pleonal rather than cephalothoracic midgut caeca (unique in Anomura but not Decapoda). Recent debate over anomuran phylogeny has focused primarily on the phenomenon of carcinization. 'Carcinization' was first coined by Borradaile in 1916 in reference to aspects of morphology of the hermit crab *Porcellanopagurus*, but it is now widely understood to denote derivation of a crab-like body form from a non-crab ancestor in clades outside of the Brachyura (the true crabs) (Wolff, 1961; Guinot, 1979; Cunningham et al., 1992; Morrison et al., 2002; Ahyong et al., 2009). Essentially, carcinization is achieved through widening of the carapace and sternum, and shortening and reduction of the pleon, which is held fully folded flat under the body. Also, the chelipeds, which are

plesiomorphically directed forwards, can be folded transversely across the anterior of the cephalothorax. The focus of most carcinization debates has been on whether or not the king crabs were derived from within the asymmetrical hermit crabs, the 'hermit to king' hypothesis (Cunningham et al., 1992). Recent molecular phylogenetic studies, however, not only support the 'hermit to king' hypothesis, but also suggest that asymmetry in hermit crabs may have multiple origins and that convergence of body form may be significantly more prevalent than previously recognized (Ahyong et al., 2009; Bracken et al., 2009; Chu et al., 2009). Accordingly, evaluation of the origins and pathways of carcinization could provide important insights into the evolution and adaptation in this morphologically and ecologically diverse group of animals. Evaluation of carcinization hypotheses of course requires robust knowledge of phylogeny.

Numerous morphological and molecular studies (e.g., Cunningham et al., 1992; Morrison et al., 2002; Tsang et al., 2008b; Ahyong et al., 2009; Chu et al., 2009) support the 'hermit to king' hypothesis and in some cases suggest hermit crab polyphyly. These hypotheses, however, are strongly opposed by some larval and adult morphological studies that reject asymmetrical hermit crab ancestry of king crabs (McLaughlin and Lemaitre, 1997; McLaughlin et al., 2004, 2007). Previous efforts to elucidate the anomuran phylogeny, based exclusively on morphology, or mtDNA and rDNA sequence data have suffered from insufficient topological robustness or taxon sampling to draw strong conclusions. To evaluate the evolution of Anomura, we generated a molecular dataset with >2,600 bp of DNA

sequences from five nuclear protein-coding gene regions across 14 of 17 recognized families. The phylogenetic relationships are well resolved at most nodes. We further mapped different morphological forms of anomurans onto the inferred phylogeny in order to reconstruct the history of body form transitions.

4.2 Materials and Methods

4.2.1 Taxon Sampling and Sequencing

A total of 46 species spanning 14 of the 17 anomuran families and five of the six hermit crab families (with the exception of monotypic Pylojacquesidae) were included in our study. We followed the most recent classification scheme of De Grave et al. (2009). The voucher information, sampling locations and GenBank accession nos. are listed in Table 4.1. Total genomic DNA was extracted from the pleopods or pereiopods of the anomuran species and the four outgroup taxa (Table 4.1) using the commercial QIAamp Tissue Kit (QIAGEN). Primers for amplifying the five genes, arginine kinase (AK), enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sodium-potassium ATPase α-subunit (NaK), and phosphoenolpyruvate carboxykinase (PEPCK), are listed in Table 4.2. The amplifications were conducted in a reaction mix containing 1-5 μl of template DNA, 1X PCR reaction buffer, 3 mM $MgCl_2$, 200 nM of each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (QIAGEN) and ddH_2O to a total volume of 25 μl. The PCR profiles were as follows: 3 min at 94°C

Table 4.1

Classification, sampling locations and voucher ID of the species and GenBank accession number of the gene sequences of the present study. New sequences are indicated in bold and "n.a." denotes missing sequence data.

					Gene				
Superfamily	Family	Species	Sampling location	Voucher ID	AK	enolase	GAPDH	NaK	PEPCK
Aegloidea	Aeglidae	*Aegla alacalufi*	Argentina	unvouchered	**GU382856**	**GU382903**	**GU382953**	**GU383035**	**GU383002**
	Aeglidae	*Aegla neuquensis*	Argentina	unvouchered	**GU382857**	**GU382904**	**GU382954**	**GU383036**	**GU383003**
Galatheoidea	Chirostylidae	*Uroptychodes grandirostris*	Taiwan	NTOU A01103	**GU382898**	**GU382948**	**GU382997**	**GU383067**	**GU383034**
	Chirostylidae	*Eumunida funambulus*	Taiwan	NTOU A01104	**GU382866**	**GU382913**	**GU382963**	**GU383041**	**GU383008**
	Galatheidae	*Cervimunida princeps*	Taiwan	MSLKHC-Crpri	**GU382859**	**GU382906**	**GU382956**	**GU383037**	**GU383004**
	Galatheidae	*Munida albiapicula*	Taiwan	NTOU A00837	**GU382874**	**GU382921**	**GU382971**	EU427119	EU427188
	Galatheidae	*Munidopsis formosa*	Taiwan	NTOU A01105	**GU382872**	**GU382919**	**GU382969**	**GU383046**	**GU383013**
	Galatheidae	*Paramunida tricarinata*	Taiwan	NTOU A00838	**GU382886**	**GU382935**	**GU382984**	EU427122	EU427191
	Galatheidae	*Shinkaia crosnieri*	Okinawa	NTOU A01106	**GU382892**	**GU382942**	**GU382991**	**GU383061**	**GU383028**
	Porcellanidae	*Neopetrolisthes maculatus*	Aquarium shop, Hong Kong	NTOU A00928	**GU382876**	**GU382923**	**GU382973**	**GU383048**	**GU383015**
	Porcellanidae	*Petrolisthes japonicus*	Hainan, China	MSLKHC-Ptjap	**GU382889**	**GU382938**	**GU382987**	EU427123	EU427192
	Porcellanidae	*Novorostrum indicum*	Taiwan	NTOU A01006	**GU382877**	**GU382924**	**GU382974**	**GU383049**	**GU383016**
Hippoidea	Albuneidae	*Albunea occultus*	Taiwan	NTOU A00839	**GU382858**	**GU382905**	**GU382955**	EU427113	EU427182
	Hippidae	*Hippa adactyla*	Taiwan	NMNS 4368-027	**GU382867**	**GU382914**	**GU382964**	**GU383042**	**GU383009**
Kiwaoidea	Kiwaidae	*Kiwa hirsuta*	South East Pacific Rise	MNHN Ga5310	**GU382869**	**GU382916**	**GU382966**	**GU383044**	**GU383011**
Lithodoidea	Lithodidae	*Lithodes turritus*	Taiwan	NTOU A01107	**GU382871**	**GU382918**	**GU382968**	**GU383045**	**GU383012**

	Lithodidae	*Neolithodes nipponensis*	Taiwan	NTOU A00845	**GU382875**	**GU382922**	**GU382972**	EU427120	EU427189
	Lithodidae	*Paralomis arae*	Taiwan	NTOU A00849	**GU382884**	**GU382933**	**GU382982**	n.a.	**GU383023**
	Lithodidae	*Paralomis dofleini*	Taiwan	NTOU A01096	**GU382888**	**GU382937**	**GU382986**	**GU383059**	**GU383026**
Lomisoidea	Lomisidae	*Lomis hirta*	South Australia	NTOU A00840	**GU382870**	**GU382917**	**GU382967**	EU427118	EU427187
Paguroidea	Coenobitidae	*Coenobita rugosus*	Taiwan	NTOU A00842	**GU382863**	**GU382910**	**GU382960**	EU427116	EU427185
	Coenobitidae	*Coenobita violascens*	Taiwan	NTOU A00841	**GU382862**	**GU382909**	**GU382959**	EU427115	EU427184
	Diogenidae	*Calcinus laevimanus*	Taiwan	NTOU A01100	**GU382860**	**GU382907**	**GU382957**	**GU383038**	**GU383005**
	Diogenidae	*Clibanarius virescens*	Hong Kong	NTOU A01092	**GU382861**	**GU382908**	**GU382958**	**GU383039**	**GU383006**
	Diogenidae	*Clibanarius humilis*	Philippines	NTOU A01094	n.a.	**GU382929**	**GU382978**	**GU383054**	**GU383021**
	Diogenidae	*Clibanarius englaucus*	Philippines	NTOU A01095	**GU382881**	**GU382930**	**GU382979**	**GU383055**	n.a.
	Diogenidae	*Dardanus impressus*	Taiwan	NTOU A00843	**GU382864**	**GU382911**	**GU382961**	EU427117	EU427186
	Diogenidae	*Dardanus setifer*	Hong Kong	NTOU A01108	**GU382865**	**GU382912**	**GU382962**	**GU383040**	**GU383007**
	Paguridae	*Icelopagurus crosnieri*	Taiwan	NTOU A01099	**GU382868**	**GU382915**	**GU382965**	**GU383043**	**GU383010**
	Paguridae	*Michelopagurus limatulus*	Taiwan	NTOU A01098	**GU382873**	**GU382920**	**GU382970**	**GU383047**	**GU383014**
	Paguridae	*Pagurodofleinia doederleini*	Taiwan	NTOU A00846	**GU382883**	**GU382932**	**GU382981**	EU427114	EU427183
	Paguridae	*Pagurus angustus*	Taiwan	NTOU A01097	**GU382880**	**GU382927**	n.a.	**GU383052**	**GU383019**
	Paguridae	*Pagurus bernhardus*	Scotland	NTOU A01091	**GU382882**	**GU382931**	**GU382980**	**GU383056**	**GU383022**
	Paguridae	*Pagurus* sp.	Hong Kong	NTOU A01093	n.a.	**GU382928**	**GU382977**	**GU383053**	**GU383020**
	Paguridae	*Propagurus obtusifrons*	Taiwan	NTOU A00847	**GU382890**	**GU382940**	**GU382989**	EU427124	EU427193
	Paguridae	*Spiropagurus profundorum*	Taiwan	NTOU A00719	**GU382893**	**GU382943**	**GU382992**	**GU383062**	**GU383029**
	Parapaguridae	*Oncopagurus orientalis*	Taiwan	NTOU A00371	**GU382878**	**GU382925**	**GU382975**	**GU383050**	**GU383017**
	Parapaguridae	*Paragiopagurus acutus*	Philippines	NTOU A01110	**GU382879**	**GU382926**	**GU382976**	**GU383051**	**GU383018**
	Parapaguridae	*Paragiopagurus boletifer*	Taiwan	NTOU A00299	**GU382887**	**GU382936**	**GU382985**	**GU383058**	**GU383025**
	Parapaguridae	*Paragiopagurus ventilatus*	Taiwan	NTOU A01111	**GU382885**	**GU382934**	**GU382983**	**GU383057**	**GU383024**

	Parapaguridae	*Strobopagurus gracilipes*	Taiwan	NTOU A00009	**GU382894**	**GU382944**	**GU382993**	**GU383063**	**GU383030**
	Parapaguridae	*Sympagurus burkenroadi*	Taiwan	NTOU A00221	**GU382895**	**GU382945**	**GU382994**	**GU383064**	**GU383031**
	Pylochelidae	*Xylocheles macrops*	Taiwan	NTOU A00848	**GU382891**	**GU382941**	**GU382990**	EU427125	EU427194
	Pylochelidae	*Pylocheles mortensenii*	Taiwan	NTOU A01101	n.a.	**GU382939**	**GU382988**	**GU383060**	**GU383027**
	Pylochelidae	*Trizocheles brachyops*	New Zealand	NTOU A01109	**GU382896**	**GU382946**	**GU382995**	**GU383065**	**GU383032**
	Pylochelidae	*Trizocheles sakaii*	Taiwan	NTOU A01102	**GU382897**	**GU382947**	**GU382996**	**GU383066**	**GU383033**
Cancroidea	Cancridae	*Cancer japonicus*	Taiwan	NTOU B00003	**GU382899**	**GU382949**	**GU382998**	EU427127	EU427196
Dromioidea	Dromiidae	*Conchoecetes artificiosus*	Taiwan	NTOU B00005	**GU382900**	**GU382950**	**GU382999**	EU427129	EU427198
Grapsoidea	Varunidae	*Eriocheir japonica*	Hong Kong	MSLKHC-EjapHK	**GU382901**	**GU382951**	**GU383000**	EU427132	EU427201
	Upogebiidae	*Austinogebia edulis*	Hong Kong	MSLKHC-AeduHK	**GU382902**	**GU382952**	**GU383001**	EU427167	EU427236

Table 4.2

Primer sequences used for PCR amplification

Gene/Primer	Sequence (5' to 3')	Source
Arginine kinase		
AK for a-1	CTC CCC TST TTG AYC CCA TCA T	this study
AK for a-2	ACC CCA TCA TTG AGG AYT AYC A	this study
AK for b	ATA GAC GAC CAC TTC CTS TTC AA	this study
AK rev 1	TGG AAC TCA GTC AGA CCC ATR CG	this study
AK rev 2	CCG CCC TCA GCC TCR GTG TGY TC	this study
Enolase		
Enol EA1	CAG CAA TCA ATG TCA TCA AYG GWG G	this study
Enol EA2	AGT TGG CTA TGC AGG ART TYA TGA T	this study
Enol ES1	ACT TGG TCA AAT GGR TCY TCA AT	this study
Enol ES2	ACC TGG TCG AAT GGR TCY TC	this study
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)		
GAPDH F2	ATG AAG CCA GAA AAC ATT CCA TGG	this study
GAPDH GA	ATG GTG TAT ATG TTC AAG TAY GAY TC	this study
GAPDH R	GAA TAG CCT AAC TCG TTG TCR TAC CA	this study
GAPDH GR	TCG CTA GAT ACA ACA TCA TCY TCR GT	this study
Phosphoenolpyruvate		

carboxykinase (PEPCK)		
PEPCK for	GTA GGT GAC GAC ATT GCY TGG ATG AA	Tsang et al., 2008b
PEPCK for2	GCA AGA CCA ACC TGG CCA TGA TGA C	Tsang et al., 2008b
PEPCK rev	GAA CCA GTT GAC GTG GAA GAT C	Tsang et al., 2008b
PEPCK rev3	CGG GYC TCC ATG CTS AGC CAR TG	Tsang et al., 2008b
Sodium-potassium ATPase α-subunit (NaK)		
NaK for-a	GTG TTC CTC ATT GGT ATC ATT GT	Tsang et al., 2008b
NaK for-b	ATG ACA GTT GCT CAT ATG TGG TT	Tsang et al., 2008b
NaK rev	ACC TTG ATA CCA GCA GAT CGG CAC TTG GC	Tsang et al., 2008b
NaK rev2	ATA GGG TGA TCT CCA GTR ACC AT	Tsang et al., 2008b

for initial denaturation, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50-60°C (depending on the primers and taxa) for 1 min, elongation at 72°C for 1.5 min, and a final extension at 72°C for 10 min. The PCR products were then purified using the QIAquick gel purification kit (QIAGEN) according to manufacturer's instructions. Sequencing reactions were carried out using the same sets of primers and the ABI Big-dye Ready-Reaction mix kit, following the standard cycle sequencing protocol. The products were analyzed using an Applied Biosystems (ABI) 3100 automated sequencer.

4.2.2 Phylogenetic Analyses

Sequences were aligned using CLUSTAL W (Thompson et al., 1994) with default parameters and confirmed by translating into amino acid sequences. The total dataset was analyzed using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) analyses. MP analysis was performed using heuristic search and tree-bisection-reconnection with 1,000 random addition sequence replicates on PAUP*4.0b10 (Swofford, 2002). Character states were unordered and equally weighted. Gaps were treated as missing data. Bootstrap (BP) support for the most parsimonious tree was evaluated using 1,000 replicates with 100 random sequence addition replicates. ML analysis was implemented with RAxML 7.0.3 (Stamatakis, 2006). The model GTRGAMMAI was used for the five partitions (genes), with individual α-shape parameters, GTR-rates and base frequencies estimated and optimized for each partition. We

conducted 1,000 BP runs and searched for the best-scoring ML tree. Bayesian analysis was conducted using MrBayes v.3.12 (Ronquist and Huelsenback, 2003) with the best-fit models of nucleotide substitution for individual genes determined by Modeltest 3.7 (Posada and Crandall, 1998). GTR+I+G was selected for AK, GAPDH, NaK and PEPCK, while GTR+G was applied to enolase. Four 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 the analysis. Chains were sampled every 500 generations. Convergence of the analyses was validated by the standard deviation of split frequencies and monitoring the likelihood values over time graphically using Tracer v1.4 (Rambaut and Drummond, 2007). The trees prior to the achievement of stationarity of the log likelihood values (2,000 trees) were discarded as burn-in. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PP).

Alternative a priori phylogenetic hypotheses from previous morphological and molecular studies were tested using the likelihood based Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) implemented in PAUP* and Bayes factor (Nylander et al., 2004). Alternative tree topologies were constructed using MacClade 3.0 (Maddison and Maddison, 1992) by rearranging the branches showing conflicting relationships between the inferred topology and the a priori hypotheses. The SH test was carried out with RELL optimization and 1,000 BP pseudoreplicates. The Bayes factors were calculated as twice the difference

in the harmonic mean -lnL scores (2ln B01) between the unconstraint topology obtained from the BI analysis with those under the constraint of a priori phylogenetic hypotheses (Nylander et al., 2004; Brandley et al., 2005). We evaluated alternative hypotheses according to the framework provided by Kass and Raftery (1995).

We reconstructed the pattern of body form evolution of anomurans by mapping the three different body forms: hermit crab, squat lobster and crab-like onto the inferred phylogeny using ML approaches described by Pagel (1999) implemented in BayesTraits v1.0 (available at www.evolution.reading.ac.uk). This approach is more appropriate than parsimony based methods, which do not consider branch lengths and models of nucleotide evolution. The likelihood of different possible ancestral states of the nodes was also estimated.

4.3 Results

The combined dataset consisted of 2,664 bp from five gene fragments (AK: 630 bp/212 (aligned length/number of parsimony informative sites); enolase: 339 bp/130; GAPDH: 534 bp/197; NaK: 612 bp/ 232; PEPCK: 549 bp/ 233; Table 4.3). A 3-bp insertion was observed in the GADPH gene of *Kiwa hirsuta* and *Sympagurus burkenroadi*, and a 3-bp deletion was found in the NaK gene of *Hippa adactyla* and *Icelopagurus crosnieri*. All these deletions/insertions did not represent frameshift mutations.

Table 4.3 Summary of parsimony results

	length	no. of variable site	no. parsimony informative sites	A/T %
NaK	612	292	232	47.5
PEPCK	549	260	233	44.5
enolase	339	148	130	51.3
GAPDH	534	217	197	47.9
AK	630	248	212	42.3

ML, MP and BI analyses of the combined dataset resulted in highly congruent and resolved topologies with strong support for most interfamilial nodes. They only differ in relative positions of genera within Paguridae and thus we present the nodal supports obtained from the three analyses together on the BI topology (Fig. 4.2). Anomura is strongly supported as monophyletic.

As currently conceived, Paguroidea and Galatheoidea are polyphyletic. Of the three anomuran superfamilies with more than one family, only Hippoidea is monophyletic. Chirostylidae, Diogenidae and Paguridae are each paraphyletic with the incursion of Kiwaidae, Coenobitidae and Lithodidae, respectively. The placement of king crabs (Lithodidae) within the asymmetrical hermit crabs, Paguridae, is consistent with previous molecular studies (Cunningham et al., 1992; Morrison et al., 2002; Tsang et al., 2008b; Ahyong et al., 2009), though not with the conclusions of McLaughlin & Lemaitre (1997) and McLaughlin et al. (2007) based on adult morphology, and McLaughlin et al. (2004) based on larval morphology. Note, however that results of McLaughlin et al. (2004) are either inconclusive (their Figure 7) or actually show lithodids to be nested within the Paguridae (their Figure 6). Pylochelidae is polyphyletic such that the two subfamilies analyzed, Trizochelinae (represented by *Trizocheles*) and Pylochelinae (represented by *Pylocheles* and *Xylocheles*) are widely dispersed. Monophyly of Pylochelidae is strongly rejected by the SH test ($p = 0.007$) and Bayes factor (BF) (68.5). Other paguroid clades are widely dispersed and an a priori hypothesis of a

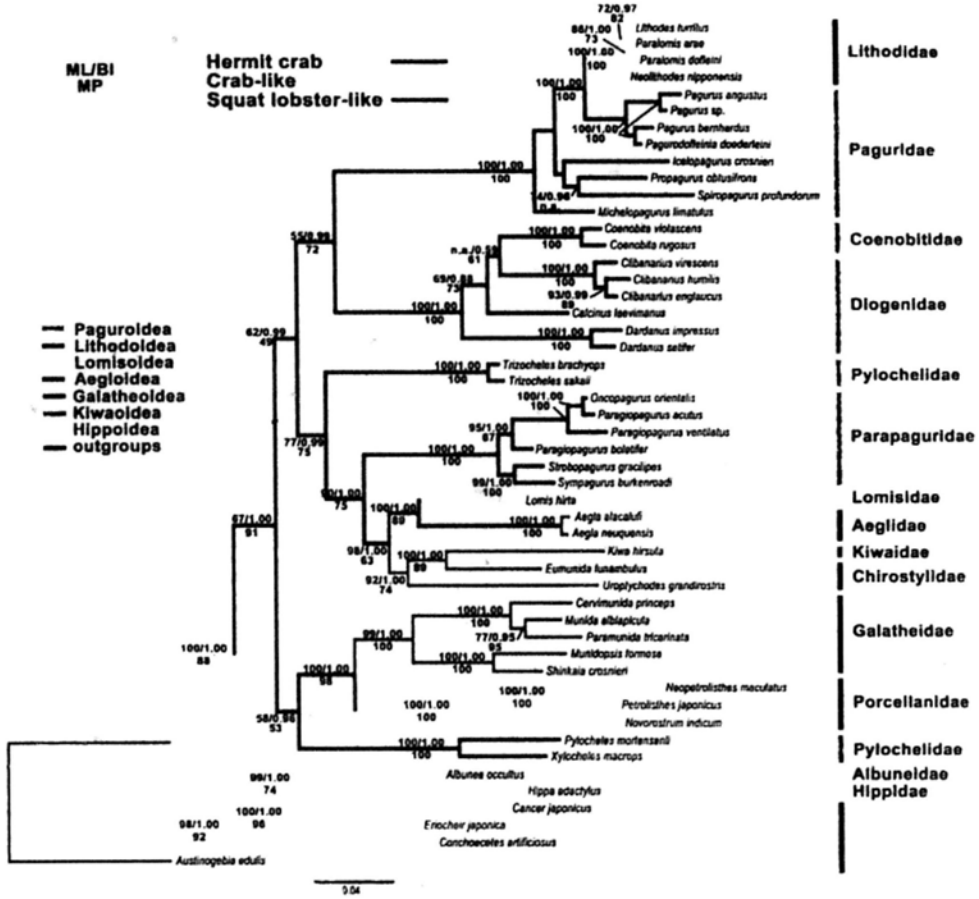

Figure 4.2. Bayesian consensus topology of the combined five-gene dataset. Nodal supports are denoted on the corresponding branches and values under 50 (for MP and ML) or 0.5 (for BI) are represented by "n.a.". The color of the branches and nodes indicate the ancestral body form inferred from BayesTraits. The superfamily and family classifications are denoted by the color bars at the right.

monophyletic hermit crab clade is significantly worse than the inferred phylogeny, irrespective of whether Lithodoidea (king crabs) is included in Paguroidea (SH test $p<0.001$, BF = 168.9) or excluded (SH test $p<0.001$, BF = 1194.8). Similarly, an alternative hypothesis of Galatheoidea monophyly is also rejected (SH test $p<0.001$, BF = 198.2). Galatheid squat lobsters and porcelain crabs (Porcellanidae) are most closely related to the symmetrical hermit crab clade Pylochelinae. The other squat lobsters (Chirostylidae, Kiwaidae and Aeglidae) and hairy stone crab (Lomisidae) are allied to the asymmetrical hermit crab clade, Parapaguridae, and the symmetrical hermit lineage, Trizochelinae.

We inferred the ancestral body form of the most recent common ancestor (MRCA) of different lineages based on the Bayesian topology. The results are indicated by the branch color in Fig. 4.2 and the probabilities of the ancestral reconstruction for the MRCA of the major clades are all over 80% except the MRCA of Anomura only with a likelihood of ~75%. The ancestral state reconstructions indicated that the hermit crab body plan evolved only once, during the divergence between Hippoidea and MRCA of the remaining anomurans, but that other body forms were derived from hermit crabs.

5.3 Discussion

5.3.1 Evolution of Hermit Crabs, Crab-like and Squat Lobster Forms

Our results not only document significant polyphyly among anomuran lineages that were long thought to be monophyletic, but they also show parallel evolution of several markedly different types of body forms in the Anomura including transitional trends towards carcinization. Moreover, each of these body forms has been derived from within clades of hermit crabs (whether symmetrical or asymmetrical). As expected, the crab-like form is achieved via a progressive broadening of the cephalothorax and shortening of the pleon, which is held partially 'tucked under', followed by a further significant reduction of the pleon, which is held fully folded under the cephalothorax. The transition proceeds from the long-tailed symmetrical hermit crab through the squat lobster or asymmetrical hermit crab form and finally to crab-like form.

The paguroids emerged early in anomuran evolution, diverging from the sister clade, Hippoidea (Pérez-Losada et al., 2002; Ahyong and O'Meally, 2004; Porter et al., 2005; Tsang et al., 2008b; Ahyong et al., 2009), by at least the early Jurassic, as evidenced by isolated fossil chelae of indeterminate familial placement. More complete hermit crab fossils, including some attributed to the symmetrical Pylochelidae, are known from the late Jurassic onwards (van Bakel et al., 2008). Galatheid squat lobsters are known from the Middle-Jurassic, chirostylids and aeglids from the Cretaceous (Feldmann

et al., 1998; Schweitzer and Feldmann, 2000), and lithodids from the Miocene (Feldmann, 1998). Thus, our results are consistent with these fossil findings, though we have not attempted to estimate the timing of hermit crab radiations. Most other major anomuran clades were probably derived from symmetrical hermit crab ancestors given that pylochelids are sister to the two major clades that contain squat lobsters and crab-like forms, and in one case also including an asymmetrical hermit crab clade (Parapaguridae). Additionally, the 'low' positions of the pylochelid clades indicate that pylochelid symmetry is plesiomorphic, rather than possibly secondarily acquired. Derivation of squat lobsters from symmetrical hermit crab ancestors is also consistent with fossil evidence. For instance, the fossil galatheid squat lobster, *Munitheites,* possesses morphological features in common with early symmetrical hermit crabs, indicating possible shared ancestry (van Bakel et al., 2008).

The *Lomis* + *Aegla* clade has been recovered by previous molecular studies (Morrison et al., 2002; Ahyong and O'Meally, 2004; Ahyong et al., 2009), though their nearest relatives have long been enigmatic; both have been variously posited as relatives of hermit crabs (and squat lobsters in the case of *Aegla*) (Martin and Abele, 1986; Pérez-Losada et al., 2002). *Aegla* resembles galatheid and especially kiwaid squat lobsters in overall body form, but its pleon is proportionally shorter and can be considered to be more highly carcinized than its marine counterparts (Fig. 4.1j). *Lomis*, the sister to *Aegla*, is strongly crab-like and is highly carcinized (Fig. 4.1q). Thus, a carcinization trend is fully consistent with a transition from

chirostylid/kiwaid and aeglid to lomisid. The common ancestor of *Lomis* and *Aegla* probably had a much reduced pleon compared to the more elongated form observed in modern Chirostylidae and Kiwaidae. Unlike most squat lobsters, which are free-living or coral associates, *Lomis* and *Aegla* live under boulders and stones, the former on intertidal rocky shores of southern Australia, and the latter in flowing freshwater creeks and streams of South America. For both animals, a short, compact pleon is probably advantageous in exploiting crevices in rocky habitats as done by other sympatric brachyuran crabs. Concordantly, the porcelain crabs, which are also derived from long-tailed ancestors, are predominantly shallow water inhabitants that also usually exploit similar habitats to *Lomis*. They may have experienced similar selective pressures as *Lomis* and *Aegla*, resulting in parallel carcinization. This phenomenon is consistent with the Morrison et al. (2002) thesis of a shallow water origin of carcinization. The multiple independent circumstances of transition offer strong evidence for the adaptive advantages of carcinization in relation to habitat type.

The king crabs (Lithodidae) are the only crab-like anomurans to be derived from asymmetrical hermit crabs (Paguridae). The porcellanids and lomisids, both of which are derived from symmetrical ancestors, retain the symmetrical pleon. Likewise, the king crabs appear to display clear traces of pagurid ancestry in pleonal and cheliped asymmetry. McLaughlin et al. (2004) argued that the pleonal asymmetry of lithodoids and paguroids is not homologous because developmental stages are not directly parallel, and the right-handedness of shared by both groups is not necessarily homologous.

However, the deeply nested position of king crabs within the asymmetrical hermit crabs strongly suggests that pleon asymmetry has homologous origins even if its precise ontogenetic expression is no longer identical to that of the common ancestor. Similarly, the phylogenetic position of the lithodoids within pagurids indicates that right-handedness is homologous. Lithodoids differ from most paguroids in having sexually dimorphic pleonal asymmetry – symmetrical in males and asymmetrical in females. It is noteworthy then that one of the 'carcinised' parapagurid hermit crabs, *Probeebei*, also exhibits sexually dimorphic pleonal asymmetry (Wolf, 1961).

5.3.2 Prevalence of Parallel Evolution in Anomura

Our phylogenetic results demonstrate that the deep sea asymmetrical hermit crab clade, Parapaguridae, is not closely related to the Paguridae and other asymmetrical hermit crabs, but closer to squat lobsters (chirostylids, kiwaids, aeglids) and crab-like lomisoids. This indicates that pleonal asymmetry and decalcification evolved independently in two different lineages, presumably to exploit ammonite shell or dextrally coiled gastropod shell habitats. Additionally, such a finding is consistent with the carcinized morphology of some very rare parapagurids, *Tylaspis* and *Probeebei*. The tendency towards acquisition of crab-like form is widespread throughout Anomura.

The squat lobster body form, exhibited by Galatheidae, Chirostylidae, Kiwaidae and Aeglidae (all formerly grouped together under

Galatheoidea), has evolved independently at least twice: once in the common ancestor of Chirostylidae, Kiwaidae and Aeglidae + Lomisidae, and once in Galatheidae. Out of these 'squat lobster' clades, two independent carcinization events have occurred: one in the porcelain crabs (Porcellanidae), which are sister to Galatheidae, and one in Lomisidae, sister to Aeglidae. The squat lobster form can be plausibly regarded as an intermediate morphology, a case of partial carcinization through the widened cephalothorax and sternal plate (in comparison to hermit crabs), and pleonal disposition, which although well-developed, is always carried folded and partially concealed by the cepahlothorax (Fig. 4.1j-m). Thus, in each case of carcinization, a transition pathway from long-tail (i.e., Pylochelidae) to squat lobster to crab-like form is consistent with the phylogeny. On the other hand, the king crab is the only crab-like anomuran to be derived from asymmetrical hermit crabs (Paguridae). In contrast to the modification of symmetrical forms, asymmetrical pleonal reduction is associated with a shift from linear to dextrally coiled carcinoecia, independently derived in Parapaguridae and remaining asymmetrical hermit crabs. The asymmetrical hermit crabs can also be considered to be partially carcinized, having undergone partial pleonal reduction. Carcinization pathways of the king crabs, are thus similar to those of symmetrically carcinized forms – a long-tailed plesiomorphic form followed by an intermediate form (pleonal reduction via adaptation to dextral shell-carrying), culminating in the crab-like form (Lithodidae). Just as the other two crab-like anomurans (Porcellanidae and Lomisidae) have symmetrical pleons, derived from symmetrical ancestors, respectively, the king crabs display clear traces of pleonal asymmetry consistent with their

pagurid ancestry.

Some authors have concluded that the 'hermit to king' hypothesis is developmentally infeasible as it would require reversal in morphology of complex characters related to dextral shell habitation, and this requires the maladaptive scenario of an asymmetrical shell-carrier to abandon the gastropod shell to expose its soft abdomen (McLaughlin and Lematire, 1997; McLaughlin et al., 2004). Yet the crab-like terrestrial coconut crab *Birgus latro* (family Coenobitidae) (Fig. 4.1e), whose nearest relatives all use gastropod shell shelters, is a good example demonstrating ontogenetic carcinization. Juvenile coconut crabs have a soft pleon like most other asymmetrical hermit crabs and reside in a gastropod shell for protection. With increasing size, the body becomes more robust and crab-like. Adult coconut crabs are free-living without dependence on a gastropod shell (Reese, 1968). Thus, within the ontogeny of a contemporary species, abandonment of the gastropod shell is already demonstrable. Furthermore, larval studies of the asymmetrical hermit crab *Clibanarius vittatus* reveal that the asymmetry is partially influenced by environment (Harvey, 1998). Juveniles are asymmetrical but in the absence of a gastropod shell, the initial asymmetry is weakened and pleonal calcification increased. The degree of pleonal asymmetry and calcification in hermit crabs is environmentally mediated (Harvey, 1998). A number of non-gastropod shell living hermit crabs of the families Paguridae (Fig. 4.1g), Diogenidae (Fig. 4.1h) and even Parapaguridae (Fig. 4.1i) that occupy cervices in coral, rock or worm tubes, bivalve and tusk shells have a symmetrical, though non-calcified, pleon.

Given that pleonal asymmetry has independent derivations within the Anomura, it is reasonable to anticipate that most, if not all, of the anomurans have retained the genetic potential for significant changes in body plan.

The independent cases of carcinization in Anomura, each with a similar possible transition series, are products of parallel evolution. This raises the question of the nature of developmental constraint in hermit crabs and allies that lead to the remarkable prevalence of parallel evolution within the group. A major question concerning the phylogenetic separation of these superficially similar groups of Anomura is whether they have arisen from convergence of different developmental pathways or through genetically homologous parallelism. Either way, body form transition is much more evolutionarily plausible than previously thought for Anomura, resulting in repeated derivation of various crab-like and squat lobster forms as well as asymmetrical forms.

The parallel derivation of multiple anomuran body types out of the hermit crabs helps account for past controversies over the sister relationships of major groups. Most non-paguroid families have, with good morphological evidence, been variously posited as sister to the hermit crabs. Little wonder that anomuran phylogeny is so contentious. Paradoxically, these contradictory hypotheses are now simultaneously plausible. With recognition that the major anomuran body forms arose from within the paguroids, it is evident all major groups of anomurans are indeed closely related to hermit crabs, just different clades of hermit crabs. Thus, rather than 'hermit to king',

evolution within the paguroids may be more aptly described as 'hermit to all', that is, to 'squatters' and 'kings'.

4.4.3 Systematic Implications

The longstanding high-level classification of Anomura dominated by Galatheoidea, Paguroidea and Hippoidea has been largely based on superficially similar body forms, though McLaughlin et al. (2007) provided further refinements. The extensive degree of parallelism in anomuran body forms, however, considerably destabilizes the current classification, chiefly the Paguroidea and Galatheoidea, neither of which are monophyletic as currently conceived. Our results indicate that the classification of the Anomura requires significant revision if it is to reflect phylogenetic relationships.

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Chapter 5

Molecular Phylogeny of the true crab, Brachyura: Origin of the Freshwater Crabs

5.1 Introduction

True crabs of the infraorder Brachyura represent one of the most diverse groups of crustaceans with almost 7000 described species in 93 families inhabiting habitats from marine, freshwater to terrestrial (Ng et al., 2008; De Grave et al., 2009). The phylogenetic relationships among the brachyuran families remain poorly understood owing to the high morphological diversity of the group. Brachyura has been divided into three sections: Podotremata, Heterotremata and Thoracotremata, according to the gonopore position (Guinot 1977, 1978, 1979). Podotremata is considered to be primitive as it retains various presumably ancestral characteristics while the Heterotremata and Thoracotremata together form the Eubrachyura with the latter being the most derived.

The monophyly of Prodotremata is, however, contentious as they are defined based on possibly pleisomorphic characters. Results of various studies based on adult and larval morphology and spermatozoa characters are mixed (reviewed by Ahyong et al., 2007; Ng et al., 2008). Ahyong et al. (2007) falsified the monophyly of Podotremata based upon their molecular phylogeny constructed with nuclear 18S gene sequences. They found that the Raninidae and Cyclodorripidae of Podotremata are more closely related to

the Eubrachyura than to the other podotremes, with the Cyclodorripdae as the sister group of Eubrachyura. This view subsequently receives support from recent morphological analysis (Scholtz and McLay, 2009). Ahyong et al. (2007) have, therefore, proposed taxonomic revision for Podotremata, which is followed by De Grave et al. (2009) in an updated classification of all extant and fossil decapods. The "Podotremata" is divided into three sections: Dromiacea, Raninoida and Cyclodorippoida.

On the other hand, the Thoracotremata is generally accepted to be monophyletic (Sternberg & Cumberlidge 2001a, b; Ng et al 2008; but see Brösing et al. 2006), probably a sister group to the Heterotremata. Yet some authors suggested that the Thoracotremata might have evolved from the paraphyletic Heterotremata and the latter is therefore a synonym of Eubrachyura (Scholtz and Richter, 1995, Sternberg and Cumberlidge, 2001a, b; Dixon et al., 2003; Brösing et al 2006). Among various heterotremes, the phylogenetic position of the freshwater crabs is undoubtedly one of the most contentious issues.

The freshwater crabs refer to the crabs that live exclusively in freshwater or terrestrial habitats and they never inbabit brackish or marine waters. They all undergo direct development in adapting to the fluctuation in availability of water. They are a very diverse group of Brachyura, with 1300+ described species distributed in five families (Pseudothelphusidae, Potamonautidae, Potamidae, Gecarcinucidae and Trichodactylidae; Ng et al., 2008; Yeo et al., 2008; Cumberlidge and Ng, 2009). The alpha taxonomy and

phylogenetic relationships among genera and species of freshwater crabs have received more attention recently due to their high diversity and conservation value (e.g. Daniels et al., 2006; Cymberlidge et al., 2008; Yeo et al., 2008; Cumberlidge and Ng, 2009; Klaus et al., 2009). Compared to our increasing understanding on their phylogeny at the generic and species level, the higher systematics of freshwater crabs is still controversial and unstable. The five families are generally considered to be divided into two major lineages: the monophyletic Trichodactylidae and a monophyletic assemblage consisting of the four remaining familes (Pseudothelphusidae, Potamonautidae, Potamidae, Gecarcinucidae). Morphological evidences point to a close affinity between Trichodactylidae and Portunoidea (Rodriguez, 1992; Sternberg et al., 1999; Sternberg and Cumberlidge, 2003) yet this hypotheis is not supported by recent molecular analysis (Schubart et al., 2009). The position of the other lineage is even more disputing. They are placed in the Heterotremata under most of the current classification schemes (Martin and Davis, 2001; Ng et al., 2008; De Grave et al., 2009), yet some authors argue that they share a number of synapomorphies with thoracotremes (Sternberg et al., 1999; Sternberg and Cumberlidge, 2001a, b). Morphological cladistic analysis further suggests that the Thoracotremata may constitute a marine sister group of the nontrichodactylid freshwater crabs and the two groups were possibly originated from some xanthoid-like progenitors (Sternberg et al., 1999). Furthermore, given the circumtropical distribution of the nontrichodactylid freshwater crabs, a single evoluationary origin would imply that the diversification and radiation of the group predated the breakup of Pangaea (~200 mya). This phylogenetic hypothesis,

however, requires an ancient origin of freshwater crabs and the Hetreotremata, which strongly contradicts the fossil records discovered so far (earliest fossil of freshwater crab dated <30 mya; Feldmann, 2007). In sum, the origin of Thoracotremata and the various freshwater crabs, and the distinction between Thoracotremata and Heterotremata remain obscure.

The morphological phylogeny of Brachyura is hampered by the large number of highly derived characters and extreme diversity of the group, whilst the molecular phylogenetic studies of Brachyura are mainly restricted to particular superfamilies/families and related taxa (e.g. Kitaura et al., 2001; Daniels et al., 2006; Schubart et al., 2006; Hultgran and Stachowicz, 2008; Wetzer et al., 2009). A comprehensive study on the overall phylogeny of Brachyura and the relationships among superfamilies and/or subsections remains lacking. The molecular study by Ahyong et al. (2007), focused chiefly on Podotremata, has included a considerate number of eubrachyuan taxa (17 families). In their topology based on 18S rRNA gene sequences, the relationships among the eubrahyuran familes are, however, poorly resolved. Many of the familes demonstrate very low interfamilial divergence in 18S gene and hence insufficient information could be provided to resolve interfamilial relationship. The mtDNA markers, on the other hand, cannot provide enough resolution concerning the higher systematics of brachyuran (e.g. Schubart et al., 2006; Wetzer et al., 2009). Hence, alternative new markers are sought to resolve the brachyuran phylogeny.

The nuclear protein-coding genes are proven to be informative in

resolving relationship across a wide spectrum of taxonomic levels, from infraordinal to inter-generic, in decapods (Tsang et al., 2008b, 2009; Chu et al., 2009; Ma et al., 2009). Recent studies also suggest their usefulness in resolving brachyuran phylogeny (Mahon and Neigel, 2008; Chu et al., 2009). Here, we attempt to reconstruct the phylogeny of Brachyura using sequences from five nuclear protein-coding genes. We aimed to resolve the following questions: 1) whether the "Podotremata" is paraphyletic; 2) whether the Heterotremata and Thoracotremata are natural groupings; and 3) the origin and sister taxa of the nontridactylid freshwater crabs.

5.2 Materials and Methods

5.2.1 Taxon sampling

Brachyura comprises of 96 extant families in 38 superfamilies (Ng et al., 2008; De Grave et al., 2009). We attempted to sample extensively from different families and genera to resolve the familial and superfamilial relationships. A total of 84 species from 44 families and 24 superfamillies, representing atmost half of the extant brachyuran families, from all of the four sections were included (Table 5.1). We attempted to analyze multiple genera from the taxonomically diverse families (e.g. Xanthidae). To evaluate the origin and phylogenetic position of nontrichodactylid freshwater crabs, we included six species from three families (Gecarcinucidae, Potamidae and Potamonautidae) in the present study. Most of the evidences suggest

Table 5.1 Classification of the species of the present study. "X" indicates obtained sequence in bold and "n.a." denotes missing sequence data.

Section	Subsection	Superfamily	Family	Species	Gene				
					enolase	GAPDH	H3	NaK	PEPCK
Dromiacea		Dromioidea	Dromiidae	*Conchoecetes artificiosus*	X	X	X	X	X
		Dromioidea	Dromiidae	*Lauridromia dehaani*	X	X	X	X	X
		Dromioidea	Dynomenidae	*Dynomene praedator*	X	X	X	X	n.a.
		Homoloidea	Homolidae	*Homola orientalis*	X	X	X	X	X
		Homoloidea	Latreillidae	*Eplumula phalangium*	X	X	X	X	X
Cyclodorippoida		Cyclodorippoidea	Cyclodorippidae	*Neocorycodus sp*	X	X	X	X	X
		Cyclodorippoidea	Cyclodorippidae	*Tymolus brucei*	X	X	X	n.a.	X
Raninoida		Raninoidea	Raninidae	*Lyreidus tridentatus*	X	X	X	X	X
		Raninoidea	Raninidae	*Ranina ranina*	X	X	X	X	X
Eubrachyura	Heterotremata	Aethroidea	Aethridae	*Aethra scruposa*	X	X	X	X	X
		Calappoidea	Calappidae	*Calappa philargius*	X	X	X	X	X
		Calappoidea	Matutidae	*Matuta planipes*	X	X	X	X	n.a.
		Cancroidea	Cancridae	*Cancer japonica*	X	X	X	X	X
		Carpilioidea	Carpilidae	*Carpilius convexus*	X	X	X	X	X
		Corystoidea	Corystidae	*Jonas distinctus*	X	X	X	X	X
		Dorippoidea	Dorippidae	*Paradorippe granulata*	X	X	X	X	X
		Dorippoidea	Dorippidae	*Heikea japonicum*	X	X	X	X	X
		Dorippoidea	Ethusidae	*Ethusa sexdentata*	X	X	X	X	X
		Eriphioidea	Eriphidiae	*Eriphia scabricula*	X	X	X	X	X
		Eriphioidea	Eriphidiae	*Eriphia sp*	X	X	X	X	X
		Eriphioidea	Menippidae	*Menippe sp*	X	X	n.a.	X	X
		Eriphioidea	Oziidae	*Epixanthus sp*	X	X	X	X	X
		Gecarcinucoidea	Gecarcinucidae	*Parathelphusa pantherina*	X	X	X	X	X
		Gecarcinucoidea	Gecarcinucidae	*Somanniathelphusa sp*	X	X	X	X	X
		Goneplacoidea	Euryplacidae	*Eucrate alcocki*	X	X	X	X	X
		Goneplacoidea	Goneplacidae	*Carcinoplax longimana*	X	X	X	X	X
		Goneplacoidea	Mathildellidae	*Mathildella rubra*	X	X	X	X	X
		Leucosioidea	Leucosiidae	*Arcania cornuta*	X	X	X	X	X
		Leucosioidea	Leucosiidae	*Tokoyo eburnea*	X	X	X	X	X
		Leucosioidea	Leucosiidae	*Urashima pustuloides*	X	X	X	X	X
		Majoidea	Epialtidae	*Doclea japonica*	X	X	X	X	X

Majoidea	Epialtidae	*Hyastenus diacanthus*	X	X	X	X	X
Majoidea	Epialtidae	*Pugettia nipponensis*	X	X	X	X	X
Majoidea	Inachidae	*Platymaia remifera*	X	X	X	X	X
Majoidea	Majiidae	*Maja squinado*	X	n.a.	X	X	X
Majoidea	Majiidae	*Mithraculus coryphe*	X	X	X	X	X
Majoidea	Majiidae	*Mithraculus forceps*	X	X	X	X	X
Orithyioidea	Orithyiidae	*Orithyia sinica*	X	X	n.a.	X	X
Parthenopoidea	Parthenopidae	*Cryptopodia fornicata*	X	X	X	X	X
Parthenopoidea	Parthenopidae	*Parthenope longimanus*	X	X	X	X	X
Pilumnoidea	Pilumnidae	*Heteropanope glabra*	X	X	X	X	X
Pilumnoidea	Pilumnidae	*Pilumnus vespertilio*	n.a.	X	X	X	X
Pilumnoidea	Tanaochelidae	*Tanaocheles bidentata*	X	X	X	X	X
Portunoidea	Portunidae	*Charybdis feriatus*	X	X	X	X	n.a.
Portunoidea	Portunidae	*Ovalipes punctatus*	X	X	X	X	X
Portunoidea	Portunidae	*Parathranites orientalis*	X	X	X	X	X
Portunoidea	Portunidae	*Portunus pelagicus*	X	X	X	X	n.a.
Portunoidea	Portunidae	*Scylla paramamosain*	X	X	X	X	n.a.
Potamoidea	Potamidae	*Isolapotamon griswoldi*	X	X	X	X	X
Potamoidea	Potamidae	*Johora tiomanensis*	X	X	X	X	X
Potamoidea	Potamidae	*Sinolapotamon anacoluthon*	X	X	X	X	X
Potamoidea	Potamonautidae	*Potamonautes perlatus*	X	X	X	X	n.a.
Retroplumoidea	Retroplumidae	*Retropluma denticulata*	X	X	X	X	X
Trapezioidea	Trapeziidae	*Philippicarcinus sp*	X	X	X	X	X
Trapezioidea	Trapeziidae	*Quadrella maculosa*	X	X	X	X	X
Trapezioidea	Trapeziidae	*Trapezia tigrina*	X	X	X	X	X
Xanthoidea	Panopeidae	*Panopeus herbstii*	X	X	X	X	X
Xanthoidea	Xanthidae	*Atergatis floridus*	X	X	X	X	X
Xanthoidea	Xanthidae	*Epistocavea cavipes*	X	X	X	X	X
Xanthoidea	Xanthidae	*Lybia tessellata*	X	X	X	X	X
Xanthoidea	Xanthidae	*Novactaea bella*	X	X	X	X	X
Xanthoidea	Xanthidae	*Palapedia sp.*	X	X	X	X	X
Grapsoidea	Gecarcinidae	*Cardisoma crassum*	X	X	X	X	X
Grapsoidea	Grapsidae	*Grapsus albolineatus*	X	X	X	X	X
Grapsoidea	Grapsidae	*Metapograpsus frontalis*	X	X	X	X	X
Grapsoidea	Plagusiidae	*Percnon sp*	X	X	X	X	X
Grapsoidea	Plagusiidae	*Plagusia squamosa*	X	X	X	X	X

	Grapsoidea	Sesarmidae	*Perisesarma bidens*	X	X	X	X	X
	Grapsoidea	Sesarmidae	*Parasesarma sp*	X	X	X	X	X
	Grapsoidea	Varunidae	*Eriocheir japonica*	X	X	X	X	X
	Grapsoidea	Varunidae	*Gaetice depressus*	X	X	X	X	n.a.
	Grapsoidea	Varunidae	*Helice formosensis*	X	X	X	X	n.a.
	Grapsoidea	Varunidae	*Hemigrapsus penicillatus*	X	X	X	X	X
	Grapsoidea	Varunidae	*Metaplax longipes*	X	X	X	X	X
	Ocypodoidea	Dotillidae	*Dotilla myctiroides*	X	X	X	X	n.a.
	Ocypodoidea	Dotillidae	*Scopimera bitympana*	X	X	X	X	X
	Ocypodoidea	Dotillidae	*Scopimera intermedia*	X	X	X	X	X
	Ocypodoidea	Dotillidae	*Scopimera proxima*	X	X	X	X	X
	Ocypodoidea	Dotillidae	*Tmethypocoelis ceratophora*	X	X	X	X	X
	Ocypodoidea	Macrophthalmidae	*Macrophthalmus erato*	X	X	X	X	n.a.
	Ocypodoidea	Mictyridae	*Mictyris brevidactylus*	X	X	X	X	X
	Ocypodoidea	Ocypodidae	*Ocypode ceraphthalma*	X	X	X	X	X
	Ocypodoidea	Ocypodidae	*Ocypode sinensis*	X	X	X	X	X
	Ocypodoidea	Ocypodidae	*Uca crassipes*	X	X	X	X	X
Outgroup	Aegloidea	Aeglidae	*Aegla alacalufi*	X	X	X	X	X
	Hippoidea	Hippidae	*Hippa adactylus*	X	X	X	X	X
	Lithodoidea	Lithiodidae	*Neolithodes nipponensis*	X	X	X	X	X
	Lomisoidea	Lomisidae	*Lomis hirta*	X	X	X	X	X

Anomura asthe sister group of Brachyura, together forming the Meiura (Scholtz and Richter, 1995; Dixon et al., 2003; Ahyong and O'Meally, 2004; Tsang et al., 2008b). Hence, four species of "crab-like" anomurans, *Aegla alacalufi* (Aeglidae), *Hippa adactylus* (Hippidae), *Lomis hirta* (Lomisidae), *Neolithodes nipponensis* (Lithodidae) were used as outgroups.

5.2.2 Sequences collection

Total genomic DNA was extracted from pleopod or pereiopod of the target species using the commercial QIAamp Tissue Kit (QIAGEN). Primers for amplifying the GAPDH, PEPCK, NaK and enolase were the same as those listed in Chapter 4 (see Table 4.2) while the primers for amplifying H3 gene were based onColgar et al. (1998). The amplifications were conducted in a reaction mix containing 1-5 μl of template DNA, 1X PCR reaction buffer, 3 mM $MgCl_2$, 200 nM of each primer, 200 μM dNTPs, 1.5 units of *Taq* polymerase (Amersham) and ddH_2O to a total volume of 50 μl. The PCR profiles were as follows: 3 min at 94°C for initial denaturation, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50-60°C depending on the primers for 30 s, elongation at 72°C for 1.5 min, and a final extension at 72°C for 10 min. The PCR products were then purified using the QIAquick gel purification kit (QIAGEN) according to manufacturer's instructions. Sequencing reactions were carried out using the same sets of primers and the ABI Big-dye Ready-Reaction mix kit, following the standard cycle sequencing protocol. The products were analyzed using an Applied

Biosystems (ABI) 3100 automated sequencer.

5.2.3 Phylogenetic analyses

Sequences were aligned using CLUSTAL W (Thompson et al., 1994) with default parameters and confirmed by translating into amino acid sequences. The total dataset was analyzed using maximum likelihood (ML), and Bayesian inference (BI) analyses. ML analysis was implemented with RAxML 7.0.3 (Stamatakis, 2006). The model GTRGAMMAI was used for the five partitions (genes), with individual α-shape parameters, GTR-rates and base frequencies estimated and optimized for each partition. We conducted 1,000 BP runs and searched for the best-scoring ML tree. Bayesian analysis was conducted using MrBayes v.3.12 (Ronquist and Huelsenback, 2003) with the best-fit models of nucleotide substitution for individual genes determined by Modeltest 3.7 (Posada and Crandall, 1998). GTR+I+G was selected for all five genes. Four 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 the analysis. Chains were sampled every 500 generations. Convergence of the analyses was validated by the standard deviation of split frequencies and monitoring the likelihood values over time graphically using Tracer v1.4 (Rambaut and Drummond, 2007). The trees prior to the achievement of stationarity of the log likelihood values (2,000 trees) were discarded as burn-in. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PP).

5.3 Results

5.3.1. Sequence variation

The combined dataset consisted of 2,312 bp from five gene fragments (Table 5.3). ML and BI analyses of the combined dataset resulted in topologies without highly conflict nodes (ML BP > 70 or BI PP > 0.95) and some of the nodes are recovered with strong support in both trees. We present the nodal supports obtained from the two analyses together on the BI topology (Fig. 5.1).

5.3.2 Higher-level relationships

The Brachyura is strongly supported to be monophyletic (Fig. 5.1), corroborating the results of previous studies (Scholtz and Richter, 1995; Dixon et al., 2003; Ahyong and O'Meally, 2004; Porter et al., 2005; Ahyong et al., 2007; Tsang et al., 2008b). The former Podotremata (currently Dromiacea, Raninoida and Cyclodorippoida) is shown to be paraphyletic in congruent with the molecular study by Ahyong et al. (2007) using the nuclear 18S gene sequence. All the four sections proposed by Ahong et al. (2007) and De Grave et al. (2009), Dromiacea, Raninoida, Cyclodorippoida and Eubrachyura are monophyletic. The Dromiacea is the most basal brachyuran lineages, while Cyclodorippoida is the sister taxon of Eubrachyura. This is consistent with the recent molecular (Ahyong et al., 2007) and morphological

Table 5.2 Summary of parsimony results

	length	no. of variable site	no. parsimony informative sites	A/T %
NaK	594	293	243	47.7
PEPCK	549	277	241	36.4
enolase	339	162	141	52.7
GAPDH	534	231	200	46.2
H3	294	117	107	40

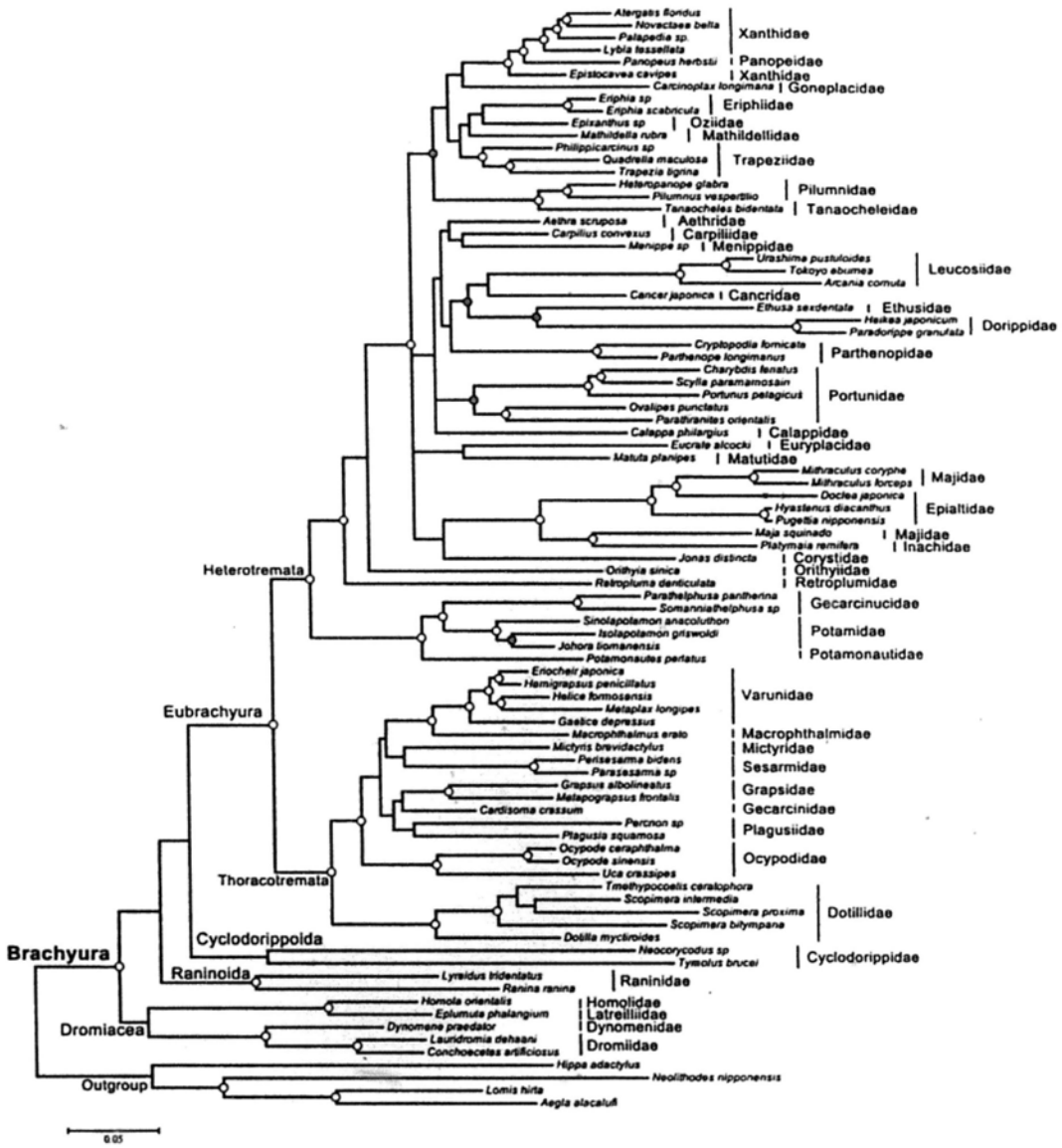

Fig. 5.1 Bayesian inference topology from the five genes combined. The nodes strongly supported by both ML (BP≥70) and BI (PP≥0.95) are indicated by open circle; nodes only strongly supported by one of the analyses are indicated with solid grey circle. The family classifications of the species are indicated on the right-hand side with the sections denoted on the corresponding branches. Please refer to Table 5.1 for superfamily classification of the species based on De Grave et al. (2009).

evidence (Scholtz and McLay, 2009). Moreover, the reciprocal monophyly of the two subsections of the Eubrachyura, Heterotremata and Thoracotremata, are confirmed with strong nodal supports (Fig. 5.1).

5.3.3 Superfamily- and family-level relationships

Most of the families with multiple exemplars included are shown to be monophyletic with the exception of Xanthidae, Inachidae and Majidae (Fig 5.1). The Xanthidae is paraphyletic with the incursion of Panopeidae. On the other hand, the Epialtidae and Majidae are found to be polyphyletic assemblages in their present composition and the genera from the two familes and are mixed by themselves and with Inachidae (represented by *Platymaia remifera*). In contrast to the families, the superfamily classification appeared to be more problematic. Calappoidea, Eriphioidea, Goneplacoidea, Ocypodoidea and Grapsoidea are all found to be polyphyletic while Potamoidea is paraphyletic with respect to Gecarcinucoidea. Only Dromioidea, Homoloidea, Dorippoidea, Majoidea, Plumnoidea and Xanthoidea are recovered to be monophyletic.

The six nontrichodactylid freshwater crab exemplars clustered together to form a monophyletic assemblage with strong nodal support. They align with other heterotremes and appeared to be the basal lineage with Heterotremata.

5.4 Discussion

In the present study, we have attempted to construct a robust phylogeny for the Brachyura based on the most comprehensive dataset, both in terms of taxon sampling and molecular markers employed, to date. In spite of poorly resolved internal relationships within the Eubrachyura, the topology has provided new insights into the evolution and systematics of the Brachyura.

5.4.1 Confirmation of the paraphyly of Podotremata and the section status for Dromiacea, Raninoida and Cyclodorippida

Our inferred topology is largely congruent with previous molecular work by Ahyong et al. (2007) in revealing the "Podotremata" as a paraphyletic group, with Raninidae and Cyclodorippidae being more closely related to the Eubrachyura with the latter two being sister taxa. The monophyly of Podotremata has long been contentious and there is accumulating evidence from morphological studies arguing for the paraphyly of the group (Brösing et al., 2002, 2006; Scholtz and McLay, 2009). In the most recent comprehensive revision of the classification of all brachyuran species, Ng et al. (2008) provisionally retains the usage of Podotremata but in a subsequent updated classification of all Decapoda genera, De Grave et al. (2009) follow the suggestion by Ahyong et al. (2007) in abandoning the Podotremata and recognizing the three sections, Dromiacea, Raninoida and Cyclodorippoida, of the former Podotremata. In the previous study by Tsang

et al. (2008b) which has employed two nuclear protein-coding involved in the present study, they recover a monophyletic Podotremata in contrast to the current topology. Yet the support is low and the number of podotremes analyzed is small, in particular missing the Cyclodorippidae. In the present study, we have expanded the taxon and gene sampling and our inferred phylogeny strongly supports the identity of the three new sections, instead of Podotremata.

5.4.2 Monophyly of Heterotremata and Thoracotremata

In the present study, we provided the first molecular evidence to demonstrate the Heterotremata and Thoracotremata are natural monophyletic assemblages. The nontrichodactylid freshwater crabs, despite sharing many characters with the thoracotremes, are shown to be more closely related to Heterotremata than to the Thoracotremata.

The monophyly of Heterotremata is challenged by cladistic analyses on morphological characters (Scholtz and Richter, 1995, Sternberg and Cumberlidge, 2001a, b; Dixon et al., 2003; Brösing et al., 2006). In this point of view, the modifications of thoracic sternum observed in thoracotremes are possibly driven by adaptation to better locomotion, and the two subsections therefore represent two extremes with a series transitional forms (Magalhães and Türkay, 1996; Sternberg and Cuberlidge, 2001b). The two subsections are, indeed, characterized respectively by two distinct morphological types, coxal male sexual apertures and sternal male sexual aperture, and no

intermediate form could be found (Sternberg and Cumberlidge, 2001b). The reciprical monophyly of the two subsections indicates that the two forms male sexual aperture arose independently in the Heterotremata and Thoracotremata, so that neither of them is the precursor of the other. This character is an apparent apomorphy to define the two groups. The previous hypothesis of a heterotreme origin of Thoracotremata is probably attributed to convergence in morphology. It is not surprising that given the high diversity and overlapping in ecological niches of many brachyurans, they would have evolved similar features. However, the present study does not include a few problematic taxa, such as the Hexapodidae and Pinnotheoroidea, the placement of which in the Heterotremata or Thoracotremata is controversial. Future study on these taxa would be fruitful to validate the distinction between Heterotremata and Thoracotremata.

5.4.3 Origin and phylogenetic position of the freshwater crabs

The phylogenetic position of nontrichodactylid freshwater crabs has long been contentious (Sternberg and Cumberlidge, 1999, 2001a, b; Sternberg et al., 1999; Ng et al., 2008; Cumberlidge and Ng, 2009). The present molecular study provides the first strongly supported topology that confirms the potamoids and gecarcinucoids are heterotremes and they diverged from the other major lineages in the early radiation of brachyurans. The ancient origin and high level of morphological convergence presented might explain the failure and difficulties in previous attempts on identifying the marine sister group of nontrichodactylid freshwater crabs. We have not

included the Pseudothelphusidae in the present study yet a majority of studies generally support the close affinity of Potamoidea and Pseudothelphusidae (Sternberg and Cumberlidge, 1999, 2001a; Daniels et al., 2006; Klaus et al., 2006). Therefore, it is reasonable to anticipate that they would cluster with the potamoids and gecarcinucoids and the circumtropically distributed nontrichodactylid freshwater crabs share a single common ancestor.

The tempo of nontrichodactylid freshwater crab divergence is no less controversial as its phylogenetic placement. Some authors have postulated the origin of freshwater crabs to exceed 120 mya (Ng and Rodriguez, 1995; Ng et al., 1995). Yet this hypothesis is challenged by other researchers since this would probably imply that the diversification of these freshwater crabs might probably predate the radiation of Heterotremata, or that the Brachyura as a whole is a much more ancient group. The oldest fossil of freshwater crabs is relatively recent, dated back to the Miocene (25-30 mya; Glaessner, 1969; Feldmann et al., 2007). The Heterotremata probably has undergone a post-Cretaceous radiation (Schram, 1986) and hence it is suspected that the freshwater crab diversified at ~ 30-65 mya. Although we have not attempted to calibrate the divergence time of the freshwater crabs, they apparently diverged from the other heterotremes in the early stage of brachyuran radiation. Brachyura contains one of the oldest decapod fossils, *Imocaris tuberculata*, dated back to Carboniferous (~300-350 mya), suggesting the ancient origin of this group. Furthermore, Porter et al. (2005) estimated the Majoidea (a heterotreme) originated at ~240 mya, based on fossil calibration of a molecular inferred topology. If the divergence time

estimated is close to the reality, the divergence time of nontrichodactylid freshwater crabs would precede that time accordingly. This would fulfill one of the critical prerequisite for the single origin of all the nontrichodactylid freshwater crabs that distributed on all continents except the Antarctica, a divergence time predating 200 mya, when the Pangaea broke up and isolated different lineages.

Most of the molecular phylogenetic studies on the freshwater crabs revealed that the phylogeny of the crabs strongly reflects the geological history of the region (Daniels et al., 2006; Klaus et al., 2009; Shih et al., 2009). Morphological characters, on the contrary, are less informative to the evolutionary history of the crabs and exhibit high level of convergence (Daniels et al., 2006; Klaus et al., 2009). Our gene tree shows that the Potamidae is more closely related to the Gecarcinucidae than to the Potamonautidae from the same superfamily. The Potamidae and Gecarcinucidae overlap in their distribution to a large extent in the Asian region while the Potamonautidae is restricted to the Afrotropical area. Therefore, our results provide further evidence supporting the importance of geology, over morphology, in the evolution of freshwater crabs. This also supports the proposal by Klaus et al. (2009) to put all Old World freshwater crabs into one singe superfamily, Potamoidea.

5.4.4 Implications to superfamilial and familial classification

The taxonomy of Brachyura has been revised and refined

continuously in recent years based upon studies on adult and larval morphology, molecular evidence and spermotzoa structure, etc. (reviewed in Ng et al., 2008). Unfortunately, the highly derived characters in many brachyurans humper the identification of synapomorphies and the inference of phylogenetic relationships among familes/genera. Therefore, many controversies remain to be settled. From the inferred gene tree in the present study, we attempt to evaluate the validity of recent changes in the brachyuran systematics.

5.4.4.1 Dromiacea

Within the Dromiacea, the familial relationship inferred in the present study is highly concordant with the molecular phylogeny built upon the nuclear 18S sequence by Ahyong et al. (2007). The Homolidae and Latreillidae are sister families while Dromiidae aligns with Dynomenidae, and the two lineages together form the monophyletic Dromiacea. However, Dromiidae and Homolidae are shown to be paraphyletic due to the incursion of Latreillidae and Dynomenidae in the 18S gene tree (Ahyong et al., 2007). In the present molecular phylogeny, the Homolidae is only represented by a single taxon so that the monophyly of the group cannot be determined. We recover the reciprocal monophyly of Dromiidae with strong statistical support. Yet we could not obtain sequences from *Hypoconcha*, the basal dromiid in the topology of Ahyong et al. (2007).

5.4.4.2 Xanthoidea sensu lato

The composition and taxonomy of Xanthoidea has been revised substantially over the years (Števčić, 2005; Karasawa and Schweitzer, 2006; Ng et al., 2008). A number of other familes, including Carpiliidae, Eriphidae, Goneplacidae, Hexapodidae, Menippidae, Pilumnidae, and Trapezidae, were once placed in the Xanthoidea until recently (Martin and Davis, 2001; Števčić, 2005; Karasawa and Schweitzer, 2006) but have been elevated and/or removed to other superfamilies (Ng et al. 2008; De Grave et al., 2009). Most of these families cluster into a big clade with moderate nodal support in the present gene tree. This provides the first clear molecular evidence for the close affinity of the Xanthoidea *sensus lato* families. Xanthoidea *sensus stricto* currently comprises of three families, Xanthidae, Panopeidae and Pseudorhombilidae. We have included two families in the present study and showed that they form a strongly supported monophyletic assemablge. Yet Panopeidae is nested within other xanthids,

Considering the validity of the newly raised superfamilies, a monophyletic Pilumnoidea is recovered. The sister relationship of Eriphidae and Oziidae only receive low statistical support and furthermore, the remaining Eriphiodea family analyzed in the present study, Menippidae, is distantly related to the other two. Accordingly, the monophyly of Eriphiodea in its current composition is obscure. Similarly, the three goneplacoid families examined are dispersed in the tree. As noted by Ng et al. (2008), the monophyly of Goneplacoidea is uncertain and the relationship among its

families remain poorly understood as many genera possess very unique features that make them warrant family status and difficult to align with each other. Therefore, our gene tree generally supports the reappraisal of most of the superfamilies proposed by Ng et al. (2008), yet further refinement is apparently needed, especially for Goneplacoidea.

5.4.4.3 Majoidea

Comprising of more than 800 extant species, majoids are a diverse group of brachyurans (Ng et al., 2008, De Grave et al., 2009). Although the monophyly of the group as a whole is generally accepted (reviewed in Ng et al., 2008; but see Brösing et al., 2006), many of the families within this superfamily is poorly defined due to a lack of thorough studies on the Indo-Pacific genera (reviewed in Ng et al., 2008). In the preswnt study, we corroborate the monophyly of Majoidea, indicating that the terminal moult upon maturity and highly shortened larval development are synapomorphies of the group. However, the reciprocal monophyly of the majority of the majoid families are falsified. Other recent molecular studies also found that most of the majoid families are, indeed, polyphyletic (Hultgran and Stachowicz, 2008; Hultgren et al., 2009). This suggests prevalence of convergence in the group and therefore the adult morphological characters currently used to unite different familes are invalid. On the other hand, the larval characters appear to be more congruent with the molecular phylogeny (Hultgran and Stachowicz, 2008; Hultgren et al., 2009). Nevertheless, given the diversity of the majoids, it is no doubt that more extensive analyses, in

particular on the Indo-Pacific genera, are needed for further taxonomic revision.

5.4.4.4 Grapsoidea and Ocypodoidea

Schubart et al (2000) presented the first molecular examination on the phylogeny of Grapsidae based on taxa collected from North America. The mitochondrial 16S rRNA gene tree reveals that the Gecarcinidae is closely related to grapsoid subfamilies and the former grapsid subfamilies Grapsinae, Plagusiinae, Sesarminae and Varuninae should be given full family ranking accordingly. The Gecarcinidae and the newly described Glyptograpsidae Schubart, Cuesta & Felder, 2002 were placed within the Grapsoidea. The superfamily Gecarcinoidea thereby lost its validity. Their suggestions were followed by Martin and Davis (2001) in their updated classification of Crustacea. Moreover, these authors included the Mictyridae, which had for a while been considered part of the Grapsoidea (see Bowman & Abele 1982), in the superfamily Ocypodoidea. The Ocypodoidea on the other hand, according to Martin and Davis (2001), still consists of a large and diverse family Ocypodidae with four subfamilies Dotillinae, Heloeciinae, Macrophthalminae and Ocypodinae. Yet this classification is challenged by subsequent molecular phylogenetic studies by Kitaura et al. (2002) and Schubart et al. (2006). Despite minor differences in the arrangements of some clades, their topologies consistently show that both Ocypodoidea and Grapsoidea are polyphyletic and families/subfamilies from the two intermingle. Summing up all these gene trees, Schubart et al. (2006) argued

against the traditional use of the Grapsoidea and Ocypodoidea as monophyletic superfamilies and treated the constituent families separately. Ng et al. (2008), however, doubted this argument as some assemblages in the gene trees strongly contradict results from morphological analyses. Thus, they have kept the two superfamilies, but give full family ranking to the former subfamilies of Ocypodidae.

In the present study, we confirm the results of previous mtDNA analyses that the two superfamilies are polyphyletic in their current composition. Some groupings revealed by Schubart et al. (2006), for example Varunidae + Macropthalmidae, is recovered in the present tree with strong support, suggesting that the overall congruence of the topology from molecular analyses using different markers. Moreover, recent molecular studies using 16S gene showed that subfamily Asthenognathinae of the Pinnotheridae is closely related to the Varunidae (Palacios-Theil et al., 2009), while Cryptochiridae may be a close ally of Grapsidae (Wetzer et al., 2009). These further challenge the validity of the Ocypodoidea and Grapsoidea. Yet the resolution concerning the interfamilial relationships remains low so that it is premature to draw any conclusion on taxonomic revision. Therefore, further studies using combined mtDNA and nuclear markers with comprehensive taxon sampling would be essential to obtain a well-resolved, robust phylogeny for a consensus on the evolutionary history and taxonomy of the Thoracotremata.

Chapter 6

General Conclusion

The phylogenetic relationships of the decapods are as contentious as ever. Despite increasing volumes of both morphological and molecular data being brought to bear on the issue, consensus is yet to be reached. In the present study, I present the first significant application of nuclear protein-coding gene sequences to high-level decapod phylogeny. A particular advantage of these gene sequences is their ease of alignment. Topologies derived from different protein-coding gene markers are highly congruent among themselves and well supported under various analytical approaches (ML, MP and BI). Moreover, they are highly informative for phylogenetic reconstruction across all taxonomic levels of the Decapoda, from infraordinal to interspecific relationships, as illustrated in the previous chapters on different decapod taxa.

Whereas the topologies remain to be corroborated by future studies, especially identification of synapomorphies of various clades, several significant phylogenetic hypotheses concerning higher decapod phylogeny are proposed: that Stenopodidea is sister to Caridea; that the old taxon, Macrura Reptantia, might be valid; that the Thalassinidea is polyphyletic and that the stem lineage reptants are thalassinidean-like. The phylogenetic positions of selected controversial taxa (e.g., Polychelidae, Enoplometopidae) are also well resolved in the gene trees.

Several controversies concerning the evolution of selected decapod groups have been resolved in the present study. The spiny lobsters are found to be originated from shallow water instead of deep sea as previously proposed. Moreover, the stridulating organ has only evolved once in the diversification of spiny lobsters. An even more surprising finding from the present study is that not only the king crabs, but also the other anomurans, including squat lobsters, porcelain crabs and hairy stone crab were all evolved from hermit crab-like ancestor. All of the asymmetric hermit crabs, the squat lobster-like and crab-like forms evolved at least twice from the symmetric hermit crab ancestors, thus indicating the unexpected high evolutionary flexibility of the hermit crab body ground plan.

The present study also provides the first molecular evidence for the monophyly of two brachyuran subsections: Heterotremata and Thoracotremata, and confirms the paraphyly of the Podotremata as shown in a previous molecular study. More importantly, I reveal that all the Old World freshwater crabs represent a monophyletic assemblage, which has been diverged from the other major lineages at the early stage of brachyuran evolution. This may partially explain why it has been difficult to identify the marine sister of freshwater crabs, simply because there is no any particular family closely related to them. Thus, these new gene markers have provided us with many new insights in the evolution of decapods and are promising for future multi-locus studies on phylogenetic reconstruction of decapods.

Results from the present study demonstrate that a large number of potential candidate genes in the genome remain unexplored in evolutionary studies. It is anticipated that this study will initiate the discovery and application of more protein-coding genes for phylogenetic analysis of the Decapoda. However, a few issues remain to be settled. First, the high genetic divergence among the target organisms and the degenerate nature in the third codon position remain the major obstacle in primer design for PCR amplification, and therefore the wide application of these markers. Taxon specific primers may be the most suitable solution toward the problem once we have built a more comprehensive sequence database. Another critical issue is, whether there is any paralog of these protein-coding gene markers present in the decapod genome. This will have significant implication in the currently inferred phylogeny. Although I do not detect any strong signal of the presence of any paralogs, further studies are necessary to to confirm that the gene markers used are single-copied. Once these uncertainties could be solved, the use of these genes as the basic repertoire in the phylogenetic toolkit in analyzing decapod relationships represents a major step towards our goal in assembling the tree of life for Decapoda.

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