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Mitochondrial gene rearrangements confirm the parallel evolution of the crab-like form

C. L. Morrison[1], A. W. Harvey[2], S. Lavery[3], K. Tieu[4], Y. Huang[4] and C. W. Cunningham[4*]

[1]*Department of Fisheries Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA*
[2]*Department of Biology, Georgia Southern University, Statesboro, GA 30460, USA*
[3]*School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand*
[4]*Department of Biology, Duke University, Durham, NC 27708, USA*

The repeated appearance of strikingly similar crab-like forms in independent decapod crustacean lineages represents a remarkable case of parallel evolution. Uncertainty surrounding the phylogenetic relationships among crab-like lineages has hampered evolutionary studies. As is often the case, aligned DNA sequences by themselves were unable to fully resolve these relationships. Four nested mitochondrial gene rearrangements—including one of the few reported movements of an arthropod protein-coding gene—are congruent with the DNA phylogeny and help to resolve a crucial node. A phylogenetic analysis of DNA sequences, and gene rearrangements, supported five independent origins of the crab-like form, and suggests that the evolution of the crab-like form may be irreversible. This result supports the utility of mitochondrial gene rearrangements in phylogenetic reconstruction.

Keywords: carcinization; parallel evolution; convergence; mitochondrial rearrangements

1. INTRODUCTION

The true crabs (Brachyura) and the Alaskan king crabs (Lithodidae, Anomura) are so similar that laymen have difficulty recognizing them as distinct groups. This is a fact even though true crabs have persisted for 320 million years (Myr), whereas the king crabs arose recently from hermit crab ancestors within the last 20 Myr (Cunningham *et al.* 1992). Although the origin of similar forms in very different lineages is commonly reported, many cases can be attributed to selection for a particular lifestyle or environment. This is true for many well-known cases of convergence, including between marine predators such as dolphins, sharks and ichthyosaurs, or between birds and bats. More enigmatic are cases in which a group of organisms seem to have a tendency to produce the same form or structures repeatedly, but in which the basis for selection is not so obvious. Such an innate tendency can be considered a form of parallel evolution (Simpson 1961).

Unlike cases of convergent evolution, the repeated evolution of the crab-like form (i.e. a broad, fully calcified carapace and a reduced abdomen tucked forward under the thorax) is not associated with any particular environment or lifestyle. Members of the three major groups of crabs are found from abyssal trenches to pelagic, freshwater and terrestrial environments. Because the crab-like form is not associated with any obvious selective forces, Borradaile (1916) argued that because most crab-like groups are found in the infraorder Anomura, '[t]he conclusion seems to be inevitable that there is in the constitution of the Anomura a disposition or tendency...to achieve that special conformation of body which constitutes a crab, and such is not the case with other Decapoda'. Unfortunately, this very tendency towards parallel evolution has made it difficult to interpret relationships among anomuran groups (McLaughlin 1983; Martin & Abele 1986). To further study the evolution of the crab-like form, we have used two sources of phylogenetic information: mitochondrial gene rearrangements and DNA sequence data.

Despite the remarkable conservation of mitochondrial gene order across most arthropods—with the primitive gene order in each of the major arthropod groups differing by only a single tRNA gene (Boore *et al.* 1998)—our laboratory has previously identified at least seven gene rearrangements within the Anomura (Hickerson & Cunningham 2000; see also Hickerson & Cunningham 2002). These rearrangements include two movements of protein-coding genes, a phenomenon unknown in the Arthropoda except for certain tick lineages (Campbell & Barker 1999). Because gene rearrangements are so rare in arthropods, they have the potential for resolving phylogenetic relationships among the groups in which they exist (Boore *et al.* 1998). We have investigated the phylogenetic distribution of four gene rearrangements in concert with a molecular phylogenetic analysis of two mitochondrial and two nuclear genes.

2. MATERIAL AND METHODS

(a) *Species list, amplification and sequencing*

For this analysis, we obtained sequences from 26 decapods, including *Procambarus clarkii, Callichirus major, Neotrypaea californiensis, Hepatus epheliticus, Raninoides louisianensis, Panulirus argus, Jaxea nocturna, Upogebia affinis, Pachycheles rudis,*

* Author for correspondence (cliff@duke.edu).

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Petrolisthes armatus, Eumunida sternomaculata, Munida quadrispina, Aegla uruguayana, Lomis hirta, Blepharipoda occidentalis, Emerita analoga, Lepidopa californicus, Cryptolithodes typicus, Discorsopagurus schmitti, Pagurus longicarpus, Paguristes turgidus, Isocheles pilosus, Calcinus obscurus, Clibanarius albidigitus, Coenobita compressus and *Birgus latro.* Genomic DNA was isolated from muscle tissue taken from the claws or walking legs of anomurans and outgroups using either phenol/chloroform or GNOME (Bio 101; Vista, CA) extractions. PCR amplification was performed by standard procedures as described by Hickerson & Cunningham (2000).

Four regions were sequenced for the phylogenetic analysis with primers from Simon *et al.* (1994), except where indicated. The mitochondrial *COII* was amplified using C2-J-3138 (5′-AGAGCTTCACCCTTAATAGAGCAA-3′) and C2-N-3661 (5′-CCACAAATTTCTGAACATTGACCA-3′); mitochondrial 16S rDNA was amplified using LR-N-13398 (5′-CGCCTG TTTAACAAAAACAT-3′) and LR-J-12887 (5′-CCGGTCTG AACTCAGATCACGT-3′); nuclear 18S rDNA was amplified using 18E-F (5′-CTGGTTGATCCTGCCAGT-3′) and 18SR3′ (5’-TAATGATCCTTCCGCAGGTT-3′); nuclear 28S rDNA was amplified using OI (5′-GTCTTTGCGAAGAAGAACA-3′) and DIB (5′-AGCGGAGGAAAAGAAACTAAC-3′; both primers, R. DeSalle, personal communication).

Following purification of PCR products (Wizard PCR kit, Promega), cycle sequencing was performed with ABI PRISM BigDye terminator chemistry and analysed on ABI 373 or 377 automated sequencers (ABI/Perkin-Elmer, Princeton, NJ).

(b) *Assaying four mitochondrial gene rearrangements*

We assayed selected taxa for four gene rearrangements. Rearrangements 1–3 (see § 3) were previously identified in the genome of the hermit crab *P. longicarpus* (Hickerson & Cunningham 2000), rearrangement 4 was identified by comparison to a partial sequence of the mitochondrial genome of the hermit crab *C. albidigitus* (4830 bases, GenBank accession AF425321; see § 3).

To assay for the rearrangements we sequenced the indicated regions. Rearrangements 1 and 2: the region spanning the 3′ end of 12S rDNA to the 5′ end of *COI* (primers 12Sair 5′-ATAATAGGGTATCTAATCCTAGTTT-3′ and COIR3 5′-GTSGARAAAARTCATCGTTTCG-3′, C.W.C. laboratory). Rearrangement 3: the region spanning ND1–16S (primers N1-J-12585 5′-GGTCCCTTACGAATTTGAATATCCT-3 and LR-J-12887, see above for sequence) and/or the region spanning *COI–COII* (primer C1-J-2183 5′-CAACATTTATTTTGAT TTTTTGG-3′ and C2-N-3661, sequence above). Rearrangement 4: we sequenced the region spanning *COI–COII* using primers 2797 and C2-N-3661 (5′-CCACAAATTTCTGAA CATTGACCA-3′).

(c) *Phylogenetic analysis*

Sequences were aligned using ClustalX (Thompson *et al.* 1997), and regions of uncertain homology were excluded from the analysis. The maximum-likelihood model used was the best-fit model found by Modeltest (Posada & Crandall 1998), which was a general time-reversible model with estimates of invariant sites and a γ-distribution estimated using Paup* 4.0 (Swofford 1999).

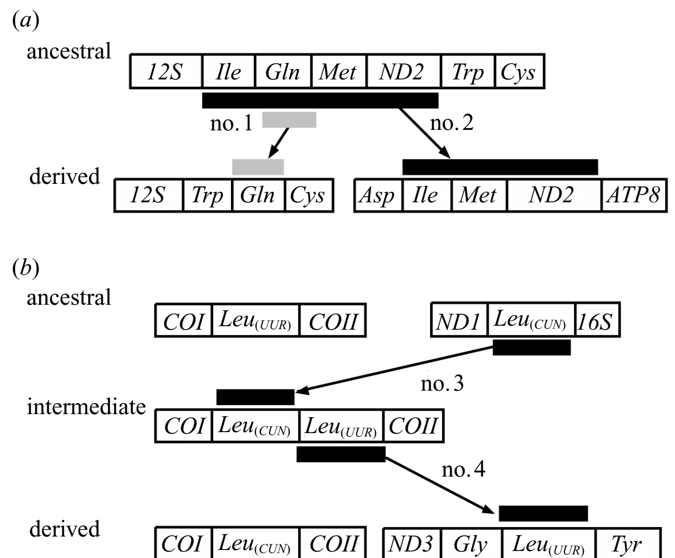

Figure 1. Four mitochondrial gene rearrangements, the distribution of which was surveyed within the Anomura and several outgroups. The ancestral gene order shown is found in several insects and crustaceans (Crease 1999). (*a*) Rearrangements 1 and 2 always appear together in the same taxa; (*b*) rearrangements 3 and 4 take place sequentially, so that the intermediate form of the two adjacent leucine (Leu) tRNAs were observed in several taxa (see figure 3).

3. RESULTS

(a) *Mitochondrial gene rearrangements*

The four rearrangements we assayed are shown in figure 1. The most parsimonious explanation for the observed pattern of gene arrangements is that each occurred only once, and that each defines specific nodes in the phylogeny. The presence or absence of rearrangements 1 and 2 was assayed for 19 taxa: *Procambarus* (AF436024), *Callichirus* (AF436025, AF436026), *Neotrypaea* (AF436027), *Hepatus* (AF436028), *Raninoides* (AF436029), *Panulirus* (AJ133049), *Jaxea* (AF436030), *Petrolisthes* (AF436031, AF436032), *Munida* (AF436033), *Aegla* (AF436034), *Lomis* (AF436035), *Blepharipoda* (AF436036), *Emerita* (AF425302), *Lepidopa* (AF436037), *Cryptolithodes* (AF425304), *Pagurus* (NC_003058), *Paguristes* (AF436038) *Calcinus* (AF436039), *Clibanarius* (AF425321).

The presence or absence of rearrangements 3 and 4 was assayed for 19 taxa: *Procambarus* (AF436040), *Callichirus* (AF436041, AF437614), *Hepatus* (AF436043, AF437616), *Panulirus* (AJ133050), *Jaxea* (AF436046, AF437618), *Upogebia* (AF436047), *Pachycheles* (AF436048), *Petrolisthes* (AF436049), *Aegla* (AF436051), *Blepharipoda* (AF436053, AF437625), *Emerita* (AF425322), *Cryptolithodes* (AF425325), *Discorsopagurus* (AF436055, AF437627), *Pagurus* (NC_003058), *Paguristes* (AF436056, AF437628), *Isocheles* (AF436057), *Calcinus* (AF437629), *Clibanarius* (AF425362), *Coenobita* (AF436059, AF437630).

(b) *Phylogenetic analysis*

We constructed a phylogeny for 26 decapod taxa based on 2764 DNA base pairs from four nuclear and mitochondrial genes not involved in the gene rearrangements (figure 2).

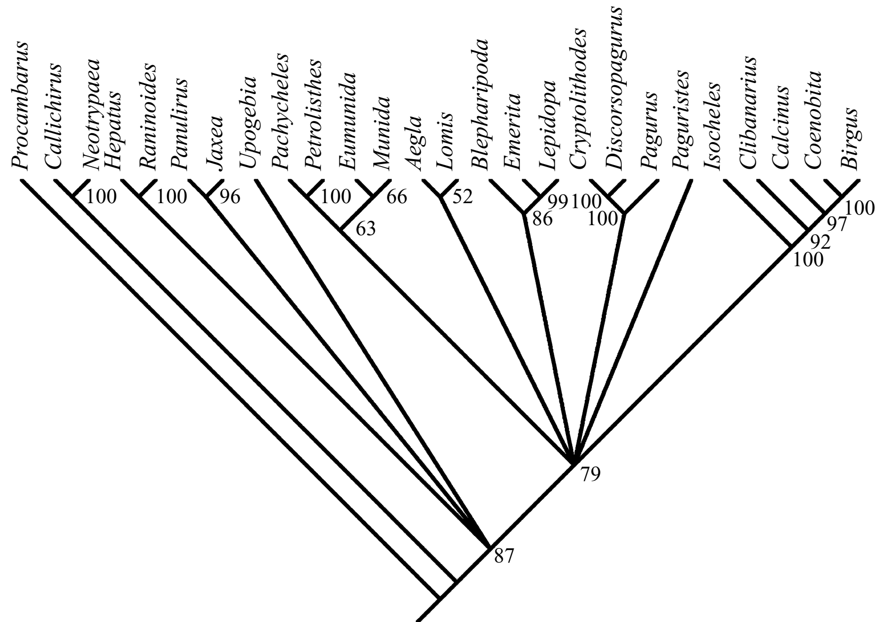

Figure 2. Unconstrained maximum-likelihood phylogeny for four genes with a best-fit model (general time-reversible with invariant sites plus γ; Yang 1994; Gu *et al.* 1995), implemented using PAUP* 4.0b4a (Swofford 1999). Full species names are given in § 2. Numbers at nodes indicate bootstrap support greater than 50% in 100 pseudoreplicates, and nodes are collapsed that show less than 50% bootstrap support.

In order to polarize the relationships of the putatively anomuran groups (Thalassinidea, Galatheoidea, Hippoidea, Paguroidea and Coenobitoidea), four outgroups were chosen from three decapod groups that had always been considered to fall outside the Anomura: the Astacidea (crayfish), Brachyura (true crabs) and Palinura (spiny lobsters). According to the morphological analysis of Scholtz & Richter (1995), the Astacidea (represented by *Procambarus*) is the sister group to the remaining taxa in this analysis, although rooting at either Brachyura or Palinura would not change the conclusions of this study. Out of these 26 taxa, 13 taxa (shown in bold in figure 3) were assayed for all four rearrangements shown in figure 1, with some taxa assayed for a subset of these rearrangements.

For our phylogenetic analysis, we obtained partial sequences for four genes (nuclear 18S (AF436001–23, U19182, AF438751) and 28S (AF435982–6000, AF425341, AF425342, AF425343, AF425345), mitochondrial *COII* (AF425361, AF425362, AF425364, NC_003058, AJ133050, AF437613–30), and 16S (AF436040–60, AF425322, AF425323, AF425325)) for all taxa, with the following exceptions: *B. latro* (*COII*, 18S, 28S), *Eumunida* sp. (*COII*, 16S), *P. armatus* (*COII*), *C. major* (28S), *D. schmitti* (28S), *I. pilosus* (28S). This alignment is available from the author.

We began by performing an unconstrained maximum likelihood analysis using the best-fit model (figure 2). The well-supported nodes in our unconstrained analysis were congruent with all four gene rearrangements. The node defined by rearrangements 1 and 2—a node that happens to support a monophyletic Anomura—also had 79% bootstrap support in our best-fit maximum-likelihood analysis (figure 2). Similarly, the node supported by rearrangement 4 also had 100% bootstrap support in this analysis (figure 2). Despite this congruence, the DNA sequence phylogeny by itself was unable to resolve any of the crucial relationships between families within the Anomura (figure 2). Here, rearrangement 3 played a crucial role, supporting the monophyly of the Paguroidea, Coenobitoidea and Hippoidea.

For our combined maximum-likelihood phylogenetic analysis, the relationships of the taxa assayed for all four gene rearrangements (shown in bold in figure 3) were constrained using a 'backbone' procedure to match the phylogeny implied by the gene rearrangements (figure 1) using PAUP* 4.0 (Swofford 1999). A 'backbone' constraint allows the DNA sequence data to place the unconstrained taxa anywhere in the phylogeny where they fit best. The numbers at each node represent the result of 200 bootstrap pseudoreplicates, with no numbers being given for the nodes defined by gene rearrangements, or for bootstrap values greater than 50%. When we constrained the maximum-likelihood analysis to conform to the gene rearrangements, anomuran relationships were largely resolved (figure 3).

(c) *Additional mitochondrial rearrangements*

In the course of assaying for the rearrangements, several additional mitochondrial rearrangements were observed, none of which affected the phylogeny shown in figure 2. In *Callichirus* and *Neotrypaea Asp* has moved in between *Gln* and *Met*. In *Hepatus*, *Trp* is missing from its usual location next to *Cys*. In *Callichirus*, $Leu_{(UUR)}$ has moved from its usual location between *ND1* and *16S*, and moved next to *COI*, to create the arrangement *COI* $Leu_{(UUR)}$ $Leu_{(CUN)}$ *COII*. This is very similar to rearrangement 3 (figure 1), except that the positions of the two *Leu* genes are reversed in *Callichirus*, strongly suggesting that this rearrangement is independent of our rearrangement 3.

In *C. albidigitus* (AF425321), a remarkable four rearrangements are observed compared with its fellow hermit crab *P. longicarpus* (NC_003058). These can be summarized in the following novel gene order (*12S*,

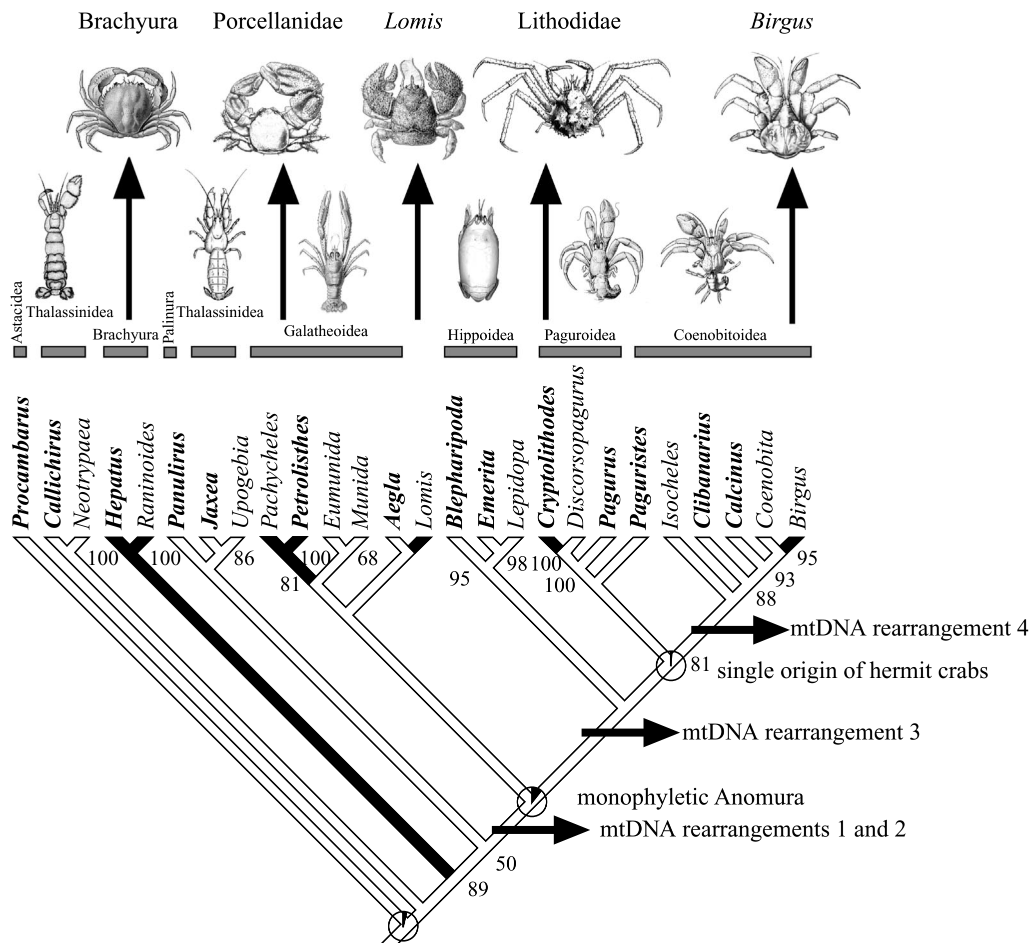

Figure 3. Constrained maximum-likelihood phylogeny for four genes with a best-fit model as described in figure 2. Full species names are given in § 2. Taxa in bold were assayed for all four gene rearrangements, and the relationships of assayed taxa were assigned a backbone constraint (explained in the text) to match the phylogeny implied by the gene rearrangements (figure 1). Numbers at nodes indicate bootstrap support greater than 50% in 100 pseudoreplicates, and no numbers are given for the nodes constrained by the gene rearrangements. Shaded branches indicate maximum-likelihood character reconstructions performed using Discrete 1.0 (Schluter *et al.* 1997; Pagel 1998). Maximum likelihood found significant support for a non-crab ancestor at several crucial nodes (the degree of support for the crab-like character state is shown in black on the pie diagrams, the remainder represents the non-crab-like state). The values shown in figure 3 are equivalent to parsimony reconstructions as all branches were assumed to be of equal length, but did not change appreciably when branch lengths were considered (results not shown). Drawings are representative of the groups shown, but do not correspond exactly to the species used in this study.

$Ser_{(AGN)}$, *Ala*, *ND3*, *Gly*, $Leu_{(UUR)}$, *Tyr*, *Trp*, *Gln*, *Cys*, *COI*). The movement of $Leu_{(UUR)}$ has already been reported above (rearrangement 4, figure 1), and the three other rearrangements in *Clibanarius* include the movement of $Ser_{(AGN)}$ from its typical position between *Asn* and *Glu*, the movement of a block of three genes *Gly*, *ND3* and *Ala* (including one protein-coding gene) from its usual location between *Lys* and *Asp*, and the movement of *Tyr* from its usual location between *Cys* and *COI*.

A similar absence of *Tyr* betweeen *Cys* and *COI* has been previously reported in *P. longicarpus* (Hickerson & Cunningham 2000), and was observed in several other taxa in our survey (*Jaxea*, *Cryptolithodes*, *Clibanarius*, *Calcinus*; see § 4).

4. DISCUSSION

In contrast to cases in which multiple origins of identical mtDNA rearrangements have been observed in obviously unrelated groups, ranging from wasps to birds (Curole & Kocher 1999), gene rearrangements seem to be providing a clear phylogenetic signal within the Anomura. First, three out of the four gene rearrangements took place at nodes that are strongly supported by independent sequence data (see § 3). The fourth (rearrangement 3) took place at a node that was not resolved by the sequences, and helped to resolve that crucial node (figure 2). Second, the rearrangements shown in figure 1 are perfectly nested, making it less likely that any single rearrangement arose independently more than once. For example, the derived rearrangements 1 and 2 (figure 1*a*) are always observed together in the same taxa, instead of in various combinations as predicted by multiple origins. Along the same lines, rearrangements 3 and 4 (figure 1*b*) only appear in a subset of the taxa that already shows rearrangements 1 and 2.

Further evidence for the phylogenetic utility of mitochondrial gene rearrangments comes from a gene movement that at first glance seems homoplastic. We observed five taxa in which the *Tyr* gene was missing from its usual

location in arthropods (see § 3). According to our phylogeny in figure 2, this would suggest three independent movements of *Tyr*, two of which appear to have taken place within the hermit crabs. Although *Paguristes* has the ancestral gene order, it is nested within hermit crabs (e.g. *Pagurus* and *Clibanarius*) in which *Tyr* has moved. It is gratifying that the ending locations of *Tyr* in the hermit crabs *Pagurus* and *Clibanarius* are completely different (see § 3; Hickerson & Cunningham 2000), confirming that they were not the same event.

Finally, rearrangements 3 and 4 are particularly interesting because they form a series that begins and ends with a single leucine tRNA gene between *COI* and *COII* (figure 1*b*)—but the intervening rearrangements have changed the identity of the tRNA gene from $Leu_{(UUR)}$ to $Leu_{(CUN)}$. Satisfyingly, we observed the intermediate stage (rearrangement 3) in which $Leu_{(UUR)}$ is found adjacent to $Leu_{(CUN)}$ (figure 1*b*). The most parsimonious explanation for the observed pattern of gene arrangements is that each occurred only once, and that each defines specific nodes in the phylogeny.

Given the robust phylogeny from our combined analysis of gene rearrangements and DNA sequences, what can we learn about morphological evolution? Our phylogenetic analysis shows five independent origins of the crab-like form, one in the Brachyura and four in the Anomura (figure 3). This result confirms the conclusions of morphologists, most of whom have suggested independent origins for all of the five crab-like groups shown in figure 3 (e.g. McLaughlin 1983; Scholtz & Richter 1995).

Maximum-likelihood estimates of ancestral states strongly support the hypotheses that the ancestors of each of these groups were not crab-like, and that these five origins are truly independent (figure 3). A statistically significant degree of support for the crab-like form is shown in the pie diagrams at three crucial nodes in figure 3. Interestingly, although transitions to the crab-like form have happened repeatedly, there is no case in which members of a crab-like group have reverted to a shrimp or lobster-like form. This suggests that the evolution of the crab-like form may be irreversible.

Why has the crab-like form appeared so many times in independent lineages? One possible explanation is that the crab-like form represents a key innovation conferring a large advantage. The huge success of the non-anomuran Brachyura, with more than 10 000 species, is consistent with this hypothesis. Within the Anomura, however, this hypothesis is not supported. For all four crab-like lineages in the Anomura, the number of species in the crab-like group is lower than the number of species in its non-crab sister group: Porcellanidae versus Chirostylidae + Galatheidae; *Lomis* versus *Aegla*; *Birgus* versus *Coenobita*; Lithodidae versus *Pagurus* (Bliss 1990).

A second explanation for the parallel evolution of the crab-like form is that there is some common developmental mechanism underlying its evolution. In fact, the morphological change from the ancestral shrimp and lobster-like forms to the crab-like form simply involves broadening the carapace, and reducing and tucking the abdomen underneath the body (see drawings in figure 3). Such changes in relative size and shape can be easily generated by a heterochronic shift in developmental timing (Blackstone 1989). For instance, in the crab-like *B. latro*—the world's largest terrestrial arthropod—juveniles occupy gastropod shells as do most hermit crabs, but as development proceeds, individuals become more crab-like and eventually lose their dependence on shells (Reese 1968). Hence, the ontogeny of *Birgus* actually recapitulates its hermit crab ancestry on its way to a crab-like adulthood. In other hermit crab lineages, a tendency towards the crab-like form is already apparent at metamorphosis (MacDonald *et al.* 1957; Blackstone 1989), suggesting a somewhat different mechanism (e.g. displacement heterochrony; Alberch *et al.* 1979; Blackstone 1989). Detailed developmental studies are required to determine whether these apparently different routes to the crab-like form share a similar genetic basis.

Although the mechanism underlying the transition to the crab-like form is not known, in a separate paper we investigate the transition from hermit crab to lithodid (Alaskan king crab), and suggest that the Lithodidae arose in the intertidal zone (Zaklan & Cunningham 2002). Interestingly, both of our monotypic crab-like forms are also found in shallow water, including the juvenile stage of the aforementioned *B. latro* and the crab-like *L. hirta*. Existing phylogenetic analyses of the remaining two crab-like groups (Brachyura and Porcellanidae) are not sufficient to test the hypothesis that these groups also arose in shallow waters.

The charismatic Anomura have a long history of being considered one of the most problematic groups in the Decapoda, but the agreement between DNA sequence data and gene rearrangements has brought us closer than ever to understanding the evolutionary pathways that brought us hermits and kings. In addition to documenting several origins of the crab-like form, this study supports the hypothesis proposed by several generations of taxonomists—beginning with Boas (1880; Borradaile 1916; MacDonald *et al.* 1957; Wolff 1961; Richter & Scholtz 1994)—that the king crabs (family: Lithodidae) are descended from hermit crab ancestors (figure 3). This conclusion, also drawn by a previously reported single-gene phylogeny (Cunningham *et al.* 1992), contradicts a recent morphological study that disputed a hermit crab ancestry for the Lithodidae (McLaughlin & Lemaitre 1997).

Our phylogeny also clarifies a number of important taxonomic issues (McLaughlin 1983; Martin & Abele 1986; Richter & Scholtz 1994), which support a single origin of the asymmetric hermit crabs, confirming the widely held view that the Thalassinidea falls outside a monophyletic Anomura and supporting the suggestion that the enigmatic crab-like *Lomis* falls well outside the hermit crabs (figure 3). The most striking disagreement with morphological analyses is our strongly supported conclusion that the morphologically homogeneous Thalassinidea may not form a monophyletic group (figure 3; see Poore (1994) and Scholtz & Richter (1995) for morphological evidence of monophyly).

Although we cannot say for certain why the crab-like form has arisen repeatedly, our phylogenetic framework for anomuran evolution points to a number of avenues for future research, including the developmental mechanism underlying the transformation and the reasons why transitions to the crab-like form may be irreversible. Finally, our study confirms the power of gene rearrangements, as

long as they are considered in the context of other phylogenetic information (Curole & Kocher 1999).

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