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Decapod Crustacean Phylogenetics

edited by

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Preface

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Decapods are undoubtedly the most recognizable of all crustaceans. The group includes the well-known "true" crabs (Brachyura), hermit crabs and their relatives (Anomura), shrimps (Dendrobranchiata, Caridea, and Stenopodidea), and lobsters (Astacidea, Thalassinidea), among other lesser known groups. They are the most species-rich and diverse group of the Crustacea, which in turn is the fourth largest assemblage or clade of animals (behind insects, mollusks, and chelicerates) on Earth (e.g., Martin & Davis 2001). Currently, the Decapoda contains an estimated 15,000 species, some of which support seafood and marine industries worth billions of dollars each year to the world's economy. Decapods also are the quintessential group of crustaceans in the public eye. Perhaps more than any other group of marine invertebrates, the crabs, lobsters, and shrimps that make up the Decapoda are familiar to nearly everyone.

In part because of the popularity of the decapods, there has been a long-standing interest in their relationships. Over the years, hypotheses of decapod relationships have relied on sources of information as varied as behavior (such as the early split between swimming or "natant" decapods and crawling or "reptant" forms), adult morphology, larval morphology, and, in more recent years, molecular sequence data. Despite these efforts, we remain largely in the dark as to the evolutionary relationships of the major decapod clades and to the relationships of decapods to other groups of crustaceans. Although there is no shortage of publications reflecting the wide variety of ideas and hypotheses concerning decapod phylogeny, there is also no obvious consensus among carcinologists working today. Additionally, prior to January 2008, the world's leading decapodologists had never assembled with the sole purpose of elucidating relationships among the major decapod lineages and between decapods and other crustaceans.

Toward rectifying this deficit, several key decapod workers (Keith Crandall at Brigham Young University (team leader), Joel Martin at the Natural History Museum of Los Angeles County, Darryl Felder at the University of Louisiana Lafayette, and Rodney Feldmann and Carrie Schweitzer at Kent State University) were funded by the National Science Foundation's "Assembling the Tree of Life" program beginning in the fall of 2005 to work toward elucidating the evolutionary relationships of the decapods. That team has been in contact with other decapod researchers all over the world, many of whom have been supplying fresh and preserved material or fossil material for our combined analysis while also collaborating on a variety of component phylogenetic studies focused on decapods. In short, interest in decapod evolution currently is at an all-time high, with most of the world's carcinologists aware of the ongoing Tree of Life project and eager to contribute in some way.

In January 2008, carcinologists from throughout the world convened at a symposium hosted by the Society of Integrative and Comparative Biology and The Crustacean Society in San Antonio, Texas, in order to (1) present methodological updates for research on the diversity and relationships (phylogeny) of the decapods, (2) present overviews on our understanding of the systematics and

relationships within some of the major decapod clades, and (3) work toward assembling and coding molecular and morphological characters toward an overall decapod phylogeny. Invited participants represented a wide variety of backgrounds and included established decapod workers as well as beginning students of decapod phylogeny. Attendees represented fourteen nations (Australia, Belgium, Brazil, China, England, France, Germany, Japan, the Netherlands, New Zealand, Singapore, Spain, Taiwan, and the United States). The chapters that follow are based on contributions to that symposium and on a few additional manuscripts from workers who could not be present at the San Antonio meeting.

The aforementioned meeting on the phylogeny of decapods, as well as this resulting volume, might seem premature at this point, not only because so much remains unknown in general but also because our Tree of Life group is still actively researching the question of decapod evolution from many different angles. Indeed, one of our primary goals is to produce a better-resolved phylogeny of the entire Decapoda than has been published to date. However, the symposium was seen as important for bringing together a majority of the world's preeminent workers, some of whom had not previously met, and for establishing our current state of knowledge with regard to the three major areas outlined above. Thus, the contributions contained herein range rather widely in scope. Some are state-of-the-art reviews of large bodies of literature and/or methodologies for elucidating decapod phylogeny (e.g., Schram on the fossil origin of decapods, Asakura on the evolution of mating and its bearing on phylogeny, Schubart on mitochondrial approaches, Scholtz on decapod "evo-devo" studies, Tudge on decapod spermiocladistics, Palero & Crandall on phylogenetic inference). Others are somewhat preliminary attempts to construct the first known phylogenetic tree for a given group of decapods (e.g., Tavares et al. on the Dendrobranchiata, Tshudy et al. on clawed lobsters, Palacios-Theil et al. on pinnotherid crabs). Several contributions present the most comprehensive analyses to date on major clades of decapods (e.g., Bracken et al. on carideans, Ahyong & Schnabel on anomurans, Robles et al. on thalassinideans, Breinholt et al. on the diversification of the crayfishes, Hultgren et al. on the crab superfamily Majoidea). Still others present data or approaches that, although not widely applied to studies of decapod evolution previously, could be used eventually to help elucidate the phylogeny of the Decapoda (e.g., Porter & Cronin on the evolution of visual elements, Bokyo & Williams on the use of decapod parasites as phylogenetic indicators). All told, we feel that the 29 contributions contained herein constitute both a fascinating overview of where we are currently in our understanding of decapod phylogeny and a tantalizing promise of what's to come.

Many people and several societies participated in supporting the symposium and/or the publication of the resulting volume, and we are indebted to all of them. For financial support of the symposium itself (including the publication of this volume), we thank the U.S. National Science Foundation (NSF grant DEB 072116), the Society of Integrative and Comparative Biology (SICB), the SICB Divisions of Invertebrate Zoology and Evolutionary and Systematic Biology, the American Microscopical Society, the Crustacean Society, and the Society of Systematic Biologists. The decapod crustacean Tree of Life project is also supported by the National Science Foundation via a series of collaborative grants to K. A. Crandall (team leader) and Nikki Hannegan (DEB 0531762), D. L. Felder (DEB 0531603), J. W. Martin (DEB 0531616), and R. Feldmann and C. Schweitzer (DEB 0531670). Our institutions (JWM: Natural History Museum of Los Angeles County; KAC: Brigham Young University; DLF: University of Lousiana, Lafayette) supported us in kind by providing space and facilities for editing the volume and by underwriting some of the research on which it is based. We are extremely grateful to the many conscientious referees who contributed their time to review the chapters on our behalf. Our promise of anonymity prevents us from listing them individually here. We especially thank Dr. Stefan Koenemann, editor of *Crustacean Issues*, for his invitation to publish the proceedings as part of that series and for his help in editing the volume, and John Sulzycki, Senior Editor of CRC Press / Taylor & Francis, for his encouragement and assistance at several stages. We also thank Paul Martin for his invaluable

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I OVERVIEWS OF DECAPOD PHYLOGENY

On the Origin of Decapoda

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ABSTRACT

We do not have stem forms in the fossil record for Decapoda, unlike what we have for some groups of crustaceans. Thus, we currently lack a clear understanding concerning the origin of the decapods based on concrete data. Furthermore, several problem areas present themselves: 1) lack of consensus on the sister group to Decapoda, 2) the advanced nature of known Paleozoic decapods, 3) a restricted paleobiogeographic and paleoecologic distribution of these fossils, and 4) possibly incorrect assumptions about what a decapod ancestor should look like. For now the situation seems hopeless, although new data, new lines of evidence, and new perspectives might provide better insight some time in the future.

1 INTRODUCTION

Decapoda stands as one of the most diverse orders of crustaceans in terms of expressed variations on its body plan. That plan includes a carapace fused to the underlying thoracic segments, the first three pairs of thoracopods modified as maxillipeds [and thus their name, "deca"-"poda," for their five pairs of pereiopods], a pleon of six segments, and frequently (but not always) a tail fan including a well-developed telson and uropods. It is a very distinctive and easily recognizable body plan. Yet the origin of the order remains obscure. Indeed, comprehending the origin of any crown group is tied to the recognition and interpretation of its stem forms. In order to offer some promise of success, that task requires preservation of such forms in the fossil record.

It is not an unreasonable hope on our part to expect to find such fossils. For some groups of crustaceans, we do in fact possess sufficient knowledge. An example occurs in the unipeltate stomatopods, the mantis shrimp, a group of crustaceans that also exhibit a highly derived, quite distinctive (one might even say extreme) body plan. Calman (1904) recognized mantis shrimp as so idiosyncratic he erected a separate superorder, Hoplocarida, to accommodate them. Unipeltata, the crown stomatopods, have a modest fossil record that indicates the major superfamilies have Mesozoic origins (Hof 1998; Schram & Müller 2004). However, in recent years sufficient fossils in the Paleozoic have come to light that present a transition series that relates to the crown group Unipeltata (Schram 2007). We effectively now have stem forms that allow us to perceive how Unipeltata evolved.

However, no such array of fossil stem taxa exists as yet that would allow us to probe the earliest evolution of Decapoda. Indeed, what we encounter is a series of problems that obscure the ancient derivations of this important order.

2 PROBLEM ISSUES

I perceive four major areas of concern. These are: 1) no clear consensus about a sister group to Decapoda [and thus no guidance to orient us toward recognizing or interpreting possible stem forms], 2) the rather derived nature of the currently known Paleozoic decapod fossils, 3) a conundrum

concerning the paleobiogeography and paleoecology of Paleozoic malacostracans, and 4) possibly incorrect assumptions concerning an "ancestor" and thus misleading hypotheses about what we might be looking for in a stem form. Let us examine each of these in turn.

2.1 *Sister group to Decapoda*

Ever since the first cladistic analysis of eumalacostracan relationships, the issue of the identity of the sister taxon to Decapoda has presented almost too many options. Schram (1981, 1984) found that his shortest trees had the decapods in a clade with Amphionidacea and Euphausiacea, and these in turn had syncarids as a sister group. However, some of the trees had unresolved polychotomies among the major clades. Many researchers consider that Euphausiacea serves as a sister taxon; Calman (1904) assumed such when he placed Euphausiacea and Decapod together within his superorder Eucarida. Some more recent cladistic analyses indeed recovered such an arrangement, e.g., Wills (1998). However, as in Schram (1984), Amphionidacea appeared as the immediate sister group of Decapoda in the analysis of Richter & Scholtz (2001: fig. 7), but in their analysis Euphausiacea emerges as well-embedded within a group they named Xenommacarida, a clade that contains all the other eumalacostracans.

Hence, while Eucarida often finds expression in the cladograms of eumalacostracan relationships, it is not a particularly robust arrangement. In some ways, the amphionidaceans might serve as a stem form, often emerging from phylogenetic analyses between the decapods and the krill. Amphionidaceans do possess a nicely developed maxilliped, and the second and third thoracopods are miniature versions of the more posterior thoracopods but are widely separated from the maxilliped. However, other aspects of their body habitus isolate Amphionidacea as a unique taxon (see Schram 1986).

Schram & Hof (1998) in some of their cladograms obtained a pattern wherein an array of the Late Paleozoic "eocarids," e.g., Belotelsonidea (Fig. 1A) and Waterstonellidea (Fig. 1B), emerge in sister status to decapods (sometimes in combination with Euphausiacea). However, perhaps one should first ask just what is an "eocarid." The group at one time found expression as a formal taxon (Brooks 1962b), but the concept has entailed problems. First, the assemblage is a hodgepodge of often incompletely preserved forms, e.g., lacking complete sets of limbs such as *Eocaris oervigi* Brooks, 1962 (Brooks 1962a: fig. 1C), and *Archangeliphausia spinosa* Dzik, Ivantsov, & Deulin, 2004 (Dzik et al. 2004: fig. 2A). Second, Brooks' definition of the order is ambiguous ["Length of thorax reduced, caridoid facies" (Brooks 1962b: 271)], and the list of implicit characters implied by "caridoid facies" is composed of plesiomorphic features. Third, some of the taxa placed within the order have proven to be highly specialized in their own right, e.g., Belotelsonidea and Waterstonellidea. Finally, some species once placed in the group have proven to be members of other higher taxa. For example, *Palaeopalaemon newberryi* (see below) was once assigned to the eocarids (Brooks 1962b) but has proven to be a true decapod (Schram et al. 1978). Other eocarid taxa yet might be reassigned to more clearly defined groups; for example, the genus *Eocaris* is probably an aeschronectidan hoplocarid, and I suspect that *Archangeliphausia* from the Devonian of northwestern Russia may in fact represent an early eucarid (see below). Hence, the concept of "eocaridacea" is meaningless, a grade rather than a clade, and should not be used.

In regard to the origin of Decapoda, all this is unfortunate. Without a clear consensus on a sister group, we can neither reliably deduce the ground pattern for Decapoda nor derive any well-grounded hypotheses concerning an ancestral form.

2.2 *Paleozoic fossils*

A complicating factor in deducing the origins of the decapods resides in the rather derived state of the known Late Paleozoic decapod fossils. Indeed, the earliest definite decapod, the Late Devonian lobster-like *Palaeopalaemon newberryi* Whitfield, 1880 (Fig. 2), is a species that is clearly a reptant

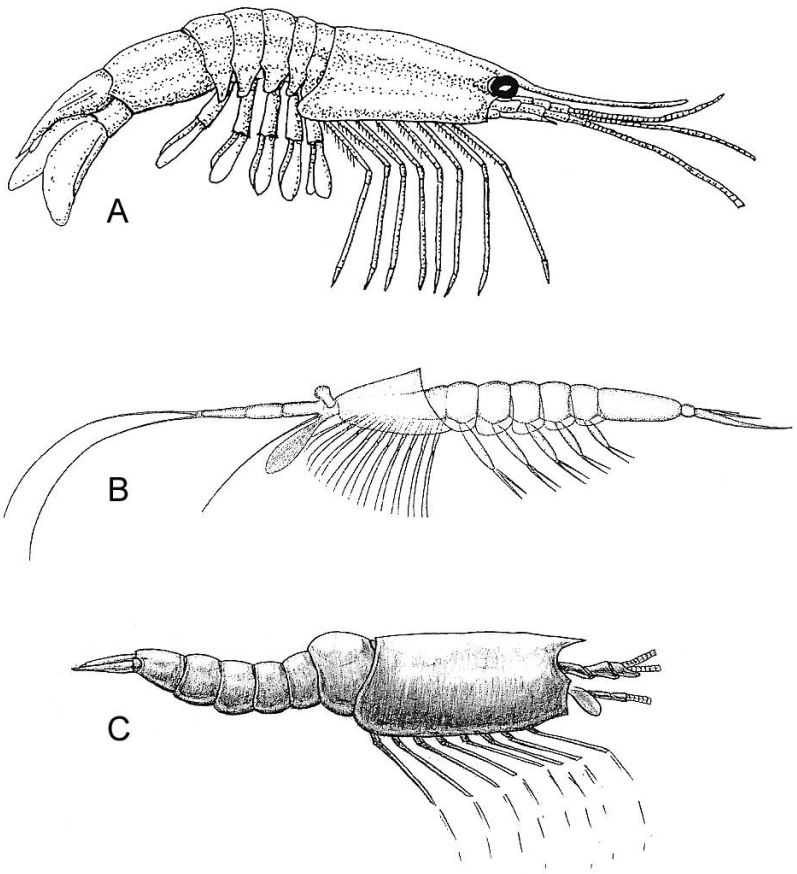

Figure 1. Examples of Late Paleozoic "eocarids." (A) *Lobetelson mclaughlinae*, a Middle Pennsylvanian belotelsonid (from Schram 2007). (B) *Waterstonella grantonensis*, the Lower Carboniferous waterstonellid (from Briggs & Clarkson 1983). (C) *Essoidea epiceron*, a Middle Pennsylvanian eumalacostracan of uncertain affinities (from Schram 1974).

(Schram et al. 1978; Hannibal & Feldmann 1984) and that in at least one analysis (Schram & Dixon 2005) emerges high in the decapod tree in a polytomy with Achelata, Anomura, and Brachyura. In any case, it is much too advanced a member of Reptantia to tell us much about decapod origins, let alone be considered an ancestor.

Another intriguing fossil is the Carboniferous genus *Imocaris* Schram & Mapes, 1984 (Fig. 3). Two species are recognized, *I. tuberculata* and *I. colombiensis.* Schram & Mapes (1984) assigned *Imocaris* to Dromiacea, i.e., suggested it belonged among podotreme brachyurans. However, only carapaces are known of this genus, and Racheboef & Villarroel (2003) chose to place *Imocaris* among the pygocephalomorph peracaridans. Resolving the affinities of *Imocaris* is a problem. The pygocephalomorphs bear a single cervical groove on the anterior part of their carapace, and the pattern in *Imocaris* appears more complex, with at least two. In addition, pygocephalomorphs typically bear a long and prominent rostrum, which *Imocaris* lacks. The species of *Imocaris* have a rather ornamented surface, such as one finds in some pygocephalomorphs such as *Tealliocaris* and *Pseudotealliocaris*, but ornamentation is a secondary feature and not particularly useful in phylogenetic comparisons. I still prefer a dromiacean assignment for *Imocaris*, but I am willing to consider other

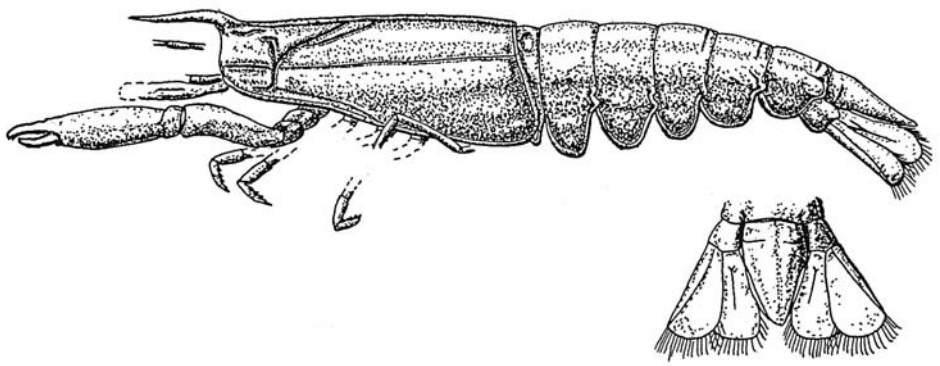

Figure 2. Late Devonian *Palaeopalaemon newberryi*, a reptant lobsteroid (modified from Schram et al. 1978; Hannibal & Feldmann 1985).

affinities for it, even with some group other than decapods or pygocephalomorphs. In any case (dromiacean, pygocephalomorph, or some other taxon), *Imocaris* tells us little about decapod origins.

One other set of fossils to consider consists of certain burrows in the Carboniferous of North America; Hasiotis (1999) believes crayfish made these. His interpretation focused on the markings on the walls of these burrows, which led him to conclude that these resemble similar features made by living crayfish in their burrows. There are no actual body fossils recovered from these tunnels. If these burrows do prove to be those of crayfish, they would again only record the presence of yet another rather derived form of reptantian in the Late Paleozoic.

The fossil record for the other major suborders of decapods essentially begins in the Mesozoic. The earliest members of Dendrobranchiata appear during the Triassic (see Garassino & Teruzzi 1995; Garassino et al. 1996), but a good fossil record for the group does not occur until the Jurassic Solnhofen Limestone (see Glaessner 1969). Fossils of Caridea are scarce; the earliest members apparently occur in the Jurassic, although those fossils are poorly preserved and of uncertain affinities (see Glaessner 1969). Reliably identified caridean fossils, however, do appear in the Cretaceous (Bravi & Garassino 1998a, 1998b; Bravi et al. 1999; Garassino 1997) with at least two families (Palaemonidae and Atyidae) represented there. Finally, Stenopodidea until recently had a problematic fossil record; Schram (1986) tentatively suggested that the Lower Jurassic form *Uncina posidoniae* might bear some relationship to the suborder. Subsequently, an apparent spongicolid, *Jilinocaris chinensis*, was identified from the Cretaceous of northern China (Schram et al. 2000), and a stenopodid, *Phoenice pasinii*, occurs in the Cretaceous of Lebanon (Garassino 2001). All of these Mesozoic decapods are more or less easily recognized members of their suborders and have nothing to tell us about decapod origins.

There are some puzzling Devonian fossils that have been recently recognized and bear consideration. Dzik et al. (2004) described *Archangeliphausia spinosa* from the Early Devonian of northeastern-most Europe (Fig. 4A). The fossils lack any preserved thoracic limbs. Nevertheless, the material suggests that the carapace was fused to the underlying thoracic segments. The fossils are flexed ventrally, but the carapaces do not appear to be lifted off the underlying thoracomeres. Furthermore, the segmental boundaries between the thoracic segments are preserved only ventrolaterally and do not extend to include the dorsal tergites—just what one would expect if the carapace were fused to the thoracomeres. The telson is not of the narrow, elongate, subtriangular form we associate with euphausiaceans and dendrobranchiates, but rather resembles the sub-quadrate form we often see in reptantians. I believe *Archangeliphausia spinosa* might in fact be at least a eucarid,

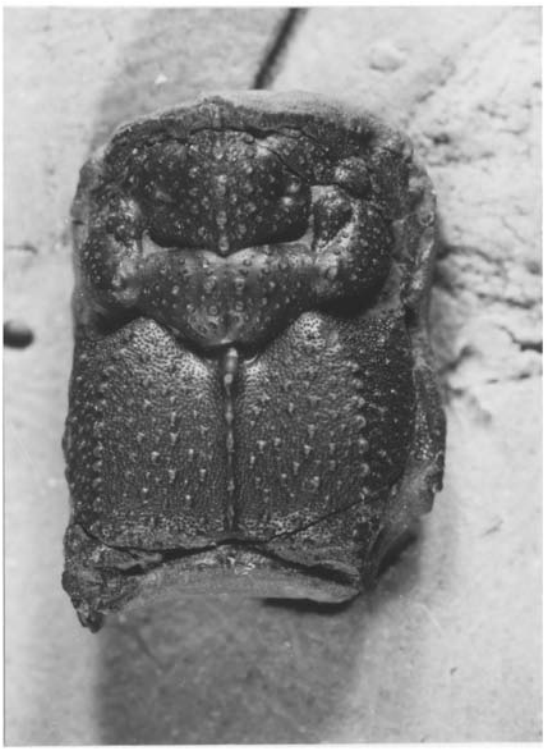

Figure 3. Lower Carboniferous *Imocaris tuberculata*, a probable dromiacean (from Schram & Mapes 1984).

and possibly another example of an advanced reptant decapod. We must wait for the collection of fossils with a full set of thoracic limbs.

Finally, another rather well-preserved, middle Paleozoic eumalacostracan is *Angustidontus seriatus* Cooper, 1936. Several species of *Angustidontus* occur in the Late Devonian and early Carboniferous across North America and Europe, and illustrate the difficulties entailed in studying early malacostracans. Originally, only the remarkable terminal segment of the maxilliped was known, and this was interpreted as a jaw of a fish. Rolfe & Dzik (2006) assembled a more extensive collection from Poland and in combination with previously collected material managed to definitively reconstruct this species as eumalacostracan (Fig. 4B). They compared *Angustidontus seriatus* to *Palaeopalaemon newberryi* and even suggested a possible synonymy of these taxa. However, *P. newberryi* is an entirely different animal, clearly a reptant decapod with the first pereiopods bearing chelate claws and the second through fifth pereiopods as walking limbs (Fig. 2). In contrast, *A. seriatus* has seven pairs of rather robust pereiopods and an elongated specialized maxilliped, a distinctly dissimilar body habitus with its singular pair of maxillipeds. What is *Angustidontus*? If we try for a link with decapods, *A. seriatus* evokes Amphionidacea with the first thoracopods as maxillipeds. *Angustidontus*, however, would seem to be a specialized benthic form rather than a mesopelagic creature like *Amphionides*. An alternative assignment of *Angustidontus* might be within Lophogastrida because *A. seriatus* has rather wide thoracic sternites, not unlike those seen in *Gnathophausia* and the pygocephalomorphs. However, no indication of fossilized oöstegites was noted on any of the fossils studied, structures that are known to occur on pygocephalomorph fossils. The wide thoracic sternites on *A. seriatus* might be akin to such sternites seen in decapods such as Achelata. Thus, whether *Angustidontus* is an early eucarid is not certain.

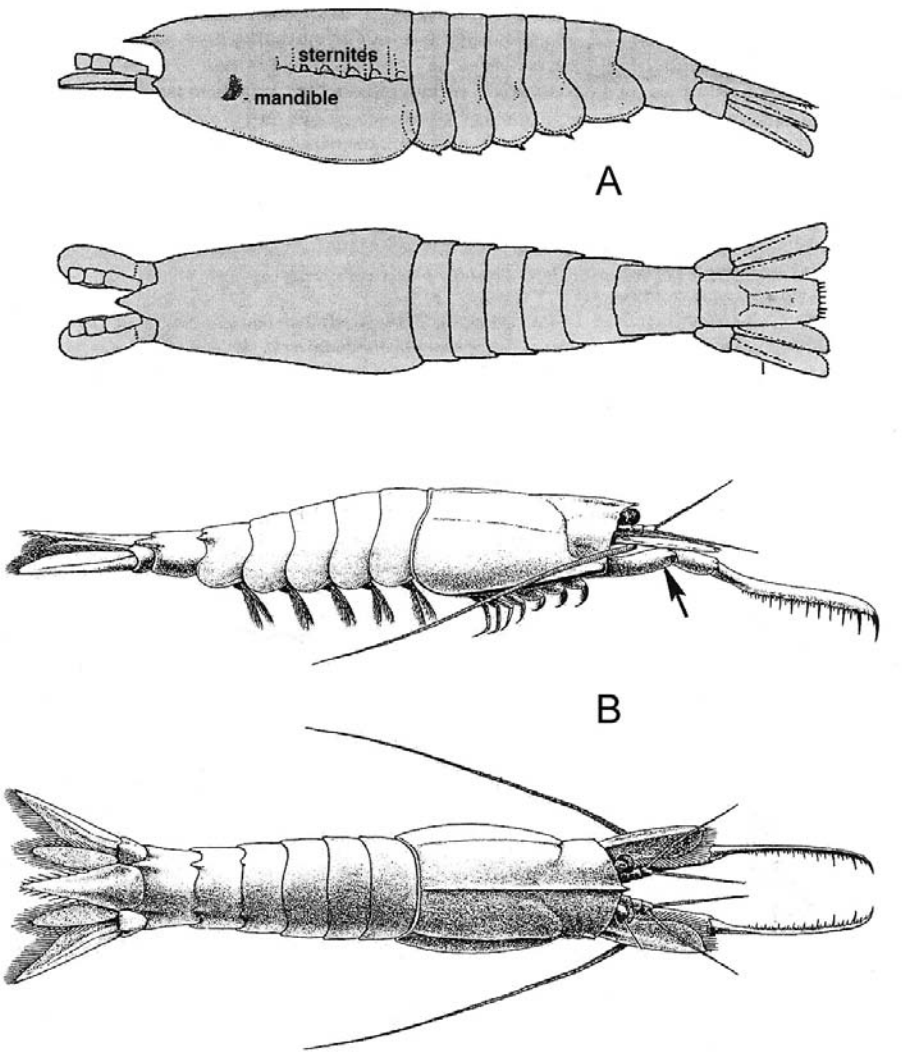

Figure 4. Lateral and dorsal reconstructions of Devonian eumalacostracans of uncertain affinities. (A) *Archangeliphausia spinosa*, a possible eucarid (modified from Dzik et al. 2004). (B) *Angustidontus seriatus*; note the large, specialized maxilliped [arrow] (modified from Rolfe & Dzik 2006).

In summary, while the fossil record of the Paleozoic decapods has interesting fossils, at present they tell us little about the origins of the group. The apparently derived nature of *Palaeopalaemon*, and possibly *Imocaris*, does indicate that there possibly was a long history of the order that extended back in time before the earliest fossils in the Late Devonian. *Angustidontus* and *Archangeliphausia* are intriguing in that they appear to indicate occurrences of at least eucarids, if not clear stem decapods, and hold out a promise of even earlier fossils relevant to decapod origins. How far back? Ordovician? Silurian? Cambrian? We cannot now say.

2.3 *Paleobiogeography and paleoecology*

One might feel better about this record if we saw an abundance of fossils from a wide array of localities across the world. However, as is the case for eumalacostracans and hoplocaridans as a whole, the Late Paleozoic record of the decapods has been up to now almost completely restricted to the equatorial island continent of Laurentia (Schram 1977). The Late Devonian *Palaeopalaemon newberryi* occurs in several localities across Ohio and Iowa. The Carboniferous *Imocaris tuberculata* was collected from Arkansas. A singular exception to this Laurentian pattern is *I. colombiensis*, which comes from what is now western Colombia on the Paleozoic continent of Gondwana. However, this site is not far paleogeographically from Arkansas during a time in which the continents were beginning to come together to form Pangaea. In a sense, it is the exception that proves the rule, since Schram (1977) postulated that a dispersal of higher malacostracan crustaceans out from Laurentia began with the formation of Pangaea. Nevertheless, compared to other malacostracans in the late Paleozoic, such as the hoplocaridans and peracaridans, the decapods have a paltry record.

Thus, what we have are three species that are decapods (possibly four, counting the elusive crayfish), from a handful of localities—clearly something is missing.

For instance, where were the decapods before the Devonian, assuming there was not a punctuation event in the Devonian or Late Silurian? The early and middle Paleozoic arthropods of the epicontinental seas of the world are not scarce. The diverse record of the trilobites needs no comment, but there was also an abundant array of xiphosurans, eurypterids, and thylacocephalans in those times. The latter two groups were effective predators. It is tempting to speculate that such an assortment of arthropods simply filled in most of the available niches on the epicontinental seas of those times. Thereafter, the late Devonian through Permian record of malacostracans is marked by an abundance of groups such as Hoplocarida, Syncarida, Peracarida (especially Pygocephalomorpha), Belotelsonidea, and Waterstonellidea. Was there too much competition from these diverse forms to allow the decapods to get established on the epicontinental seas of Laurentia? Such a conclusion would seem peculiar, since we live in a time when decapods have so completely dominated their habitats. Was it an instance of first come, first served?

Of course there are lots of places in the early and middle Paleozoic world where decapods might have lived. The decapods could have been denizens of the deep sea; the Panthallasic and Tethys Oceans were extensive. Or, taking a clue from the amphionidaceans, the decapods of that time may have been in the pelagic realm. Or, it is possible that decapods inhabited extremely cryptic habitats on the continents themselves such as interstitial, groundwater, and cave habitats. In regards to this last possibility, we should not overlook that small, cryptic forms were often important in the origin and early evolution of many groups, even phyla such as the mollusks (Mus et al. 2008). Discovery of the right sort of Lagerstätte in the pre-Devonian might provide us some material of significance in this regard.

2.4 *Incorrect assumptions concerning "ancestors"*

Implicit in all of the above is an assumption that a decapod "ancestor" will essentially be a caridoid with a well-developed pleon of 6 (maybe 7) somites, a carapace fused to the thorax, at least some kind of incipient specialization of the anterior thoracopods towards a maxillipedal condition, and

eggs shed freely into the water column. Such an animal, or series of animals, might yet emerge. We do have fossils of caridoids such as *Archangeliphausia, Belotelson, Essoidea, Lobetelson, Waterstonella,* and others, but as mentioned above just what some of these fossils represent is not always clear.

Another deeply embedded assumption about the evolution of Malacostraca is that the 7-segment pleon of the phyllocarids was in some way the precursor of the 6-segment pleon of hoplocaridans and eumalacostracans. However, this supposition seems quite unwarranted. For example, Scholtz (1995) clearly showed in the crayfish *Cherax destructor* that the expression of *engrailed* (a marker for segment boundaries in the arthropod trunk) displays nine, rather than six (or even seven), *engrailed* stripes in the pleon. The ninth stripe is faint and quickly fades to leave eight stripes; the sixth through eighth eventually merge to produce the final 6-segment pleon of the crayfish.

Moreover, this is not a unique pattern. Knopf et al. (2006) recorded in the early development of the amphipod *Orchestia cavimana* eight clearly delineated segmental blocks of cells in the early differentiation of the pleon. In fact, the eighth *Anlage* gives rise to a pair of lateral bulges, and as the seventh and eighth somites are slowly incorporated into the growing sixth pleomere, the bulges continue to grow into distinct lobes that migrate dorsad and mediad to eventually form the so-called bifurcated telson. The adult amphipod pleon clearly begins as a series of eight segmental units.

Finally, in four species of the hermit crab genus *Porcellanopagurus*, a peculiar condition is seen in the urosomal region (cf. McLaughlin 2000). For example, in *P. nihonkaiensis* (Fig. 5), an elongate area of non-sclerotized cuticle separates the tergite of the sixth pleomere and the small telson (Komai & Takeda 2006). This region is clearly not a proximal section of the telson, which retains its characteristic form. From consideration of the larval development of *Porcellanopagurus*, it is obvious that the anus appears initially on the ventral surface of the telson *Anlage* and migrates to a terminal position by the adult stage; hence, this non-sclerotized region has nothing to do with the telson. McLaughlin (personal communication) thinks that this area might somehow be a posterior extension of the sixth pleomere. A similar arrangement is seen in some species of *Solitariopagurus*. Nevertheless, such an extension of a sixth somite posterior to the attachment of the pleopods would be unique. So, what is this? Might this non-sclerotized region be a vestige of additional somites between the sixth pleomere and the telson? The only data that might speak against this as a remnant of such somites are that the area grows in size with growth of an individual. In the examples cited above from *Cherax* and *Orchestia*, the tissues attributed to the putative seventh and eighth somites decrease in size and disappear as the individuals grow. As an alternative hypothesis to consider, I suggest that this tissue does represent remnants of post-sixth somite pleomeres and is worthy of further investigation.

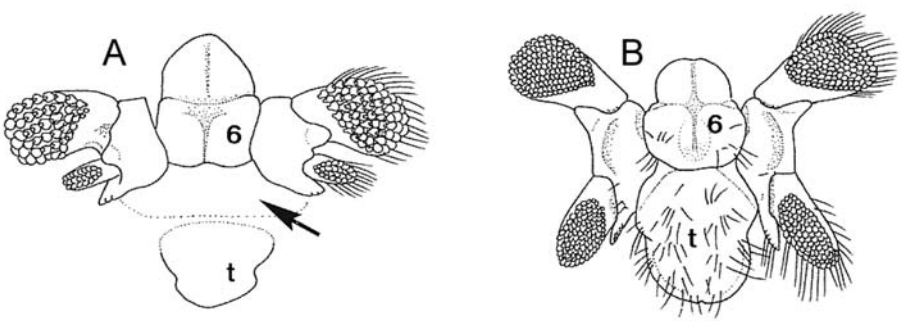

Figure 5. Pleon terminus of pagurid hermit crabs of the genus *Porcellanopagurus* (from Komai & Takeda 2006). (A) *P. nihonkaiensis*; note non-sclerotized region [arrow] between uropod-bearing sixth pleomere [6] and telson [t]. (B) *P. japonicus*, with a more typical anatomy of the urosome.

Just how all this impinges on ground patterns within Eumalacostraca is not clear at this time. However, instead of a 7-to-6 pattern long assumed to be the case, there are now alternative hypotheses to be entertained, viz., 8-to-7-to-6, or even separate scenarios of 8-to-7 and 8-to-6. What is clear is that we should not be surprised to find somewhere in the early or middle Paleozoic fossils of eumalacostracan-like creatures with more than the "expected" number of pleomeres.

Another line of evidence that impinges on hypotheses about ancestors arises from a consideration of the central nervous systems of various arthropods. Harzsch (2004) summarizes a series of detailed investigations of brain anatomy. Characteristic patterns of olfactory-globular tracts with chiasmata, olfactory neuropils with glomeruli, and lateral mechano-sensory antenna 1 neuropils suggest a set of synapomorphies shared by Malacostraca and Remipedia. A set of further unique features in regard to the specializations of the protocerebrum and the enervation of the compound eyes draws Hexapoda into this clade. These latter characters would seem to exclude at least the living remipedes, but it is quite possible the fossil enantiopodan remipedes, such as *Tesnusocaris goldichi*, which had very well-developed compound eyes, possessed protocerebral chiasmata as well. Since this complex CNS anatomy could be interpreted as too complicated to be anything other than shared apomorphies, those groups that possess these features might be related. That would mean that the insects, malacostracans, and remipedes form a monophyletic clade, with remipedes and malacostracans as sister groups.

This is a fascinating hypothesis, and it parallels the independent analysis of Schram & Koenemann (2004), which focused on matters of *Bauplan* in crustaceans such as locations of gonopores, *Hox*-gene expression, and numbers and types of trunk segments. They, too, obtained from their cladistic analysis a pattern wherein Remipedia emerged as the sister group to Malacostraca, as well as the core Maxillopoda. In the Schram & Koenemann scenario, we could envision an ancestor with a 16-segment trunk that gave rise to a more derived form bearing an 8-segment thorax and 8-segment pleon, which in turn laid the ground pattern for a line leading to malacostracans.

How all this might bear on the origins of decapods I don't know. On the one hand, the decapods probably emerged after the events suggested above. On the other hand, what comes early has to affect what comes later, and clearly what we had always assumed about caridoid ancestors must be tempered by what we know now. Perhaps we should be willing to consider a non-caridoid ancestor for decapods with weak differentiation between anterior (thorax) and posterior (pleon), a pleon with more than 6 somites, with incipient differentiation of the anterior three thoracopods (putative maxillipeds), and from a cryptic habitat such as groundwater or caves.

3 CONCLUSIONS

It would have been nice to suggest a simple little scenario here for the origin of Decapoda with a sequence of fossils at hand that would fill in the details. Unfortunately, this is not now the case. Even when we have such details, such as that seen in the wide array of Paleozoic pre-mantis shrimp relevant to scenarios about the origins of unipeltate Stomatopoda, the pattern derived is not entirely straightforward. In that example, Schram (2007) could arrange the fossils in a row wherein the increasing specialization and enlargement of the ballistic second maxilliped could be explained. However, the actual cladistic analysis of all the scored characters on these fossils indicated that this expected straight-line pattern had to be tempered by information related to the parallel evolution of the stomatopod pleon, and especially the telson.

One has to take the data as they present themselves. I suspect that while we can hope to see fossils someday that display a series of specializations of the maxillipeds toward a decapod condition, we may have to moderate our expectations. As in the stomatopods, we might have to take into account the evolution of the pleon and its urosome, or even some other aspects of the decapod body plan, to arrive at a complete understanding of the origins of this fascinating group.

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I wish to thank Dr. Pat McLaughlin for showing me the wonders of hermit crab morphology and for reading an early version of the text and making some constructive comments. Prof. Rod Feldmann and Dr. Carrie Schweitzer convinced me that one should express some caution about the possible affinities of *Imocaris.*

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Decapod Phylogenetics and Molecular Evolution

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ABSTRACT

Decapoda is the most species-rich group of crustaceans, with numerous economically important and morphologically diverse species leading to a large amount of research. Our research groups are attempting to estimate a robust phylogeny of the Decapoda based on molecular and morphological data to resolve the relationships among the major decapod lineages and then to test a variety of hypotheses associated with the diversity of decapod morphological evolution. Thus, we have developed a database of molecular markers for use at different scales of the evolutionary spectrum in decapod crustaceans. We present potential mitochondrial and nuclear markers with an estimation of variation at the genus level, family level, and among infraorders for Decapoda. We provide a methodological framework for molecular studies of decapod crustaceans that is useful at different taxonomic levels.

1 MOLECULAR TAXONOMY

There are several competing hypotheses concerning the relationships of the major lineages of Decapoda based on morphological estimates of phylogeny. Early taxonomy of the decapods was largely based on the mode of locomotion; taxa were divided into the swimming lineages (Natantia) and the crawling lineages (Reptantia) (Boas 1880). Morphological and molecular studies suggest Natantia is paraphyletic; it is presently classified based on gill structure (Burkenroad 1963, 1981) dividing Decapoda into the suborders Dendrobranchiata (penaeoid and sergestoid shrimps) and Pleocyemata (all other decapod crustaceans). Relationships within Pleocyemata are still controversial and remain unresolved. As morphological data, both recent and fossil, and genetic data continue to accumulate, we are moving towards phylogenetic resolution of these controversial relationships. Here we present a progress report for the Decapoda Tree of Life effort and the tools with which we will continue our analysis of decapod crustacean phylogenetic relationships.

Several recent hypotheses based on combined analysis of morphological and molecular data or molecular data alone suggest that resolving the systematics of this group is a difficult task (see Fig. 1). There is agreement among these studies that Dendrobranchiata represents a basal lineage within the decapod crustaceans and that within Pleocyemata the Caridea and Stenopodidea are basal infraorders (Porter et al. 2005; Tsang et al. 2008). Molecular research also supports the removal of polychelids from Palinura following Scholtz and Richter (1995) and its establishment as a separate infraorder (Polychelida) (Tsang et al. 2008; Ahyong this volume). Relationships among reptant decapods remain unresolved by the addition of molecular data. Several recent phylogenetic analyses incorporating mitochondrial and nuclear data (Robles et al. this volume) or nuclear data alone (Tsang et al. 2008; Chu et al. this volume) suggest Thalassinidea are not monophyletic but rather may represent several infraorders. The timeline of diversification among the reptant decapods or specifically whether Astacidea (Porter et al. 2005) or the Anomura/Brachyura lineages (Ahyong & O'Meally 2004; Tsang et al. 2008) are the most recently derived lineages remains a question of interest.

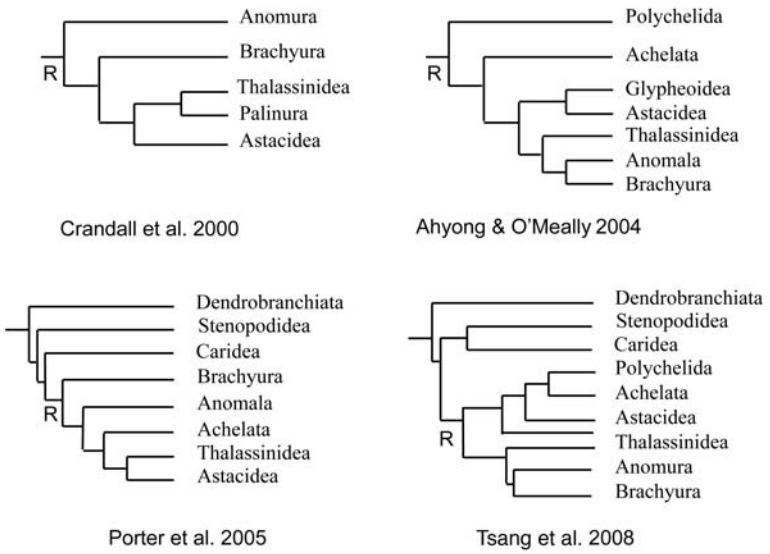

Figure 1. Hypotheses of decapod evolutionary relationships based on molecular data. R shows the position of the reptant decapods.

2 DEVELOPING GENETIC MARKERS FOR MOLECULAR PHYLOGENY

The order Decapoda includes roughly 175 families (extant and extinct) and more than 15,000 described species. Complicating things further are the estimated 437 million years since the origin of the Decapoda with the major lineages estimated to have been established by 325 million years ago (Porter et al. 2007). Constructing a molecular phylogeny across such breadth of taxa and depth of timescale requires serious consideration of markers that have enough variation to reconstruct relationships at the fine scale (at and within the family level) as well as being conservative enough to be used across infraorders representing these deeper timescales. Our approach is to accumulate molecular sequence data for different gene regions including both mitochondrial and nuclear genes, coding and non-coding. In this way, we will be able to maximize data at deeper nodes where alignment of sequence data is most difficult while retaining information among families and between the most recently diverged taxa.

There are two molecular approaches to amplifying sequence data for use in phylogenetic studies. (1) Isolation of RNA from tissues, coupled with reverse transcription-polymerase chain reaction (RT-PCR) to amplify target genes or gene fragments, reduces problems associated with amplification of pseudogenes (non-coding duplicated gene segments) and sequencing through large introns. The main limitation of RNA work is that fresh tissues, or at least tissues collected in an RNA preserving agent such as RNA*later*, require rapid transfer to $-80°$C storage. (2) Phylogenetic work using genomic tissue extractions and amplifications is still favored over RNA techniques due to lower costs, ease of field sampling, and the ability to use previously collected specimens in ethanol. To reduce the risk of sequencing multiple copy genes or pseudogenes, gene fragments are first cloned to identify the number of copies that a primer set amplifies. Although this is not the focus of this paper, in the course of looking for useful phylogenetic markers, we have sequenced a number of multigene families such as hemocyanin, actin, and opsins. These markers may be phylogenetically useful if a single gene is isolated and amplified. They also have many uses when looking at genome evolution and the expression of these genes in Decapoda (e.g., Porter et al. 2007; Scholtz this volume). However, one must be certain that the same copy is being amplified across taxa for useful phylogenetic results.

Introns or highly variable regions need to be considered when sequencing as they can be large (greater than 1000 base pairs in length) and include repeat regions in some taxa, making amplification and sequencing difficult. Often there is too much variation in the intron among taxa to be aligned and included in the analysis. Introns can be avoided by first identifying their position and then designing primer sets within the exon to remove the introns. Here we redesigned primers for elongation factor 2 (EF-2) and transmembrane protein (TM9sf4) to exclude regions of high variability of approximately 300 base pairs in EF2 and 500–1000 base pairs in TM9sf4. Although this reduced the total length of sequence amplified, the highly variable regions produce a greater noise-to-signal ratio at the higher phylogenetic relationships, our principal focus. Of course, these more variable introns might become very useful for population genetic and species level phylogenetic work, and we continue to explore their utility at these lower levels of diversity.

3 THE GENES AND THEIR DIVERSITY

3.1 *Mitochondrial genes: 12S, 16S, and COI*

Mitochondrial ribosomal genes 12S and 16S and coding genes such as COI have been extremely useful in population genetic and systematic studies. Mitochondrial markers have been favored in studies for several reasons (see Schubart, this volume, for details and proposed primer sets for decapod mtDNA amplification). The high copy number of mitochondria in tissues makes them relatively easy to isolate. They are haploid and maternally inherited and consequently are one quarter the effective population size of nuclear genes (Moritz et al. 1987), thus allowing population level studies and systematic studies among recently diverged taxa. Possibly the most important reason to use mitochondrial genes is the availability of universal mtDNA primer sets that have minimized laboratory time in the initial setting up of a project. Finally, there is already an extensive set of nucleotide sequences from these genes in GenBank, as they have been the staple for crustacean molecular phylogenetic work since its inception.

To provide a comparison of gene utility, we have included uncorrected divergence estimates between pairs of taxa: between species, between genera, between families, and between infraorders/suborders for a number of genes. We also included COI on each graph as a reference (see Figs. 2–5). The ribosomal mitochondrial genes show similar levels of divergence to each other across all comparisons. In 12S, divergence estimates range from 3.9% among *Euastacus* species,

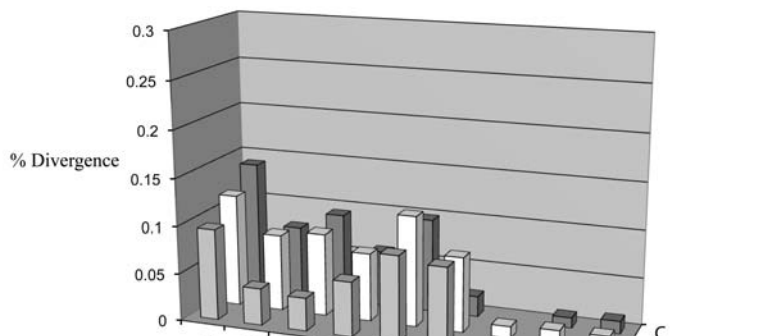

Figure 2. Pairwise divergence estimates between species of *Euastacus* (Astacidea) for mitochondrial and nuclear genes. Species are A: *E. eungella* and *E. spinichelatus*, B: *E. robertsi* and *E. eungella*, C: *E. robertsi* and *E. spinichelatus*.

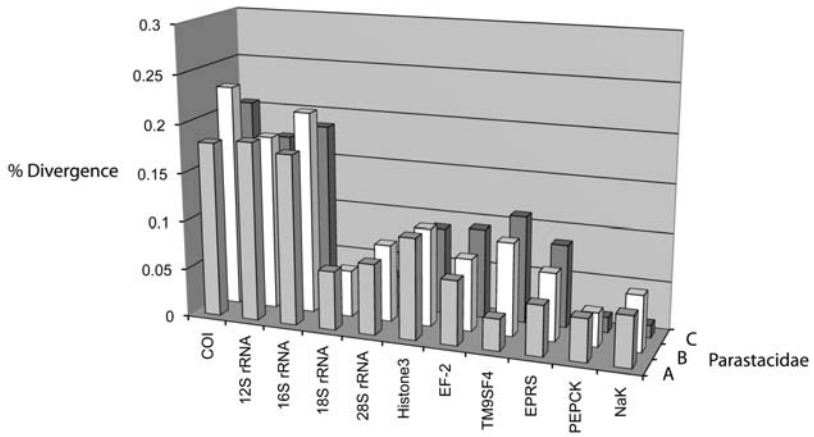

Figure 3. Pairwise divergence estimates between species of Parastacidae (Astacidea) for mitochondrial and nuclear genes. For genes COI, 12S, 16S, 18S, 28S, H3, EF-2, TM9SF4, EPRS the species are A: *Euastacus robertsi* and *Astacoides betsileoensis*, B: *E. robertsi* and *Parastacus defossus*, C: *A. betsileoensis* and *P. defossus*. Species for genes PEPCK and NaK are A: *Homarus gammarus* and *Nephropides caribaeus*, B: *H. gammarus* and *Nephropsis stewarti*, C: *N. caribaeus* and *N. stewarti*.

18% among genera within Parastacidae, 18.6% among families of Astacidea, and up to 24.2% among infraorders of Pleocyemata. Divergence of 16S ranges from 3.5% among species, 17.6% among genera, 23.5% among families, and up to 26.2% among infraorders of Pleocyemata. The coding mitochondrial gene COI is highly variable among species, thus making it a good candidate at lower levels. High divergence estimates were found above and including the family level, suggesting that this gene may have problems of nucleotide saturation above this level. This gene may still be useful for phylogenetic inference for resolving deeper nodes; however, it is important to test for

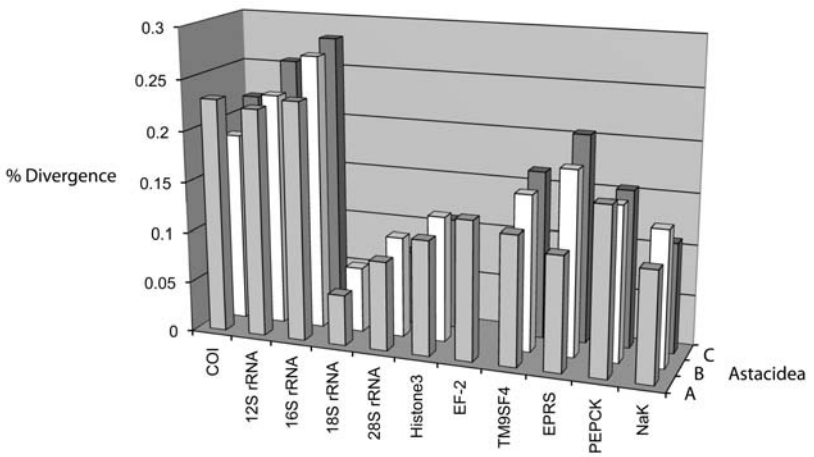

Figure 4. Pairwise divergence estimates among family representatives of Astacidea for mitochondrial and nuclear genes. For genes COI, 12S, 16S, 18S, 28S, H3, EF-2, TM9SF4, EPRS the species are A: *E. robertsi* and *Procambarus clarkii* (TM9SF4: *Orconectes virilis*), B: *E. robertsi* and *Nephropsis aculeata* (COI: *Homarus americanus*), C: *P. clarkii* (TM9SF4: *Orconectes virilis*) and *N. aculeate* (COI: *Homarus americanus*). Species for genes PEPCK and NaK are A: *H. gammarus* and *Cherax quadricarinatus*, B: *H. gammarus* and *P. clarkii*, C: *C. quadricarinatus* and *P. clarkii*.

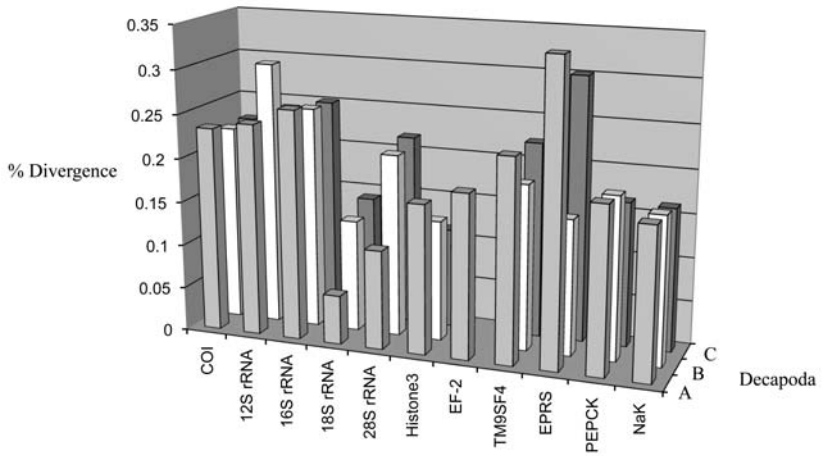

Figure 5. Pairwise divergence estimates among representatives of Decapoda for mitochondrial and nuclear genes. For genes COI, 12S, 16S, 18S, 28S, H3, EF-2, TM9SF4, EPRS the species are A: *E. robertsi* and *Calappa gallus* (COI: *Praebebalia longidactyla*), B: *C. gallus* (COI: *P. longidactyla*) and *Penaeus* sp., C: *E. robertsi* and *Penaeus* sp. Species for genes PEPCK and NaK are A: *H. gammarus* and *Calappa philargius*, B: *C. philargius* and *Penaeus monodon*, C: *H. gammarus* and *P. monodon*.

saturation and consider this in the analysis (i.e., use a model of evolution that incorporates multiple mutations at the same site — see Palero & Crandall this volume). A disadvantage of mitochondrial markers is that they are effectively a single locus, and, when used alone, they may not represent the true species tree.

Another problem of some mitochondrial genes such as COI is the presence of pseudogenes (nuclear copies of mitochondrial genes) in some species of decapods (Song et al. 2008).

3.2 *Nuclear genes*

Use of nuclear genes in addition to mitochondrial genes adds to the number of independent markers in a dataset, thus increasing the chances of reconstructing the true species phylogeny. In addition, a larger effective population size, and, on average, a lower substitution rate (Moriyama & Powell 1997), results in nuclear genes evolving slower than mitochondrial genes. Consequently, they may be better at resolving deeper phylogenetic nodes (see Chu et al. this volume). There are several considerations when choosing nuclear markers. There are at least two copies of each gene, although this is not usually a problem for phylogenetic studies as variation within an individual is less than between species. However, as mentioned previously, many genes belong to multigene families where duplications have resulted in genes or domains with a similar nucleotide sequence. In order to establish a single copy or at least the amplification of one dominant copy for new primer sets (EF-2, EPRS, TM9sf4) presented here, we analyzed 16–24 clones in several taxa representing Pleocyemata (Astacidea (*Homarus americanus*), Brachyura (*Cancer* sp.)) and Dendrobranchiata (*Penaeus* sp.). Low variation among some of the clones was observed. This could be attributed to *taq* polymerase error assuming an error rate of 1.6×10^{-6} to 2.1×10^{-4} per nucleotide per cycle (Hengen 1995) or to very low variation of a diploid gene.

The ribosomal nuclear genes 18S rDNA and 28S rDNA have been extensively used in arthropod systematics including several decapod studies (e.g., Ahyong & O'Meally 2004; Porter et al. 2005; Mitsuhashi et al. 2007; Ahyong et al. 2007). Rates of evolution vary among and within these genes, making them valuable phylogenetic tools at different taxonomic levels (Hillis & Dixon 1991). We found divergence rates for 18S were consistently moderate among species (5.8–7.2%) and

Table 1. Gene regions and primer sets selected for reconstructing the phylogeny of decapod crustaceans. For each primer, details of position (3') and a reference sequence are given. NR (nested reaction) refers to the primers used in the first reaction (1) and subsequent hemi-nested reaction (2).

Gene Region	Primer Name	Primer Sequence (5' – 3')	NR	Position	Reference Sequence	Primer Reference
Mitochondrial Genes						
12S rRNA	12sf	GAA ACC AGG ATT AGA TAC CC		390	AY659990	Mokady et al. 1994
	12sr	TTT CCC GCG AGC GAC GGG CG		778	AY659990	Mokady et al. 1994
16S rRNA	16s-1472	AGA TAG AAA CCA ACC TGG		99	AF200829	Crandall & Fitzpatrick 1996
	16sf-cray	GAC CGT GCK AAG GTA GCA TAA TC		552	AF200829	Crandall & Fitzpatrick 1996
COI	LCO1-1490	GGT CAA CAA ATC ATA AAG ATA TTG		*		Folmer et al. 1994
	HCO1-2198	TAA ACT TCA GGG TGA CCA AAA AAT CA		*		Folmer et al. 1994
Nuclear Genes						
18S rRNA	18s 1f	TAC CTG GTT GAT CCT GCC AGT AG		*		Whiting et al. 1997, Whiting 2002
	18s b3.0	GAC GGT CCA ACA ATT TCA CC		*		Whiting et al. 1997, Whiting 2002
	18s a0.79	TTA GAG TGC TYA AAG C		*		Whiting et al. 1997, Whiting 2002
	18s bi	GAG TCT CGT TCG TTA TCG GA		*		Whiting et al. 1997, Whiting 2002
	18s a2.0	ATG GTT GCA AAG CTG AAA C		*		Whiting et al. 1997, Whiting 2002
	18s 9R	GAT CCT TCC GCA GGT TCA CCT AC		*		Whiting et al. 1997, Whiting 2002
28S rRNA	28s-rD1.2a	CCC SSG TAA TTT AAG CAT ATT A		*		Whiting et al. 1997, Whiting 2002
	28s-rD3a	AGT ACG TGA AAC CGT TCA GG		*		Whiting et al. 1997, Whiting 2002
	28s-rd3.3f	GAA GAG AGA GTT CAA GAG TAC G		*		Whiting et al. 1997, Whiting 2002
	28sA	GAC CCG TCT TGA AGC ACG		*		Whiting et al. 1997, Whiting 2002
	28s-rD4.5a	AAG TTT CCC TCA GGA TAG CTG		*		Whiting et al. 1997, Whiting 2002
	28S rD5a	GGY GTT GGT TGC TTA AGA CAG		*		Whiting et al. 1997, Whiting 2002
	28s-rD4b	CCT TGG TCC GTG TTT CAA GAC		*		Whiting et al. 1997, Whiting 2002
	28S B	TCG GAA GGA ACC AGC TAC		*		Whiting et al. 1997, Whiting 2002
	28s-rD5b	CCA CAG CGC CAG TTC TGC TTA C		*		Whiting et al. 1997, Whiting 2002
	28s-rD6b	AAC CRG ATT CCC TTT CGC C		*		Whiting et al. 1997, Whiting 2002
	28S rD7b1	GAC TTC CCT TAC CTA CAT		*		Whiting et al. 1997, Whiting 2002
	28s3.25a	CAG GTG GTA AAC TCC ATC AAG G		602	AY210833	this study
	28s4.4b	GCT ATC CTG AGG GAA ACT TCG		1594	AY210833	this study

Table 1. continued.

Gene Region	Primer Name	Primer Sequence (5' – 3')	NR	Position	Reference Sequence	Primer Reference
H3	H3 AF	ATG GCT CGT ACC AAG CAG ACV GC		321	AB044542	Colgan et al. 1998
	H3 AR	ATA TCC TTR GGC ATR ATR GTG AC		694	AB044542	Colgan et al. 1998
EF-2	EF2a IF2	TGG GGW GAR AAC TTC TTY AAC		824	EF426560	Porter ML pers. comm.
	EF2a 1R2	ACC ATY TTK GAG ATG TAC ATC AT		1236	EF426560	Porter ML pers. comm.
	EF2a-F978	TGG ANA CBC TGA ARA TCA A	1,2	978	EF426560	this study
	EF2-R1435	GTT ACC HGC TGG VAC RTC TTC	2	1435	EF426560	this study
	EF2-R1536	GAC ACG NWG AAC TTC ATC ACC	1	1536	EF426560	this study
EPRS	192fin1f	+GAR AAR GAR AAR TTY GC		6874	U59923	www.umbi.umd.edu/users/jcrlab/
	192fin2r	+TCC CAR TGR TTR AAY TTC CA		7316	U59923	www.umbi.umd.edu/users/jcrlab/
TM9SF4	3064fin6f	CAR GAR GAR TTY GGN TGG AA	1	1198	NM_014742	www.umbi.umd.edu/users/jcrlab/
	3064fin7r	AAN CCR AAC ATR TAR TA		1841	NM_014742	www.umbi.umd.edu/users/jcrlab/
	3064-F1204	+GAA TTT GGR TGG AAG CTG GT	2	1204	NM_014742	this study
	3064-R1697	+CTG GGN ATY TGG TTG GTT CG	1,2	1697	NM_014742	this study

" $*$ " see primer reference for primer positions. " $+$ " addition of M13 primers to the 5' end improves PCR amplification (Regier & Shi 2005).

among infraorders (5.6%) within Pleocyemata but were higher among the suborders Pleocyemata and Dendrobranchiata (12.8% and 14.1%). Two hypervariable regions of 28S were identified and removed to avoid inflated estimates of divergence among poorly aligned repeat regions. 28S divergence estimates were higher than 18S among species (9.1–11.6%), within Pleocyemata (11.3%), and among the suborders (20.8–21.8%). Levels of divergence were lower for the intermediate taxon levels, among genera (3.4–8.0%), and among families (7.3–9.9%), and possibly represented a shorter nucleotide alignment due to indels (insertions or deletions) that are absent among species (within a genus).

Two nuclear protein coding genes that are currently used in arthropod systematics are histone 3 (H3) (e.g., Porter et al. 2005) and elongation factor 2 (EF-2) (e.g., Regier & Shultz 2001). Primer sets already developed for H3 (Colgan et al. 1998) amplify the target fragment across a range of decapod crustaceans and show moderate levels of divergence among species (2.2–8.4%), suggesting they are useful nuclear protein coding markers for relationships within a genus. It should be noted that *Euastacus* is relatively older than some decapod genera (see Breinholt et al. this volume) and consequently H3 may not be appropriate for phylogenetic analyses among recently diverged species. Divergence within and among families is also moderate (8.9–12.4%), with a higher level of divergence between *Euastacus robertsi* and *Calappa gallus* within Pleocyemata (17%).

Although we were able to amplify genomic fragments of the EF-2 gene with currently designed primer sets (see Table 1), an intron was located at base pair position 860 relative to mRNA in *Libinia emarginata* (GenBank accession AY305506). The intron may be useful for species/genera level studies, although preliminary analysis suggests it is fewer than 300 base pairs in caridean (Hippolytidae) and brachyuran (Calappidae, Leucosiidae, Goneplacidae, Majidae, Cyclodorippidae) decapods. A new forward primer was designed to exclude the intron, and GenBank sequences were downloaded and aligned to design reverse primers 400–500 base pairs downstream of the forward primer. Using different primer sets, we were able to isolate two copies of EF-2. The two copies were more similar within an individual than between species of *Euastacus* crayfish. Two similar copies of EF-2 are present in *Drosophila melanogaster* (Lasko 2000). The divergence estimates for the longer fragment are presented in figure 2 and were low among species of *Euastacus* (1.3%). Percent divergence within Parastacidae (6.7–9.3%) and between families of Astacidea (13.6%) was moderate. High divergences were noted within Pleocyemata between *E. robertsi* and *C. gallus* (18.7%).

The EPRS locus is a potentially useful nuclear gene for reconstructing phylogenetic relationships among the deeper nodes of decapod crustaceans. The EPRS locus encodes a multifunctional aminoacyl tRNA synthetase, glutamyl–prolyl–tRNA synthetase (Cerini et al. 1991). The two proteins are involved in the aminoacylation of glutamic acid and praline tRNA in *Drosophila* (Cerini et al. 1991; Cerini et al. 1997). Few phylogenetic studies have used EPRS, although a recent study of *Paramysis* (Crustacea: Mysida) demonstrates its usefulness in reconstructing relationships among genera of mysids (Audzijonyte et al. 2008). We found divergence levels were low among species of *Euastacus* (0.8–1.5%) but moderate for within the family Parastacidae (5.2–8.6%) and high between some families of Astacidea (11.3–20.5%). This locus showed high divergences within Pleocyemata between *E. robertsi* and *C. gallus* (33.9%) and between *E. robertsi* and *Penaeus* sp. (15.5–30.1%). The different levels of divergence at different taxonmic levels suggest this marker may be useful among genera up to order level for phylogenetic estimation.

Transmembrane 9 superfamily protein member 4, or TM9sf4, is a small molecule carrier or transporter. Our study is the first to present divergence estimates and phylogenetic results using this gene. Uncorrected pairwise divergence results suggest it has potential as a valuable gene for reconstructing family to order level relationships. Divergence among species within *Euastacus* was low (0.7–1.5%), suggesting this marker may be less informative than other nuclear protein coding markers such as Histone 3 when reconstructing relationships among species. As with EPRS, this marker shows greater divergences (18.8–23%) at the deeper level (among infraorders/suborders)

than Histone 3. High levels of divergence are often considered indicative of saturation; however, we found increasing divergence with increasing evolutionary distance, suggesting saturation may not have been reached even among the deeper nodes, indicating the utility of this gene to infer phylogenetic relationships at these higher levels of divergence.

4 PHYLOGENY BASED SYSTEMATICS

Reconstructing the evolutionary relationships among decapod crustaceans using molecular data has taken two directions: using only protein coding genes, which are phylogenetically informative at deeper nodes, or incorporating as much molecular information available including both ribosomal RNA and protein coding genes in a family level supertree. We have taken the latter approach and reconstructed Decapoda relationships using a total of eight genes and 46 taxa (see Table 2) including representatives of seven infraorders of Pleocyemata and a representative of Dendrobranchiata (*Penaeus* sp.) as an outgroup. Pleocyemata representatives include Astacidea, Achelata, Polychelida, Thalassinidea, Brachyura, Anomura and Caridea. Non-decapod crustaceans, *Lysiosquillina maculata* (Lysiosquillidae: Stomatopoda), were also included in the analysis as outgroups to all the decapods. Rather than focus on representing all lineages equally, we were interested in reconstructing relationships at many levels from among species within genera, among families, and among infraorders within decapod crustaceans. Therefore, we focused on sampling the Astacidea to demonstrate the usefulness of these genes for reconstructing phylogenies at these various taxonomic levels.

The genes included in our analyses were 12S, 16S, 18S, 28S, H3, EF-2, EPRS, and TM9sf4. A second analysis was run on the four nuclear protein-coding genes. Use of nuclear rRNA 18S and 28S data has been criticized for ambiguities noted in alignments (Tsang et al. 2008). The difficulties in aligning highly variable data may be overcome by using sophisticated methods of alignment employed in recently developed programs such as DIALIGN-T (Subramanian et al. 2005) and MAFFT (Katoh et al. 2002; Katoh et al. 2005). These programs produce more accurate alignments than ClustalW with increasing evolutionary distance (e.g., MAFFT, Nuin et al. 2006) or when gaps are present (indels) in the resulting alignment of sequence data (e.g., DIALIGN-T and MAFFT, Golubchik et al. 2007). To further improve the alignment, GBlocks can be used to identify and exclude ambiguous regions of sequence data (Castresana 2000; Talavera & Castresana 2007). We used MAFFT to align all gene fragments and subsequently ran each dataset through GBlocks (retaining half gap positions) to recover the most useful sequence data. As an example, this reduced the 28S MAFFT alignment from 4489 to 1254 base pairs. Our resulting alignment for the eight-gene dataset was 5104 nucleotides.

Maximum likelihood phylogenies were constructed with RAxML (Stamatakis 2006; Stamatakis et al. 2008) at the CIPRES portal assuming a GTR+G+I model and estimation and optimization of α-shape parameters, GTR-rates, and empirical base frequencies for each gene. We allowed the program to choose the number of bootstrap replicates, and for the eight-gene dataset, 150 bootstrap replicates were run before termination. For the smaller nuclear protein coding alignment, 250 bootstrap replicates were run before the program terminated. The estimated parameters are presented in Table 3.

The relationships within Astacidea were well resolved, with bootstrap support in 11 of 14 nodes supported by 95% or greater and all nodes supported greater than 80% (see Fig. 6). As a comparison, the ML phylogeny based on the four-gene dataset (nuclear protein coding) constructed a similar topology within Astacidea although the nodes were not as strongly supported. Only six nodes were supported greater than 95%, with an additional five nodes supported greater than 70%. This result suggests that although the nuclear coding genes have the power to resolve relationships within an infraorder, additional data from ribosomal genes adds to the information available for reconstructing relationships across the whole of decapod diversity. Our group continues to add genes and taxa to achieve our goal of reconstructing a robust phylogenetic estimate for the decapod crustaceans.

Table 2. Taxonomy and accession numbers of decapod samples and outgroup included in this study. Accession numbers in bold were obtained from GenBank.

Taxon	Voucher ID	12S rRNA	16S rRNA	18S rRNA	28S rRNA	H3	EF-2	EPRS	TM9SF4
Decapoda Latreille, 1802									
Dendrobranchiata Bate, 1888									
Penaeoidea Rafinesque, 1815									
Penaeus sp. Fabricius, 1798	KCpen	EU920908	EU920934	EU920969	EU921005-EU921006	EU921075	—	—	EU921109
Pleocyemata Burkenroad, 1963									
Anomura MacLeay, 1838									
Galatheoidea Samouelle, 1819									
Aegla alacalufi (Jara & López, 1981)	KAC798	**AY050012**	**AY050058**	EU920958	**AY595958**	EU921042	EU921009	EU910098	EU921077
Eumunida funambulus (Miyake, 1982)	KC3100	EU920892	EU920922	EU920957	EU920984	EU921056	EU921032	EU910124	EU921089
Kiwa hirsute (Jones & Segonzac, 2005)	KC3116	—	—	EU920942	EU920987	EU921065	EU921035	EU910128	EU921097
Munidopsis rostrata (Milne-Edwards, 1880)	KC3102	EU920898	EU920928	EU920961	EU920985	EU921066	EU921034	EU910126	EU921100
Lomisoidea Bouvier, 1895									
Lomis hirta (Lamarck, 1810)	KAClohi	**AY595547**	**AY595928**	**AF436013**	**AY596101**	**DQ079680**	EU921040	EU910131	EU921098
Paguroidea Latreille, 1802									
Pomatocheles jeffreysii (Miers, 1879)	KC3097	EU920903	EU920930	EU920965	EU920983	EU921070	EU921031	EU910123	EU921105
Astacidea Latreille, 1802									
Astacoidea Latreille, 1802									
Astacus astacus (Linnaeus, 1758)	KC702	EU920881	**AF235983**	**AF235959**	**DQ079773**	**DQ079660**	EU921008	—	EU921078
Barbicambarus cornutus (Faxon, 1884)	KC1941	EU920883	EU920913	EU920951	EU920993	EU921045	EU921017	EU910106	EU921080
Orconectes virilis (Hagen, 1870)	KC709	EU920900	**AF235989**	**AF235965**	**DQ079804**	**DQ079693**	EU921041	—	EU921102
Procambarus clarkii (Girard, 1852)	KC1497	EU920901	**AF235990**	EU920952	EU920970	EU921067	EU921011	EU910100	—
Parastacoidea Huxley, 1879									
Astacoides betsileoensis (Petit, 1923)	KC1822	EU920882	EU920912	EU920955	EU920992	EU921044	EU921014	EU910103	EU921079
Cherax cuspidatus (Riek, 1969)	KC1175	**DQ006421**	**DQ006550**	EU920960	EU920996	EU921048	EU921010	EU910099	EU921083
Euastacus eungella (Morgan, 1988)	KC2671	**DQ006464**	**DQ006593**	EU920964	EU92100-EU921002	EU921055	EU921018	EU910109	EU921088
Euastacus robertsi (Monroe, 1977)	KC2781	**DQ006507**	**DQ006633**	EU920962	EU920988	EU921058	EU921019	EU910110	EU921091
Euastacus spinichelatus (Morgan, 1997)	KC2631	**DQ006512**	**DQ006638**	EU920963	EU920989	EU921059	—	EU910108	EU921092
Gramastacus insolitus (Riek, 1972)	KC640	EU920895	EU920926	EU920968	EU920994	EU921062	EU921007	EU910097	EU921094
Ombrastacoides huonensis (Riek, 1967)	KC611	EU920905	**AF135997**	EU920956	EU920995	EU921072	—	EU910096	EU921106
Parastacus defossus (Faxon, 1898)	KC1515	EU920902	**AF175243**	EU920953	EU920991	EU921068	EU921012	EU910101	EU921103
Parastacus varicosus (Faxon, 1898)	KC1529	EU920907	EU920933	EU920954	EU920990	EU921074	EU921013	EU910102	EU921108

Table 2. continued.

Taxon	Voucher ID	12S rRNA	16S rRNA	18S rRNA	28S rRNA	H3	EF-2	EPRS	TM9SF4
Nephropoidea Dana, 1852									
Homarus americanus (Milne-Edwards, 1837)	KAChoam	**DQ298427**	**HAU11238**	**AF235971**	**DQ079788**	**DQ079675**	—	—	EU921095
Nephropsis aculeate (Smith, 1881)	KC2117	EU920899	**DQ079727**	**DQ079761**	**DQ079802**	**DQ079691**	—	EU910107	EU921101
Brachyura Latreille, 1802									
Calappoidea Milne-Edwards, 1837									
Cycloes granulose (de Haan, 1837)	KC3082	EU920887	EU920917	EU920943	EU920976	EU921050	EU921025	EU910116	EU921085
Calappa gallus (Herbst, 1803)	KC3083	EU920886	EU920916	EU920947	EU920977	EU921049	EU921026	EU910117	EU921084
Dorippoidea MacLeay, 1838									
Ethusa sp. (Roux, 1830)	KC3088	—	EU920925	EU920966	EU920980	EU921061	EU921029	EU910120	EU921093
Grapsoidea MacLeay, 1838									
Cyclograpsus cinereus (Dana, 1851)	KC3417	EU920884	EU920914	EU920945	EU920997	EU921046	EU921038	EU910130	EU921081
Leucosioidea Samouelle, 1819									
Ebalia tuberculosa (Milne-Edwards, 1873)	KC3085	EU920894	EU920924	EU920944	EU920978	EU921060	EU921027	EU910118	—
Praebebalia longidactyla (Yokoya, 1933)	KC3086	EU920904	EU920931	EU920946	EU920979	EU921071	EU921028	EU910119	—
Majoidea Samouelle, 1819									
Chorilia longipes (Dana, 1852)	KC3089	EU920889	EU920919	EU920948	EU920981	EU921052	EU921039	EU910121	EU921087
Raninoidea de Haan, 1839									
Cosmonotus grayi (White, 1848)	KC3092	EU920888	EU920918	EU920949	EU920982	EU921051	EU921030	EU910122	EU921086
Caridea Dana, 1852									
Palaemonoidea Rafinesque, 1815									
Anchistioides antiguensis (Schmitt, 1924)	KC3051	EU920880	EU920911	EU920936	EU920971	EU921043	EU921020	EU910111	—
Coutierella tonkinensis (Sollaud, 1914)	KC3068	EU920890	EU920920	EU920937	EU920975	EU921053	EU921024	EU910115	—
Crangonoidea Haworth, 1825									
Crangon crangon (Linnaeus, 1758)	KC3052	EU920885	EU920915	EU920938	EU920972	EU921047	EU921021	EU910112	EU921082
Bresilioidea Calman, 1896									
Discias sp. (Rathbun, 1902)	KC3108	EU920891	EU920921	EU920941	EU920986	EU921054	—	EU910127	—
Alpheoidea Rafinesque, 1815									
Hippolyte bifidirostris (Miers, 1876)	KC3059	EU920896	EU920927	EU920939	EU920974	EU921063	EU921023	EU910114	—
Eualus gaimardii (Milne-Edwards, 1837)	KC3056	EU920893	EU920923	EU920940	EU920973	EU921057	EU921022	EU910113	EU921090

Table 2. continued.

Taxon	Voucher ID	12S rRNA	16S rRNA	18S rRNA	28S rRNA	H3	EF-2	EPRS	TM9SF4
Achelata Scholtz & Richter, 1995									
Palinuroidea Latreille, 1802									
Jasus edwardsii (Hutton, 1875)	KC3209	—	**DQ079716**	**AF235972**	**DQ079791**	EU921064	EU921036	EU910129	EU921096
Palinurus elephas (Fabricius, 1787)	KC3210	—	EU920929	EU920959	EU920999-EU921000	EU921069	EU921037	—	EU921104
Polychelida de Haan, 1941									
Polycheles typhlops (Heller, 1862)	KC3101	EU920906	EU920932	EU920950	EU921003-EU921004	EU921073	EU921033	EU910125	EU921107
Thalassinidea Latreille, 1831									
Callianassoidea Dana, 1852									
Lepidophthalmus louisianensis (Schmitt, 1935)	KAC1852	EU920897	**DQ079717**	**DQ079751**	**DQ079792**	**DQ079678**	EU921015	EU910104	EU921099
Sergio mericeae (Manning & Felder, 1995)	KAC1865	EU920909	**DQ079733**	**DQ079768**	**DQ079811**	**DQ079700**	EU921016	EU910105	EU921110
Outgroup									
Stomatopoda Latreille, 1817									
Lysiosquilloidea Giesbrecht, 1910									
Lysiosquillina maculata (Fabricius, 1793)	KC3832	EU920910	EU920935	EU920967	EU920998	EU921076	—	—	EU921111

Table 3. Empirical base frequencies for each gene region and associated model parameters estimated from the sequence data in RAxML.

	A	C	G	T	alpha	pinvar
12S rRNA	0.3670	0.0981	0.1726	0.3622	0.6030	0.1934
16S rRNA	0.3399	0.1116	0.2027	0.3458	0.6235	0.2879
18S rRNA	0.2502	0.2342	0.2780	0.2377	0.9231	0.4940
28S rRNA	0.2501	0.2357	0.3161	0.1981	0.7772	0.2735
H3	0.2152	0.3172	0.2654	0.2022	1.0618	0.5882
EF-2	0.2364	0.2469	0.2655	0.2512	1.4067	0.4872
EPRS	0.2857	0.2159	0.2523	0.2460	1.6197	0.3690
TM9SF4	0.1587	0.2784	0.2455	0.3174	0.9592	0.4982

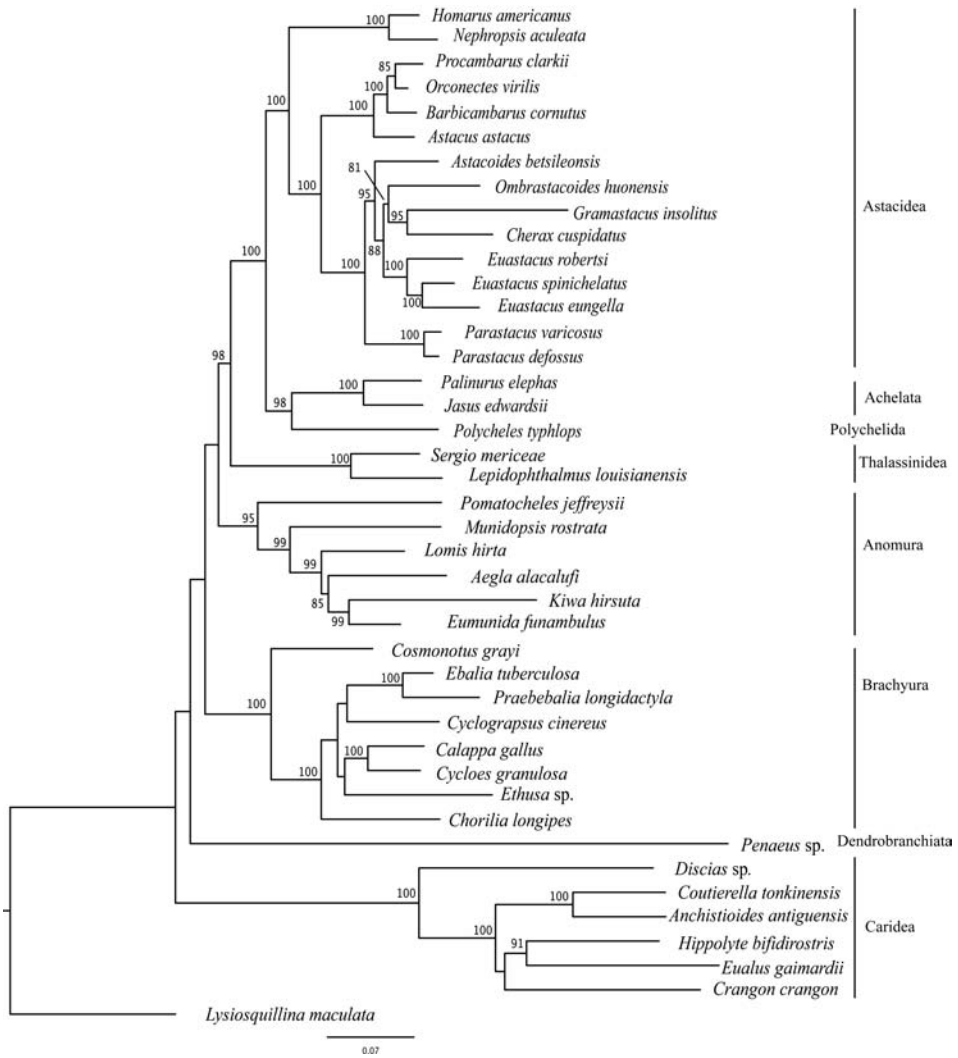

Figure 6. Maximum likelihood phylogeny based on two mitochondrial and six nuclear genes constructed in RAxML. Values at nodes represent bootstrap support greater than 70%.

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Development, Genes, and Decapod Evolution

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ABSTRACT

Apart from larval characters such as zoeal spines and stages, developmental characters are rarely used for inferences on decapod phylogeny and evolution. In this review we present examples of comparative developmental data of decapods and discuss these in a phylogenetic and evolutionary context. Several different levels of developmental characters are evaluated. We consider the influence of ontogenetic characters such as cleavage patterns, cell lineage, and gene expression on our views on the decapod ground pattern, on morphogenesis of certain structures, and on phylogenetic relationships. We feel that developmental data represent a hidden treasure that is worth being more intensely studied and considered in studies on decapod phylogeny and evolution.

1 INTRODUCTION

The morphology of decapod crustaceans shows an enormous diversity concerning overall body shape and limb differentiation. On the two extreme ends, we find representatives such as shrimps with an elongated, laterally compressed body, muscular pleon, and limbs mainly adapted to swimming, and groups like the Brachyura exhibiting a dorsoventrally flattened, strongly calcified, broad body with a reduced pleon and uniramous walking limbs. In addition, hermit crabs show a peculiar asymmetric soft and curved pleon, and among all larger decapod taxa there are species with limbs specialized for digging, mollusc shell cracking, and all other sorts and numbers of pincers and scissors. These few examples indicate that the decapod body organization is varied to a high degree. It is obvious that this disparity has been used to establish phylogenetic relationships of decapods and that it is a challenge for considerations of decapod evolution (e.g., Boas 1880; Borradaile 1907; Beurlen & Glaessner 1930; Burkenroad 1981; Scholtz & Richter 1995; Schram 2001; Dixon et al. 2003). One major example for the latter is the controversial discussion about carcinization—the evolution of a crab-like form, which, as the most derived body shape and function, desires an explanation at the evolutionary level (e.g., Borradaile 1916; Martin & Abele 1986; Richter & Scholtz 1994; McLaughlin & Lemaitre 1997; Morrison et al. 2002; McLaughlin et al. 2004).

A closer look at decapod development shows a similarly wide range of different patterns as is found in adult morphology (e.g., Korschelt 1944; Fioroni 1970; Anderson 1973; Schram 1986; Weygoldt 1994; Scholtz 1993, 2000). One can observe decapod eggs with high and low yolk content, with total cleavage and superficial cleavage types, with a distinct cell division and cell lineage pattern, and without these determinations. There are different kinds of gastrulation, ranging from invagination to immigration and delamination, and multiple gastrulation modes and phases within a species. In addition, the growth zone of the embryonic germ band is composed of different numbers of stem cells in the ectoderm, the so called ectoteloblasts (Dohle et al. 2004). Even at the level of

gene expression patterns, the few existing publications on decapods reveal some differences between species (e.g., Averof & Patel 1997; Abzhanov & Kaufman 2004). Some groups hatch as a nauplius larva, whereas others hatch at later stages (such as zoea larvae) or exhibit direct development with hatchlings looking like small adults (Scholtz 2000).

With the notable exception of zoeal larval characters (e.g., Gurney 1942; Rice 1980; Clark 2005, this volume), surprisingly little attention has been paid to this developmental diversity and to decapod development in general when the phylogenetic relationships or evolutionary pathways have been discussed.

Here we present some examples of how ontogenetic data, such as cleavage, cell division, and gene expression patterns, can be used to infer phylogenetic relationships and evolutionary pathways among decapod crustaceans. It must be stressed, however, that this is just the beginning. Most relevant data on decapod ontogeny have yet to be described.

2 CLEAVAGE PATTERN, GASTRULATION, AND THE DECAPOD STEM SPECIES

It is now almost universally accepted that the sister groups Dendrobranchiata and Pleocyemata form the clade Decapoda (Burkenroad 1963, 1981; Felgenhauer & Abele 1983; Abele & Felgenhauer 1986; Christoffersen 1988; Abele 1991; Scholtz & Richter 1995; Richter & Scholtz 2001; Schram 2001; Dixon et al. 2003; Porter et al. 2005; Tsang et al. 2008). The monophyly of dendrobranchiates is largely based on the putatively apomorphic shape of the gills, which are highly branched, and perhaps on the specialized female thelycum and male petasma (Felgenhauer & Abele 1983). Nevertheless, the monophyly of Dendrobranchiata has been doubted based on characters of eye morphology (Richter 2002). Dendrobranchiata contains sergestoid and penaeoid shrimps, which have a largely similar life style (Pérez Farfante & Kensley 1997). In contrast to this, the pleocyematans include shrimp-like forms, such as carideans and stenopodids, but also the highly diverse reptants, which include lobsters, crayfishes, hermit crabs, and brachyuran crabs among others. When Burkenroad (1963, 1981) established the Pleocyemata, he stressed the characteristic brood-care feature of this group, namely, the attachment of the eggs and embryos to the maternal pleopods. With few exceptions, such as *Lucifer*, which attaches the eggs to the 3rd pleopods (Pérez Farfante & Kensley 1997), dendrobranchiates simply release their eggs into the water column. The monophyly of Pleocyemata is furthermore supported by brain characters (Sandeman et al. 1993).

The early development is quite different between Dendrobranchiata and Pleocyemata. Dendrobranchiates show relatively small, yolk-poor eggs with a total cleavage, a stereotypic cleavage pattern resulting in two interlocking cell bands, a determined blastomere fate, and a gastrulation initiated by two large cells largely following the mode of a modified "invagination" gastrula (e.g., Brooks 1882; Zilch 1978, 1979; Hertzler & Clark 1992; Hertzler 2005; Biffis et al. in prep) (Fig. 1). They hatch as nauplius larvae (Scholtz 2000). Pleocyematans mostly possess relatively large, yolky eggs with a superficial or mixed cleavage, no recognizable cell division pattern, and an immobile embryonized egg-nauplius (see Scholtz 2000; Alwes & Scholtz 2006). There are a few exceptions found in some carideans, hermit crabs, and brachyurans among reptants, which display an initial total cleavage (e.g., Weldon 1887; Gorham 1895; Scheidegger 1976), but these cleavages never show a consistent pattern comparable to that of Dendrobranchiata. The gastrulation is highly variable, and very often it implies immigration and no formation of a proper blastopore (Fioroni 1970; Scholtz 1995). The question is, which of these two types of developmental pathways—the one exhibited by the Dendrobranchiata or the less specified type exhibited by the Pleocyemata—is plesiomorphic within the Decapoda? This can only be answered with an outgroup, since two sister groups with two alternative sets of character states cannnot tell us which states are plesiomorphic. The answer to this question allows inferences on the origin and ground pattern of decapods; in particular, it might inform us as to whether the ancestral decapod was a swimming shrimp-like animal of the dendrobranchiate type or a benthic reptant. A pelagic lifestyle in malacostracan Crustacea is not necessarily

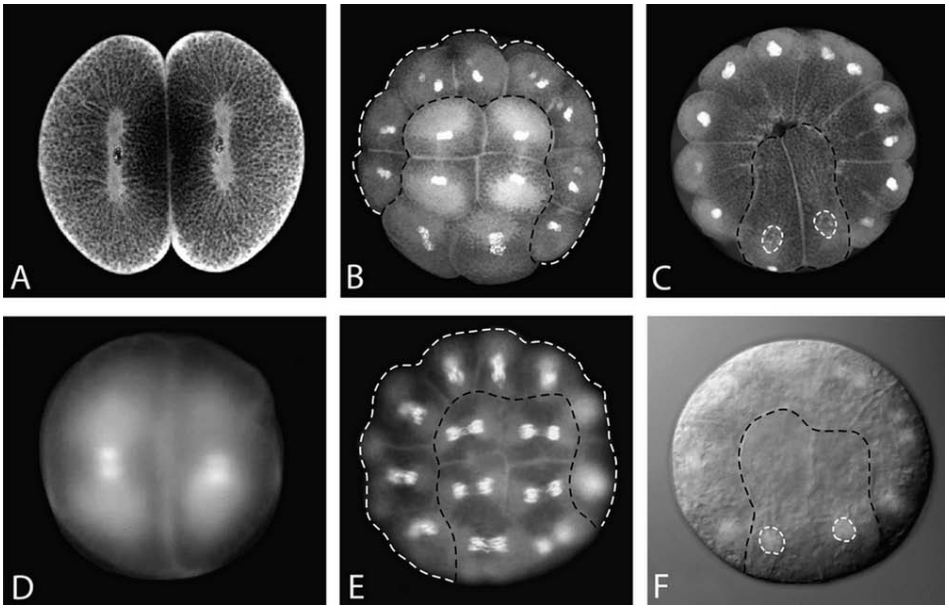

Figure 1. Different stages during early development of the dendrobranchiate shrimp *Penaeus monodon* (A-C) and of the euphausiacean *Meganyctiphanes norvegica* (D-F) stained with fluorescent dyes (Sytox A-C; Hoechst D-F). In F the fluorescence is combined with transmission light. The eggs show a low yolk content and total cleavage with a characteristic size and arrangement of the blastomeres. A and D: 2-cell stage. B and E: 32-cell stage. A stereotypic cleavage pattern leads to two interlocking cell bands, a "tennis ball pattern" (surrounded by white and black broken lines each). In B, the mitoses of the previous division are just completed, while in E the cells show the anaphase of the next division. C and F: 62-cell stage. Notice the center of the egg with two differently sized large mesendoderm cells (black broken lines), which arrest their division and initiate gastrulation.

combined with, but facilitates, the absence of brood care, whereas benthic malacostracans always show some degree of investment into the embryos and early larvae.

A comparison with the early development of Euphausiacea helps to polarize the developmental characters of Dendrobranchiata and Pleocyemata. Euphausiacea are either the sister group (Siewing 1956; Christoffersen 1988; Wills 1997; Schram & Hof 1998; Watling 1981, 1999) or are more remotely related to Decapoda (Richter 1999; Scholtz 2000; Jarman et al. 2000; Richter & Scholtz 2001). The Euphausiacea studied show remarkable similarities to dendrobranchiate decapods concerning their early embryonic and larval development (Taube 1909, 1915; Alwes & Scholtz 2004). They also release their eggs into the water column and show no brood care, with some apparently derived exceptions (Zimmer & Gruner 1956). Furthermore, they exhibit a corresponding cleavage pattern, arrangement and fate of blastomeres, and mode of gastrulation (Fig. 1). Like Dendrobranchiata, Euphausiacea hatch as a free nauplius. In particular, the formation of two interlocking germ bands, the origin and fate of the two large mesendoderm cells that initiate the gastrulation, and the formation of distinct cell rings (crown cells) at the margin of the blastopore find a detailed correspondence between dendrobranchiates and euphausiids (Hertzler & Clark 1992; Alwes & Scholtz 2004; Hertzler 2005) (Fig. 1). It must be stressed, however, that the nauplius larvae of dendrobranchiate decapods and Euphausiacea might be the result of convergent evolution (Scholtz 2000). It is furthermore not clear when this type of cleavage and early development evolved within malacostracans. The similarities in early development might indicate that euphausiaceans are the sister group to decapods (see Alwes & Scholtz 2004) (Fig. 2), in agreement with previous suggestions (e.g., Siewing 1956; Christoffersen 1988; Wills, 1997; Schram & Hof 1998; Watling

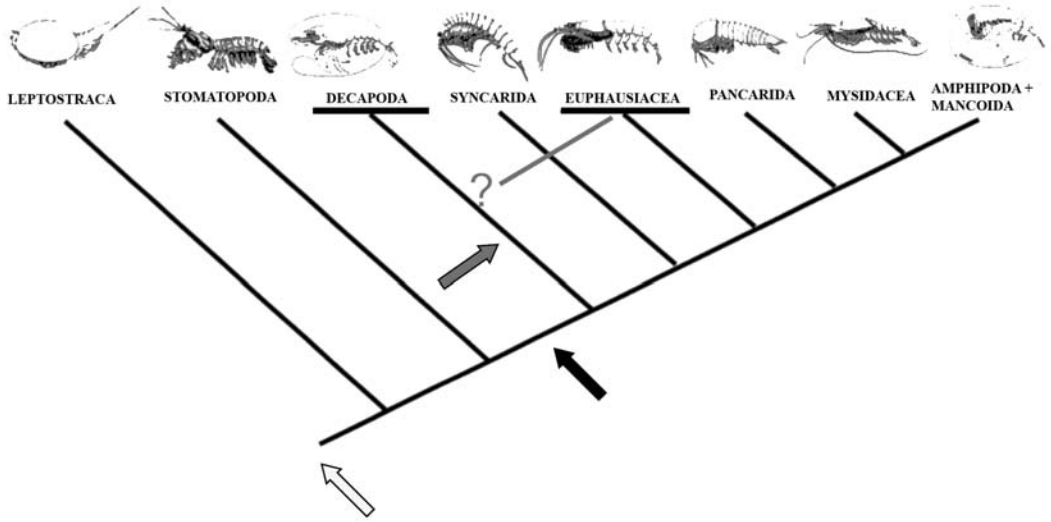

Figure 2. Malacostracan phylogeny according to Richter & Scholtz (2001). The arrows indicate the three possibilities for the evolution of the characteristic early development shared by Euphausiacea and Dendrobranchiata (Decapoda). The black arrow shows the possibility that the cleavage pattern evolved in the lineage of Caridoida. The grey arrow indicates a shared evolution of the cleavage pattern for Decapoda and Euphausiacea in combination with the view of a sister group relationship between these two groups (Eucarida), as is indicated with a question mark and light grey line. The white arrow symbolizes an older origin of the developmental pattern, perhaps even in non-malacostracans.

1981, 1999). On the other hand, if we accept the analysis of Richter and Scholtz (2001), the pattern must have evolved in the stem lineage of Caridoida (Fig. 2). However, it might be even older since similar patterns occur in some non-malacostracan crustaceans (Kühn 1913; Fuchs 1914, see Alwes & Scholtz 2004) (Fig. 2).

In either case, this corresponding early development of euphausiids and dendrobranchiate decapods to the exclusion of Pleocyemata strongly suggests that originally decapods did not care for the brood but released their yolk-poor eggs freely into the water. Furthermore, these eggs developed via a stereotypic cleavage pattern with largely determined cell fates and a specific mode of gastrulation. All of this indicates that the early development of Dendrobranchiata is plesiomorphic within Decapoda. In addition, this allows for the conclusion that the ancestral decapod was a more pelagic shrimp-like crustacean.

The oldest known fossil decapod is the late Devonian species *Palaeopalaemon newberryi* (see Schram et al. 1978). According to these authors, this fossil is a represenative of the reptant decapods (see also Schram & Dixon 2003). This was disputed by Felgenhauer and Abele (1983), who claimed that the shrimp-like scaphocerite instead indicates an affinity to dendrobranchiates or carideans. Our conclusions, based on ontogenetic data, might lead to reconsidering the affinities of *Palaeopalaemon* as a dendrobranchiate-like decapod. At least there is no morphological structure that contradicts this assumption. This interpretation would furthermore fit with the ideas of Schram (2001) and Richter (2002) who independently concluded, based on eye structure and other arguments, that it is likely that decapods originated in deeper areas of the sea.

3 WAS THE ANCESTRAL DECAPOD A DECAPOD?

One of the apomorphies for Malacostraca is the possession of eight thoracic segments and their corresponding eight thoracopods (Richter & Scholtz 2001). In the various malacostracan groups, the thoracopods are diversified to different degrees, with the most conspicuous transformation being

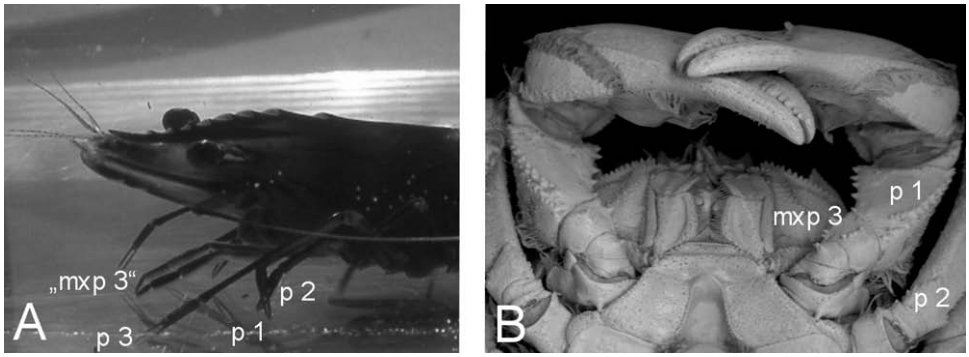

Figure 3. Evolution of 3rd maxillipeds in decapods. (A) The dendrobranchiate shrimp *Penaeus monodon* with pediform 3rd maxillipeds (mxp 3), which are not very different from the 1st anterior pereopods (p1 to p3). (B) The 3rd maxilliped (mxp3) of the brachyuran *Eriocheir sinensis* is highly transformed compared to the first two pereopods (p1, p2).

the modification of anterior thoracic limbs to secondary mouthparts, the maxillipeds. Depending on the number of thoracopods transformed to maxillipeds, the number of walking limbs (pereopods) varies. In most malacostracans we find either none (Leptostraca, Euphausiacea), one (e.g., Isopoda, Amphipoda, Anaspidacea) to two (Mysidacea), and sometimes three (Cumacea, most Decapoda) or even five (Stomatopoda) pairs of maxillipeds, which correspondingly means eight, seven, six, five, or three pairs of pereopods (Richter & Scholtz 2001). It is quite safe to assume that the plesiomorphic condition in malacostracans was the absence of any maxillipeds and that the number increased convergently in the course of malacostracan evolution. Only the anteriormost maxilliped might be homologous between those malacostracan taxa that possess it (Richter & Scholtz 2001). Decapods, as the name indicates, are characterized by five pairs of pereopods, which lie posterior to three pairs of maxillipeds. However, the concept of what has to be considered a maxilliped is not very sharp, because it relates to a combination of morphological deviation and different function from a locomotory limb, which is assumed to represent the ancestral throracopod state. Indeed, the locomotory pereopods of malacostracans are often also involved in food gathering and processing of some sort, and the large chelipeds of a lobster, for instance, are seldom used for locomotion. On the other hand, the morphology of some, in particular the posteriormost, maxillipeds is not very different from that of the pereopods. For instance, the 3rd maxillipeds of lobsters are more leg-like than those of most brachyuran crabs in which these form the operculum covering the mouth field (Scholtz & McLay this volume) (Fig. 3).

In particular, in some dendrobranchiates the 3rd maxillipeds are morphologically not really discernible from the pereopods (Fig. 3). They have the same length and segment number as the pereopods and are not kept closely attached to the mouth field. Accordingly, the question arises as to whether the stem species of decapods was equipped with only two pairs of maxillipeds and hence six pairs of pereopods (see Scholtz & Richter 1995; Richter & Scholtz 2001)—in other words, whether it was a dodecapod (dodeka: Greek for twelve) rather than a true decapod.

In their seminal work, Averof and Patel (1997) developed a new molecular criterion for maxillipeds. They found that the Hox gene ultrabithorax (UBX) is expressed in thoracic regions with pereopods, whereas in segments bearing maxillipeds, this gene is not expressed. UBX is needed to differentiate trunk segments, and the absence of UBX expression allows the transformation towards mouthparts (Averof & Patel 1997). This is true for all crustaceans investigated in this respect. Interestingly enough, the two decapod species studied by Averof and Patel (1997) differed slightly in the anterior margin of UBX expression depending on the degree of deviation from a pereopod-like appearance of the 3rd maxillipeds (see Fig. 5). In the lobster, with a more pediform 3rd maxilliped

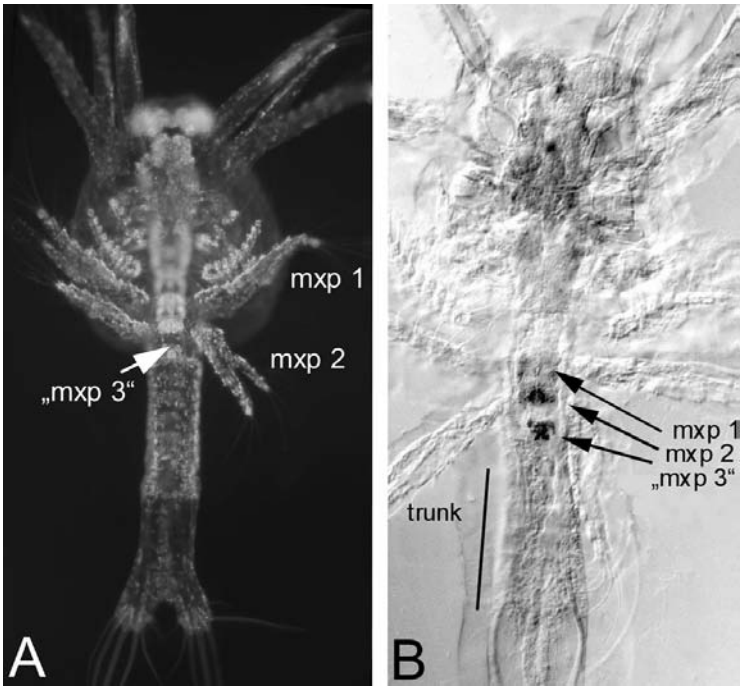

Figure 4. Expression of the UBX-AbdA protein in the protozoea of *Penaeus monodon* as seen with the antibody FP6.87. (A) 1st protozoea stained with the nuclear dye Hoechst, showing the overall shape, the limbs, and the central nervous system. The two anterior pairs of maxillipeds (mxp1, 2) are present and the corresponding ganglion anlagen are recognizable. The 3rd maxilliped pair is not yet differentiated but the ganglion is forming (mxp3). (B) 1st protozoea showing UBX expression in the ganglia of the 2nd and 3rd maxillipeds (mxp2, 3) and in the posterior part of the ganglion of the 1st maxilliped segment (mxp1). The anterior expression boundary of UBX is parasegmental. In addition, there is a weak expression in the forming trunk segments. No limbs are stained, which might be due to penetration problems through the well-developed cuticle.

(concerning length, overall shape, and the occurrence of five endopodal articles), the expression, at least in early stages, was also seen in this body segment. However, in the caridean shrimp, with a derived 3rd maxilliped (stout and only three endopodal articles; see, e.g., Bruce 2006), the anterior boundary of UBX expression was always behind the segment bearing the 3rd maxilliped. To test this phenomenon in dendrobranchiate decapods, we used the same antibody against the UBX-AbdA product (FP6.87) as Averof and Patel (1997) to study the expression of UBX in *Penaeus monodon* (Fig. 4). This species is characterized by a pediform 3rd maxilliped that still shows five endopodal segments and that is, compared to most pleocyemate species, still long and slender (Motoh 1981) (Fig. 3). In *Penaeus monodon* protozoea larvae, we find an anterior expression boundary of UBX in the forming nervous system slightly anterior to the 2nd maxilliped segment, which is the anteriormost expression found in a decapod to date (Figs. 4, 5). This result indicates that the specification of the 3rd maxilliped in dendrobranchiates has not reached the degree found in the other decapods and that most likely a 3rd maxilliped in the true sense was absent in the decapod stem species. It furthermore suggests that a true 3rd maxilliped evolved convergently several times within Decapoda. Interestingly enough, a closer look at the situation in the Amphionida, a possible candidate as the sister group to decapods (Richter and Scholtz 2001), supports this conclusion. This group possesses a well-defined maxilliped on the 1st thoracic segment and a reduced 2nd thoracic limb that nevertheless resembles the maxilliped in its overall shape. The 3rd to 8th thoracic appendages are all pereopods with a different morphology (Schram 1986).

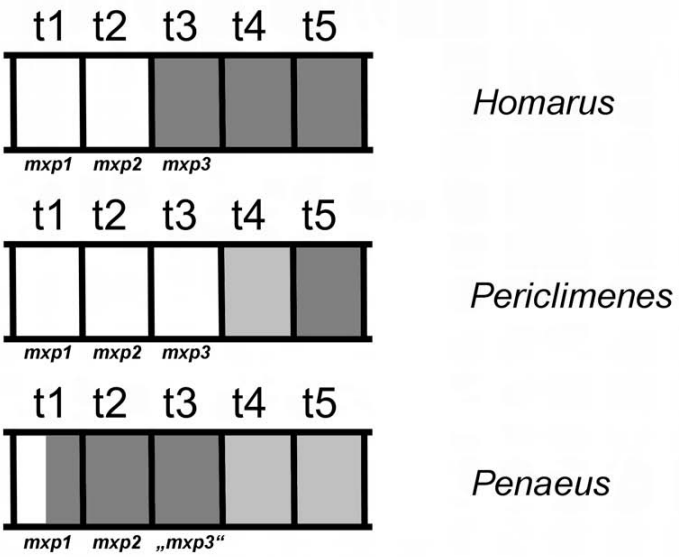

Figure 5. Scheme of the anterior expression of the UBX-AbdA protein in three decapod representatives with different degrees of pediform 3rd maxillipeds. *Homarus* and *Penaeus* with more pediform 3rd maxillipeds show a more anterior UBX expression boundary. *Penaeus* with the most pereopod-like 3rd maxilliped reveals the most anterior boundary in the 1st thoracic segment. *Homarus* and *Periclimenes* after Averof & Patel (1997), *Penaeus* this study. Light grey = weak expression, dark grey = strong expression. (mxp1,2,3 =1st to 3rd maxillipeds, t1 to t5 = 1st to 5th thoracic segments).

4 THE ORIGIN OF THE SCAPHOGNATHITE

The scaphognathite is a large flattened lobe at the lateral margin of the 2nd maxillae of decapods and amphionids (Fig. 6). The scaphognathite is equipped with numerous plumose setae at its margin and is closely fitted to the walls of the anterior part of the branchial chamber. This allows it to create a water current through the branchial chamber depending on the movement of the 2nd maxilla. This current supplies the gills with fresh oxygen-rich water for breathing. Hence, the scaphognathite is a crucial element of the gill/branchial chamber complex that is apomorphic for Decapoda (including Amphionida). The morphological nature and origin of this important structure, however, have been a matter of debate for more than a century. This relates to the general difficulty in assigning the elements of the highly modified decapod mouthparts to the parts of biramous crustacean limbs, such as the endopod, exopod, or epipods. Accordingly, several authors claim that the scaphognathite is a composite structure formed by the fusion of the exopod and epipod of the 2nd maxilla (Huxley 1880; Berkeley 1928; Gruner 1993). Huxley (1880) even discusses the alternative that it is exclusively formed by the epipod. In contrast to this, carcinologists such as Calman (1909), Giesbrecht (1913), Hansen (1925), Borradaile (1922), and Balss (1940) interpret the scaphognathite as of solely exopod origin. These different traditions are still expressed in recent textbooks (see Gruner 1993; Gruner & Scholtz 2004; Schminke 1996; Ax 1999). But Kaestner (1967: 1073) and Schram (1986: 245), discussing the morphology of decapod 2nd maxillae, state that “Homologie noch unklar!” (homology not clear) and “This appendage is so extensively modified that to suggest homologies with the various components of other limbs is a questionable exercise.”

We studied the development of the 2nd maxillae in the embryos of a freshwater crayfish, the parthenogenetic Marmorkrebs (Scholtz et al. 2003; Alwes & Scholtz 2006), applying the means

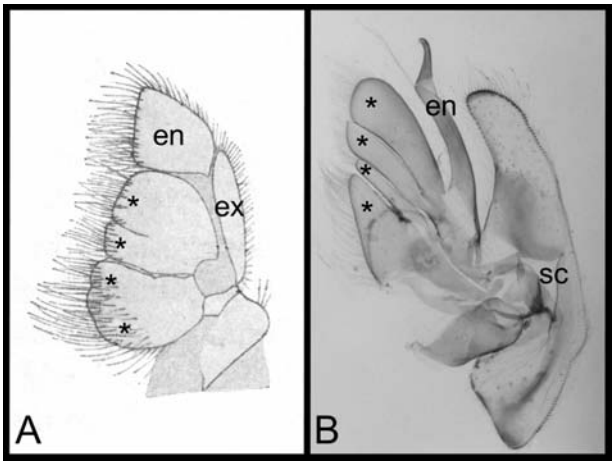

Figure 6. The shape and elements of the 2nd maxillae. (A) The 2nd maxilla of the euphausicaean *Meganyctiphanes norvegica* (after Zimmer & Gruner 1956). (B) The 2nd maxilla of the decapod *Axius glyptocereus*. The maxillae of both species show an endopod (en) and four enditic lobes (asterisks). The scaphognathite (sc) characteristic for decapods has such a special shape and function that the homology to the exopod (ex) in euphausiaceans and other malacostracans is controversial.

of histology, scanning electron microscopy, and immunochemistry (Distal-less) to clarify the issue of scaphognathite origins (Fig. 7). The Distal-less gene is involved in the adoption of a distal fate of limb cells in arthropods and is thus a marker for the distal region of arthropod limbs (e.g., Panganiban et al. 1995: Popadic et al. 1998; Scholtz et al. 1998; Williams 1998; Olesen et al. 2001; Angelini & Kaufman 2005). The early limb bud of the 2nd maxilla is undivided. After a short period, the tip of the bud shows a slight cleft that deepens with further development. This process is typical for the early development of crustacean biramous limbs (Hejnol & Scholtz 2004; Wolff & Scholtz 2008). The tips of the undivided limb buds, as well as the later-forming two separate tips, express Distal-less. Again, this is characteristic for biramous crustacean limbs and indicates that the two tips represent the exopod and endopod, since epipods do not express Dll (with the notable exception of the transient expression in epipods of *Artemia* and *Nebalia*, Averof & Cohen 1997; Williams 1998). With further development, the outer branch widens and grows in anterior and posterior directions, eventually adopting the characteristic lobed shape of the adult decapod scaphognathite (Fig. 7). In these later stages endopod and exopod still express Dll (Fig. 7D). A forming epipod is not recognizable at any stage of development, as is also revealed by the comparison to other limb anlagen which are equipped with an epipod.

Our results clearly support the idea that the scaphognathite of decapods is a transformed exopod and that an epipod is not involved in its formation. A comparison with other malacostracans reveals that in no case is the 2nd maxilla equipped with an epipod, but just endopods and exopods with different degrees of deviation from a "normal" limb branch. In addition, the overall shape of the scaphognathite is not so unusual for an exopod if we consider the shape of the exopods of phyllobranchious thoracic limbs in Branchiopoda and Leptostraca (Pabst & Scholtz 2009).

5 EMBRYONIC CHARACTERS HELP TO CLARIFY FRESHWATER CRAYFISH MONOPHYLY

Freshwater crayfish, Astacida, show a very disparate geographical distribution. In the Northern Hemisphere, the Cambaridae are found in East Asia and in the eastern part of North America, whereas the Astacidae occur in western Asia, Europe, and in the western parts of North America.

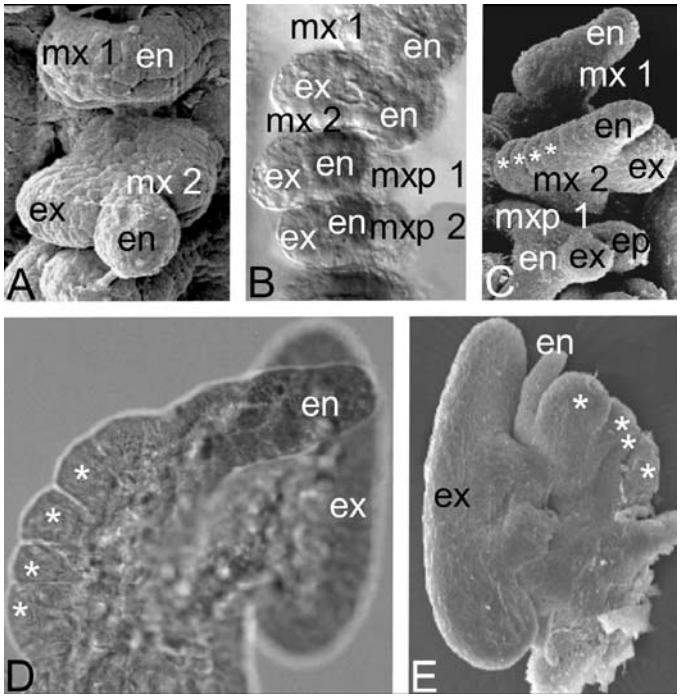

Figure 7. Development of the 2nd maxilla and the scaphognathite in the parthenogenetic Marmorkrebs (Astacida). (A) SEM image of the early 1st and 2nd maxillae (mx1, mx2) showing the forming two branches of the endopod (en) and exopod (ex) in the 2nd maxilla. (B) Expression of Distal-less (Dll) in early limb anlagen. Dll is expressed (darker areas) in the tips of the endopods (en) and exopods (ex) of the 2nd maxilla and the maxillipeds (mxp1, 2). The uniramous bud of the 1st maxilla (en) also expresses Dll. (C) SEM image showing the further differentiation of the parts of the 2nd maxilla (mx2). The four enditic lobes are forming (asterisks), and the exopod (ex) begins to form a lobe structure. The 1st maxilliped (mxp1) differentiates an epipod (ep), which finds no correspondence in the two maxillae. (D) Dll expression in an advanced stage. The expression (darker areas) is found in the tip of the endopod and around the margin of the exopod. The asterisks indicate the forming four enditic lobes. (E) SEM image of a 2nd maxilla shortly before hatching. The general shape of the adult maxilla is present (compare with Fig. 6).

Even if both groups, Astacidae and Cambaridae, are not monophyletic as has recently been suggested (Scholtz 1995, 2002; Crandall et al. 2000; Rode & Babcock 2003; Braband et al. 2006; Ahn et al. 2006), this distribution pattern is difficult to explain. The Parastacidae of the Southern Hemisphere live in Australia, New Zealand, some parts of South America, and Madagascar. Crayfish are absent from continental Africa. This is also true for the Indian subcontinent, and in more general terms, there is a crayfish-free circum-tropical zone. To explain this disparate distribution of freshwater crayfish, several hypotheses on the origin and evolution of crayfish have been discussed during the last 130 years. Most authors favored the idea that freshwater crayfish had multiple origins from different marine ancestors, i.e., are polyphyletic, and that they independently invaded freshwater many times (e.g., Huxley 1880; Starobogatov 1995; for review see Scholtz 1995, 2002). This view is based on the fact that freshwater crayfish do not tolerate higher salinities and that an explanation is needed for the occurrence of Astacida on most continents without the possibility of crossing large marine distances. Only Ortmann (1897, 1902) suggested a common origin for freshwater crayfish and a single invasion into freshwater habitats. He hypothesized East Asia as the center of origin from which Astacida spread all over the world, using assumed low sea levels to migrate to other continents (since the concept of continental drift was unknown at that time).

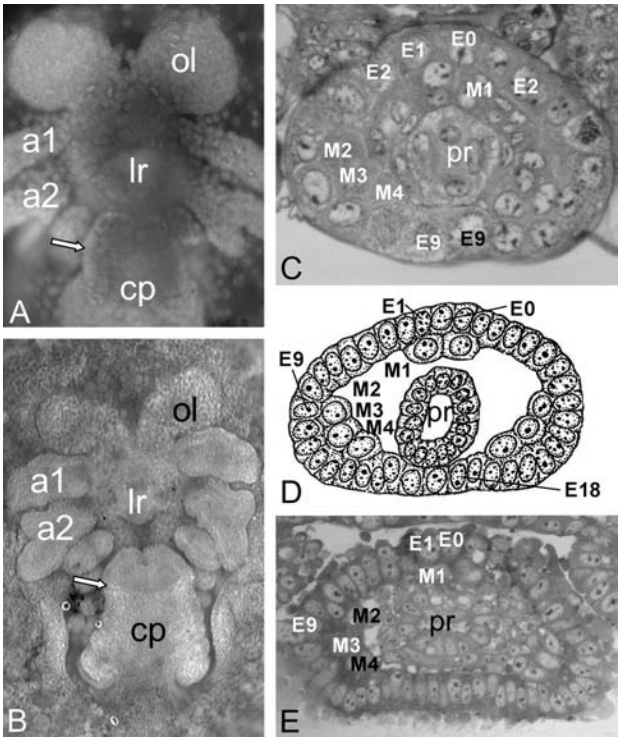

Figure 8. Teloblasts in decapod embryos. (A) Ventral view of the germ band of an embryo of the thalassinid *Callianassa australiensis*. The arrow indicates the area where the teloblasts form a ring (ectoderm and mesoderm) around the ventrally folded caudal papilla (cp). (a1, a2 = 1st and 2nd antennae, lr = labrum, ol = optic lobe). (B) Ventral view of the germ band of an embryo of the crayfish *Cambaroides japonicus* (labels as in A). Note the higher number of cells compared to A. (C) Transverse section through the caudal papilla of the American lobster *Homarus americanus* at the level of the teloblast rings; 19 ectoteloblasts (one unpaired E0 and nine paired E1 to E9 teloblast cells) and 8 mesoteloblast (four pairs in a specific arrangement) surround the forming proctodaeum (pr). (D) Transverse section through the caudal papilla of the Australian crayfish *Cherax destructor* at the level of the teloblast rings. In contrast to *Homarus*, there are about 40 teloblasts in the ectoderm. The mesoteloblasts show the same pattern as in the lobster. (E) Transverse section through the caudal papilla of the Japanese crayfish *Cambaroides japonicus* at the level of the teloblast rings. The pattern in this Northern Hemisphere crayfish is the same as in the Southern Hemisphere representative *Cherax* (after Scholtz 1993; Scholtz & Kawai 2002).

The investigation on cell division patterns in the germ band of embryos of the Australian freshwater crayfish *Cherax destructor* produced the surprising result that the growth zone of this species differs from that of all other malacostracan crustaceans studied so far in this respect (Scholtz 1992). The growth zone of malacostracans is situated in the posterior region of the embryo, immediately anterior to the telson anlage. It is formed by large specialized cells, the teloblasts, which bud off smaller cells only toward the anterior (see Dohle et al. 2004) (Fig. 8). This stem-cell-like cell type occurs in the ectoderm (ectoteloblasts) and the mesoderm (mesoteloblasts), and both sets of teloblasts produce most of the ectodermal and mesodermal material of the post-naupliar germ band. In the ground pattern of Malacostraca, we find 19 ectoteloblasts and 8 mesoteloblasts in circular arrangements (Dohle et al. 2004) (Fig. 8C). These figures are also present in most decapods studied in this respect, such as caridean shrimps, Achelata, Homarida, Thalassinida, Anomala, and Brachyura (Oishi 1959, 1960; Scholtz 1993). In contrast to this, in the freshwater crayfish *Cherax destructor* an individually variable number of more than 40 ectoteloblasts occurs, whereas the 8 mesoteloblasts

are conserved (Fig. 8D). Subsequent studies in other crayfish species from the Northern and Southern Hemispheres covering Astacidae, Cambaridae, and Parastacidae revealed that the pattern found in *Cherax* is a general freshwater crayfish character (Scholtz 1993) (Fig. 8E). This different growth zone pattern is hence a clear apomorphy of the Astacida, strongly indicating their monophyly.

This result is corroborated by a number of other developmental, in particular postembryonic, characters (see Scholtz 2002). In addition, phylogenetic analyses based on molecular datasets strongly support the monophyly of Astacida (e.g., Crandall et al. 2000; Ahyong & O'Meally 2004; Tsang et al. 2008). The question of freshwater colonization can now be addressed anew based on the strong support for Astacida monophyly. Monophyly alone is, of course, no proof for a single invasion into freshwater habitats, but parsimony and, in particular, several apomorphic freshwater adaptations strongly argue for a crayfish stem species already living in freshwater (see Scholtz 1995, 2002; Crandall et al. 2000). The modern and almost worldwide distribution of Astacida is thus best explained by the assumption of a freshwater colonization during the Triassic or even earlier before the break-up of Pangaea, which started in the Jurassic (Scholtz 1995, 2002).

6 CONCLUSIONS

With these examples, we demonstrate the different levels of impact on our views on decapod evolution resulting from comparative developmental studies (see Scholtz 2004). Including developmental characters in phylogenetic analyses expands our suite of characters for phylogenetic inference. In some cases, ontogenetic characters can be decisive in resolving phylogenetic relationships that cannot be inferred from adult characters alone. An example of this is the resolution of the common origin of astacoidean and parastacoidean crayfish. However, based on ontogenetic data, far-reaching conclusions can be drawn. For instance, the morphological "nature" of adult structures can be clarified with developmental analyses. This touches the core of morphology as a science. Morphological structures are transformed in the course of evolution; they change form and function to various degrees. In addition, new structures (novelties) emerge. These are, however, formed by pre-existing morphological precursors. Developmental analyses offer the possibility to trace these transformations and novelties. The analyses presented here of the 3rd maxillipeds and the scaphognathite of the 2nd maxillae in decapods provide examples for this approach. In the latter case, a century-old controversy was resolved and the evolutionary flexibility of limb structures was shown. In the former case, the correlation between an evolutionary shift of gene expression and altered morphology and function is revealed. Furthermore, evolutionary scenarios can be inferred based on ontogenetic data. This is shown by the timing of the gene expression shift. The transformation of a thoracic limb to a mouthpart takes place at the morphological and functional levels before gene expression has changed to the same degree (see Budd 1999). As is the case in adult structures, several ontogenetic characters are correlated with a certain lifestyle. If these characters are shared between an outgroup and part of the ingroup, it is possible to deduce the ancestral lifestyle of a given taxon. This approach is exemplified by the analysis of the early development of Dendrobranchiata. Yolk-poor eggs with a distinct cleavage pattern are found in shrimp-like crustaceans with a more pelagic lifestyle and a lack of brood care, such as euphausiaceans and, to a certain degree, anaspidaceans. This allows the conclusion that the decapod stem species was a pelagic shrimp-like animal rather than a benthic reptantian and thus strongly corroborates inferences based on the morphology of adults.

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Mitochondrial DNA and Decapod Phylogenies: The Importance of Pseudogenes and Primer Optimization

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ABSTRACT

Not much more than fifteen years ago, the first decapod phylogenies based on mitochondrial DNA (mtDNA) sequences revolutionized decapod phylogenetics. Initially, this method was accepted only reluctantly. However, a wider understanding of the methods, and the realization that credibility of specific branching patterns can be measured by statistic confidence values, allowed the recognition of molecular systematics as just another phylogenetic approach, in which homologous characters are compared and interpreted in terms of apomorphic or plesiomorphic status, and best possible trees are calculated based on distances, parsimony, or likelihoods. Similar to morphological characters, some of the shared molecular characters can result from convergence, but the large quantity of potential characters to be compared (15,000–17,000 in mtDNA) promises to reveal phylogenetic signal. For many years, preference was given to mitochondrial genes among the molecular markers, because of the relative ease with which they can be amplified (stable and numerous copies per cell) and interpreted (because they are only maternally inherited and lack introns and recombination), and because of higher mutation rates and thus greater variability than nuclear DNA. More recently, some of these apparent advantages were interpreted as shortcomings of mtDNA, and the discovery of selective sweeps, mitochondrial introgressions, and nuclear copies of mtDNA (numts) have questioned the credibility of phylogenies based exclusively on mtDNA. Here, I revisit the history and importance of mtDNA-based phylogenies of decapods, present two examples of how numts can produce erroneous phylogenies, and emphasize the need for primer optimization for better PCR results and avoidance of numts. Mitochondrial DNA has distinct advantages and disadvantages and, if used in combination with other phylogenetic markers, is still a very effective tool for phylogenetic inference. In most cases, and when used with the necessary care, phylogenies and phylogeographies based on mtDNA will render absolutely reliable results that can be tested and confirmed with other molecular and non-molecular approaches.

1 INTRODUCTION

Only a few years after the first publications announced the potential use of mitochondrial DNA for animal phylogenetics and population studies (e.g., Avise et al. 1987; Cann et al. 1987; Moritz et al. 1987) and the mitochondrial genome organization in *Artemia* was described (Batuecas et al. 1988), Cunningham et al. (1992) and Knowlton et al. (1993) published the first mtDNA-based phylogenies for Crustacea. It is noteworthy that these studies were based on sequences of the genes corresponding to the large ribosomal subunit 16S rRNA (16S; Cunningham et al. 1992) and the cytochrome oxidase subunit 1 (Cox1; Knowlton et al. 1993). Up to now, sequences of these genes continue to predominate in molecular phylogenetic studies of Crustacea, even though in many other animal taxa (including humans) other genes, like cytochrome b or the variable mitochondrial control region, have experienced at least a similarly wide use.

The proposal of Cunningham et al. (1992) that king and stone crabs (Anomura: Lithodidae) not only evolved from within the hermit crabs, but from within the genus *Pagurus*, cast a lot of doubt on the methodology and did not help to make the approach very popular among decapod crustacean systematists, causing a lot of skepticism concerning molecular phylogenies in general. For many years, it appeared that evolutionary biologists with molecular methods and taxonomists with morphological methods would continue their research separately. Consequently, there were only a few decapod molecular phylogenies published in the following years, most of them dealing with specific groups with special life history traits (Levinton et al. 1996; Patarnello et al. 1996; Sturmbauer et al. 1996; Tam et al. 1996; Kitaura et al. 1998; Schubart et al. 1998a; Tam & Kornfield 1998), rather than with phylogeny and taxonomy per se. Only in Crandall et al. (1995) and Crandall & Fitzpatrick (1996), and in subsequent papers on crayfish systematics and phylogeny (Ponniah & Hughes 1998; Lawler & Crandall 1998), was there an explicit goal to establish molecular systematics, which only Spears et al. (1992) had undertaken previously for decapods, by proposing phylogenetic relationships among brachyuran crabs using nuclear 18S.

This slowly changed as species descriptions became based on, or were accompanied by, mitochondrial DNA data (Daniels et al. 1998; Schubart et al. 1998b, 1999; Gusmão et al. 2000; Macpherson & Machordom 2001, Daniels et al. 2001; Guinot et al. 2002; Guinot & Hurtado 2003; Gillikin & Schubart 2004; Lin et al. 2004, and later papers), when species were synonymized based on mtDNA in the absence of morphological characters (Shih et al. 2004; Robles et al. 2007; Mantelatto et al. 2007), and especially when phylogenetic relationships within genera and families were reconstructed with mtDNA in order to establish new taxonomic classifications (Schubart et al. 2000a, 2002; Kitaura et al. 2002; Tudge & Cunningham 2002; Chu et al. 2003; Lavery et al. 2004; Klaus et al. 2006; Schubart et al. 2006). Only recently, mtDNA has been used as part of multi-locus studies to reconstruct phylogenies at higher levels within decapod Crustacea (Ahyong & O'Meally 2004; Porter et al. 2005; Daniels et al. 2006).

For this kind of higher-level taxonomy, the exclusive use of mitochondrial DNA as a molecular marker is inappropriate (see Schubart et al. 2000b). This is due to the fact that mtDNA is characterized by a relatively high mutation rate, which makes it very useful at low taxonomic levels (intraspecific to intrafamilial levels) but causes increasing saturation when older splits are analyzed. When that occurs, the ratio between "phylogenetic noise," mostly caused by molecular convergence (homoplasy), and phylogenetic signal becomes more and more unfavorable and restricts the use of mtDNA at these levels. Therefore, and because of other potential problems of mtDNA (see Discussion), today the combination of mtDNA with more conserved nuclear markers is essential when reconstructing higher order phylogenies.

mtDNA still has many advantages over nuclear DNA. First, its ring-shaped structure makes it a more stable molecule than the chromosomes in the nucleus. Furthermore, there are hundreds to thousands of mitochondrial genomes per cell (with up to 10 copies per mitochondrion, see Wiesner et al. 1992), whereas there is only one nuclear genome per cell. This makes mtDNA much easier to amplify than nuclear DNA (nDNA), and DNA quality becomes a less critical issue than it is for nDNA. As a result, it is now possible to sequence mtDNA from museum specimens that were preserved in ethanol 150 years ago (e.g., Schubart et al. 2005) or longer, something that would be much more difficult with nDNA. mtDNA is also characterized by the absence of introns, so that basically all DNA is informative. Nevertheless, mutation rates are much higher in mtDNA than in nDNA, allowing phylogenetic signal to accumulate at shorter time frames. The fact that mtDNA appears to not have recombination, and in most cases is only maternally inherited, makes its interpretation much easier and allows for extrapolation, as for example in the calibration of molecular clocks. More recently, the increasing number of multiple gene sequencing of mitochondrial genomes (many of them complete) and their comparison allows the detection of gene rearrangements that may be used to support phylogenetic conclusions (mitogenomics) (e.g., Hickerson & Cunningham 2000; Kitaura et al. 2002; Morrison et al. 2002).

After having listed these well-known and traditionally accepted advantages of mtDNA, below I will discuss potential disadvantages of mtDNA for the reconstruction of decapod crustacean phylogenies. This will be exemplified by the presentation of new data on pseudogenes and a subsequent discussion of their consequences and ways of avoiding them.

2 MATERIALS & METHODS

Samples of three species of the genus *Cardisoma* (Brachyura: Thoracotremata: Gecarcinidae) were collected or obtained between 1996 and 2005 from both tropical American coastlines and from western Africa (Table 1). The goal was to establish genetic differentiation between the western African species *C. armatum* Herklots, 1851, and both American species, *C. guanhumi* Latreille, 1828 (western Atlantic), and *C. crassum* Smith, 1870 (eastern Pacific). In a second study, we used single specimens of *Geryon trispinosus* (Herbst, 1803), *G. longipes* (A. Milne-Edwards, 1882), and *Chaceon granulatus* (Sakai, 1978) as part of a study investigating phylogenetic relationships within the Geryonidae and the superfamily Portunoidea (see Schubart & Reuschel this volume). Molecular studies were carried out at the University of Regensburg. DNA was extracted with the Gentra Systems buffer combination. After discovering multiple copies and strongly deviating products in some of our sequencing products, mtDNA enrichment techniques were applied during extractions, such as differential centrifugation in a saccharose gradient and a Triton X-100 treatment (see Burgener & Hübner 1998 and discussion below). This allowed us to work with two separate fractions from the same individual, one with potentially enriched mtDNA, the other with enriched nDNA. Selective amplification of an approximately 580-basepair region of the mitochondrial large ribosomal subunit 16S rRNA was carried out by PCR. Primers used were 16L29, 16L12, 1472, 16H10, 16H12 (see Tables 2, 3). In order to obtain clean sequences from otherwise mixed PCR products in *Cardisoma*, we designed specific primers for the presumed mtDNA (16L13J: 5'-TGTAGATATAAAGAG TTTAA-3') and the presumed nuclear derivate (16L13P: 5'-TGTAGATATAAAGAGTTTAG-3') for PCR and sequencing reactions. These primers differ only in the last nucleotide (3'-end) and should preferentially anneal to one of the two available products.

PCR amplifications were carried out with four minutes denaturation at 94°C, 40 cycles, with 45 s 94°C, 1 min 48°C, 1 min 72°C, and 10 min final denaturation at 72°C. PCR products were purified with Microcon 100 filters (Microcon) or Quick-Clean (Bioline) and then sequenced with the ABI BigDye terminator mix followed by electrophoresis in an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). Forward and reverse strands were obtained for most products. New sequence data were submitted to the European molecular database EMBL (see Table 1 for accession numbers). In addition, the following sequences from the molecular database were included in our analyses: *Cardisoma guanhumi* (Z79653, from Levinton et al. 1996), *Cardisoma crassum* (AJ130805, from Schubart et al. 2000b), *Chaceon quinquedens* (Smith, 1879) and *C. fenneri* (Manning & Holthuis, 1984) (AY122641 to AY122646 from Weinberg et al. 2003) and *Chaceon affinis* (A. Milne-Edwards & Bouvier, 1894) (AF100914 to AF100916 from Weinberg et al. 2003 and previously unpublished by J. Bautista and Y. Alvarez).

Sequences were aligned and corrected manually with BioEdit (Hall 1999) or XESEE 3.2 (Cabot and Beckenbach 1989). The model of DNA substitution that best fit our data was determined using the software MODELTEST 3.6 (Posada and Crandall 1998). Reconstruction of phylogenetic trees with the corresponding models (TrN+I for *Cardisoma*; TVM+I+G for Geryonidae) in a Bayesian inference analysis (BI) with MrBayes v. 3.0b4 (Huelsenbeck and Ronquist 2001) and without models in a maximum parsimony analysis (MP) with PAUP* (Swofford 2001) revealed that the majority of genetic differences at the interindividual level were so small that the position of most operational taxonomic units was unresolved in major consensus clades. Therefore, a distance-based reconstruction with minimum evolution (ME) (Rzhetsky & Nei 1992) and Maximum Composite Likelihood as implemented in MEGA4 (Tamura et al. 2007) was carried out with 2000 bootstrap pseudoreplicates

Table 1. Crab specimens used for phylogenetic reconstruction of pseudogenes with locality of collection, museum catalogue number for vouchers, and genetic database accession numbers.

Species	Collection Locality	Coll. Date	voucher	mtDNA	numt
Cardisoma					
Cardisoma guanhumi R40	Jamaica (St. Ann): Priory	8 Oct. 2000	SMF 32773	n.a.	FM 208132
Cardisoma guanhumi CA1	Jamaica (Hanover): Negril	14 Oct. 2005	leg	FM 208123	FM 208133-35
Cardisoma guanhumi CA2	Jamaica (Hanover): Negril	14 Oct. 2005	SMF 32745	FM 208123	FM 208136-37
Cardisoma guanhumi CA3	Jamaica (St. James): Montego Bay	Oct. 2005	leg	FM 208124	FM 208132
Cardisoma guanhumi CA21	Jamaica (Trelawny): Glistening W.	22 March 2003	SMF 32772	FM 208124	n.a.
Cardisoma guanhumi CA27	Jamaica (Hanover): Negril	14 Oct. 2005	leg	FM 208123	n.a.
Cardisoma guanhumi	Cuba (Pinar de Río): El Rosario	21 Sept. 1999	SMF 25747	FM 208123	n.a.
Cardisoma guanhumi	Honduras (Islas de la Bahía): Utila	18 Aug. 2000	SMF 26006	FM 208123	n.a.
Cardisoma guanhumi	Panama (Caribbean): La Galeta	3 March 1996	ULLZ 3796	FM 208123	FM 208129-31
Cardisoma armatum tradeSG	West Africa (from aquarium trade)	1992	ZRC 1996.121	FM 208125	208127
Cardisoma armatum tradeD	West Africa (from aquarium trade)	2000	leg	FM 208126	208128
Cardisoma armatum R13	Ghana: Elmina	3 July 2001	SMF 27534	FM 208125	n.a.
Cardisoma crassum	Costa Rica: Rincón	18 March 1996	SMF 24543	AJ130805	n.a.
Geryonidae					
Geryon longipes	Spain (Ibiza): Sta. Eulalia fish market	28 March 2001	SMF 32747	FM 208120	FM 208119
Geryon trispinosus	North Sea: Flade Grounds	2000	SMF 32746	FM 208121	
Chaceon bicolor	Singapore fish market	2000	ZRC 2000.2830	FM 208122	
Chaceon granulatus	Japan		SMF 32762	FM 208775	

SMF: Senckenberg Museum, Frankfurt a.M.; ULLZ: University of Louisiana at Lafayette Zoological Collection, Lafayette.
ZRC: Zoological Reference Collection, Raffles Museum, National University of Singapore.

Table 2. Decapod-specific primers used for amplification of the 16S rRNA–tRNA$_{\mathrm{Leu}}$–NDH1 complex and of the Cox1 gene.

16S towards NDH1:
16L2: 5′–TGCCTGTTTATCAAAAACAT–3′ (Schubart et al. 2002)
16L12: 5′–TGACCGTGCAAAGGTAGCATAA–3′ (Schubart et al. 1998)
16L12b: 5′–TGACYGTGCAAAGGTAGCATAA–3′ (new)
16L15: 5′–GACGATAAGACCCTATAAAGCTT–3′ (Schubart et al. 2000c)
16L29: 5′–YGCCTGTTTATCAAAAACAT–3′ (Schubart et al. 2001 as "16L2")
16L6: 5′–TTGCGACCTCGATGTTGAAT–3′ (new)
16L37: 5′–TTACATGATTTGAGTTCARACCGG–3′ (new)
16L11: 5′–AGCCAGGTYGGTTTCTATCT–3′ (new)
16LLeu: 5′–CTATTTTGKCAGATDATATG–3′ (new)

NDH1 towards 16S:
NDH4: 5′–CAAGCYAAATAYATYARCTT–3′ (new)
NDH2: 5′–GCTAAATATATWAGCTTATCATA–3′ (new)
NDH5: 5′–GCYAAYCTWACTTCATAWGAAAT–3′ (new)
NDH1: 5′–TCCCTTACGAATTTGAATATATCC–3′ (new)
16HLeu: 5′–CATATTATCTGCCAAAATAG–3′ (new)
16H10: 5′–AATCCTTTCGTACTAAA–3′ (new)
16H11: 5′–AGATAGAAACCRACCTGG–3′ (new)
16H37: 5′–CCGGTYTGAACTCAAATCATGT–3′ (Klaus et al. 2006)
16H6: 5′–TTAATTCAACATCGAGGTC–3′ (new)
16H12: 5′–CTGTTATCCCTAAAGTAACTT–3′ (new)

Cox1 forward (L) and reverse (H):
COL6: 5′-TYTCHACAAAYCATAAAGAYATYGG-3′ (new, substitute COL1490)
COL14: 5′-GCTTGAGCTGGCATAGTAGG-3′ (Roman & Palumbi 2004, unnamed)
COL19: 5′-ATAGTAGAAAGAGGRGTWGG-3′ (new)
COL7: 5′-GGTGTKGGMACMGGATGAACTGT-3′ (new)
COL8: 5′-GAYCAAATACCTTTATTTGT-3′ (new)
COL4: 5′-TAGCHGGDGCWATYACTAT-3′ (new)
COL12: 5′-GCHATTACTATACTTCTWACWGAYCG-3′ (new)
COL1b: 5′-CCWGCTGGDGGWGGDGAYCC-3′ (new, substitute for COIf)
COL3: 5′-ATRATTTAYGCTATRHTWGCMATTGG-3′ (Reuschel & Schubart 2006)
COH7: 5′-TGWARAGAAAAAATTCCTA-3′ (new)
COH14: 5′-GAATGAGGTGTTTAGATTTCG-3′ (Roman & Palumbi 2004, unnamed)
H7188: 5′-CATTTAGGCCTAAGAAGTGTTG-3′ (Knowlton et al. 1993)
COH6: 5′-TADACTTCDGGRTGDCCAAARAAYCA-3′ (Schubart & Huber, 2006, substitute HCO2198)
COI(10): 5′-TAAGCGTCTGGGTAGTCTGARTAKCG-3′ (Baldwin et al. 1998)
COH3: 5′-AATCARTGDGCAATWCCRSCRAAAAT-3′ (Reuschel & Schubart 2006)
COH8: 5′-TGAGGRAAAAAGGTTAAATTTAC-3′ (new)
COH4: 5′-GGYATACCRTTDARTCCTARRAA-3′ (Mathews et al. 2002)
COH12: 5′-GGYATACCRTTTARTCCTAARAA-3′ (new, substitute for COH4)
COH1b: 5′-TGTATARGCRTCTGGRTARTC-3′ (new, substitute for COIa)
COH18: 5′-CTA TGG AAG ATA CGA TGT TTC-3′ (Reuschel & Schubart 2007)
COH16: 5′-CATYWTTCTGCCATTTTAGA-3′ (new)