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Model-based multi-locus estimation of decapod phylogeny and divergence times

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Abstract

Phylogenetic relationships among all of the major decapod infraorders have never been estimated using molecular data, while morphological studies produce conflicting results. In the present study, the phylogenetic relationships among the decapod basal suborder Dendrobranchiata and all of the currently recognized decapod infraorders within the suborder Pleocyemata (Caridea, Stenopodidea, Achelata, Astacidea, Thalassinidea, Anomala, and Brachyura) were inferred using 16S mtDNA, 18S and 28S rRNA, and the histone H3 gene. Phylogenies were reconstructed using the model-based methods of maximum likelihood and Bayesian methods coupled with Markov Chain Monte Carlo inference. The phylogenies revealed that the seven infraorders are monophyletic, with high clade support values (bp > 70; pP > 0.95) under both methods. The two suborders also were recovered as monophyletic, but with weaker support (bp = 70; pP = 0.74). Although the nodal support values for infraordinal relationships were low (bp < 50; pP < 0.77) the Anomala and Brachyura were basal to the rest of the 'Reptantia' in both reconstructions and using Bayesian tree topology tests alternate morphology-based hypotheses were rejected (P < 0.01). Newly developed multi-locus Bayesian and likelihood heuristic rate-smoothing methods to estimate divergence times were compared using eight fossil and geological calibrations. Estimated times revealed that the Decapoda originated earlier than 437 MYA and that the radiation within the group occurred rapidly, with all of the major lineages present by 325 MYA. Node time estimation under both approaches is severely affected by the number and phylogenetic distribution of the fossil calibrations chosen. For analyses incorporating fossils as fixed ages, more consistent results were obtained by using both shallow and deep or clade-related calibration points. Divergence time estimation using fossils as lower and upper limits performed well with as few as one upper limit and a single deep fossil lower limit calibration.

Keywords: Bayes; Decapoda; Reptantia; Molecular phylogeny; Fossil calibration; Divergence time estimation; Likelihood; Molecular clock

1. Introduction

Estimated to contain more than 15,000 species, the decapods are the most species-rich group of Crustacea, including well-known groups such as shrimp (Caridea, Stenopodidea, and Thalassinidea), crabs (Anomala and Brachyura), and crayfish and lobsters (Astacidea and Achelata) and a wide array of lesser-known groups (Bowman and Abele, 1982). Accordingly, the decapods are the subject of more published papers than have all other crustaceans combined, due in part to their species richness, economic importance, and morphologic diversity (Martin and Davis, 2001). Decapod species have served as laboratory model organisms in studies of physiology, morphology, and behavior for over a century (Huxley, 1880). Hence, given the prevalence of decapods in the public and scientific mind, our lack of understanding of the evolutionary history of this significant crustacean group is impressive.

Current estimates of decapod evolutionary histories are based on fossil and morphological data. The

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decapod fossil record begins in the Late Devonian (354–364 MYA; Schram et al., 1978), with representation of almost all of the major lineages. In particular, the Reptantia have the best fossil record, as well as the oldest, of the decapods (see Glaessner, 1969). However, although all the main extant taxonomic groups have fossil representatives, the decapod record through time is incomplete (Schram, 2001). While the majority of the described decapod fossils extend into the Cretaceous (Schram, 2001), a large gap exists between these and the earliest known fossils, *Palaeopalaeomon newberryi* (Late Devonian, Schram et al., 1978) and *Imocaris tuberculata* (Lower Carboniferous, Schram and Mapes, 1984). Recently, a number of studies of decapod relationships have incorporated both fossil and extant taxa into a phylogenetic framework to examine evolutionary relationships and patterns of diversity through time (Amati et al., 2004; Rode and Babcock, 2003; Schram and Dixon, 2003; Tshudy and Sorhannus, 2003). However, while these studies have made great progress in understanding the evolution of the decapod form and the phylogenetic affiliations of fossil groups, they are limited to lineages where well-preserved fossils make comparisons of morphological characters with extant taxa possible.

Molecular phylogenetic methods can overcome these issues by combining sequence data with fossil dates, allowing the estimation of divergence times across the entire gene tree of a group by incorporating fossils into the analysis as calibration points. In the past this has been accomplished assuming a molecular clock, that is, constancy of evolutionary rates across lineages (Zuckerkandl and Pauling, 1965). Under this assumption, the estimated branch lengths can be converted into absolute divergence times using fossil calibration. However, most datasets appear to violate the clock model (Graur and Martin, 2004), which can cause serious bias in divergence time estimation (e.g., Rambaut and Broham, 1998; Soltis et al., 2002). Consequently, in the last few years several methods have been proposed within Bayesian (Thorne and Kishino, 2002) and likelihood (Yang and Yoder, 2003; Yang, 2004) frameworks that account for rate variation when estimating divergence times and incorporate multiple genetic loci and multiple fossil calibration points. Both of these methods assume a phylogenetic hypothesis of evolutionary relationships, which must be estimated separately.

Unfortunately, there are as many hypotheses of decapod phylogenetic relationships as there are experts with opinions (Schram, 2001), with no consensus in sight (Fig. 1). Historically, the decapod crustaceans were divided into two groups based on mode of locomotion: the Natantia (the 'swimming' lineages) and the Reptantia (the 'crawling' lineages) (Boas, 1880). However, early on the 'Natantia' were recognized as a paraphyletic group and accordingly the Decapoda were reorganized into the suborders Dendrobranchiata (penaeid shrimp and their relatives) and Pleocyemata (all other decapods) by Burkenroad (1963, 1981). This taxonomic restructuring is supported by several defining morphological characters (i.e., dendrobranchiate gill structure and pleocyemate brooding of eggs on the female's pleopods) and phylogenetic studies showing the 'natant' decapods to be a paraphyletic assemblage (Abele and Felgenhauer, 1986; Abele, 1991; Felgenhauer and Abele, 1983). Most of the phylogenetic studies investigating the relationships among the major decapod lineages have been based on morphological characters, which due to the extreme diversity of form makes it difficult to study the group as a whole (Schram, 1986). Moreover, there has been a surprising paucity of molecular phylogenetic studies investigating ordinal level relationships in this group. Until recently, those molecular studies focused on only part of the order (i.e., the 'Natantia') and have not included adequate taxon sampling within the Reptantia

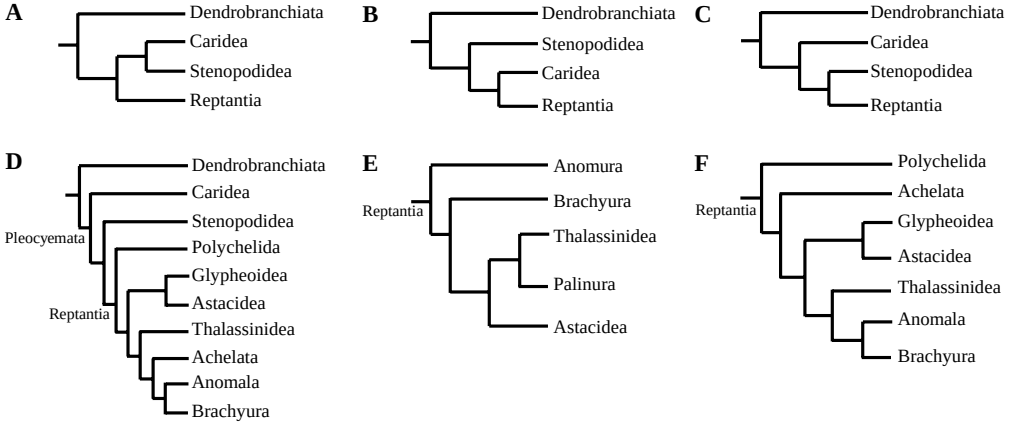

Fig. 1. Previous hypotheses of decapod relationships, with A–C illustrating morphologic hypotheses relative to 'natant' lineage relationships, D illustrating morphologic hypotheses including 'reptant' lineage relationships, and E and F illustrating molecular-based hypotheses of 'reptant' lineages: (A) Burkenroad (1963, 1981); (B) Christoffersen (1988); (C) Abele and Felgenhauer (1986), Abele (1991), and Schram (1986); (D) Dixon et al. (2003); (E) Crandall et al. (2000); and (F) Ayhong and O'Meally (2004).

to evaluate the relationships of the major infraorders (Abele, 1991; Kim and Abele, 1990). The first comprehensive study utilizing both morphological and molecular characters to investigate infraordinal relationships among reptant lineages was only recently published (Ayhong and O'Meally, 2004). However, no attempts at a comprehensive phylogenetic assessment from a molecular perspective of the entire order have ever been undertaken.

Even with a large number of conflicting hypotheses regarding decapod phylogenetic relationships, there appears to be general agreement on the monophyly of the suborder Pleocyemata and the informal 'Reptantia.' Towards the goal of investigating the divergence times of the major decapod radiations, particularly for these two consistently monophyletic clades, we will first construct a model-based phylogeny of the major decapod infraorders. This will be the first study to use molecular data to evaluate relationships among all the Decapoda infraorders. The combination of our molecular phylogeny with multiple fossil calibration points will be used for divergence time estimation under Bayesian and likelihood approaches to provide insights into the timing of the major decapod evolutionary radiations and into the relative performance of these two different methods in real data analyses.

2. Methods

2.1. Taxon sampling

The most updated classification of the recent Crustacea (Martin and Davis, 2001) was used to determine the taxonomy of the major lineages within the Decapoda with two exceptions. First, the infraorder 'Palinura,' which historically included the polychelids, palinurids, and glypheoids, has been shown to be polyphyletic, with the glypheoids clustering within Astacidea, and the polychelids shown to be basal reptants (Ayhong and O'Meally, 2004; Amati et al., 2004; Dixon et al., 2003; Scholtz and Richter, 1995; Schram and Dixon, 2003). Therefore, we chose to use the term 'Achelata' as suggested by Scholtz and Richter (1995) and Dixon et al. (2003) to represent the extant families Scyllaridae, Synaxidae, and Palinuridae. Second, the 'Anomura' lineage as described by Borradaile (1907) included both anomuran crabs and thalassinids. The distinction of the thalassinids as a lineage separate from the Anomura has been documented in numerous studies (Ayhong and O'Meally, 2004; Crandall et al., 2000; Dixon et al., 2003; Schram, 2001; Schram and Dixon, 2003); therefore, following the resurrection by others (Dixon et al., 2003; Scholtz and Richter, 1995), we chose to replace 'Anomura' with the Anomala of Boas (1880). Species used for these analyses included representatives from the Dendrobranchiata and from all of the major infraorders in the Pleocyemata (Table 1). All specimens were preserved in 95–100% ethanol and are housed in the crustacean collection at the Monte L. Bean Life Science Museum, Brigham Young University, Provo, Utah. Based on previous hypotheses of Eumalacostraca relationships, two species of Euphausiacea were used to root the tree (Christoffersen, 1988; Dixon et al., 2003; Schram, 1986).

2.2. DNA extraction, PCR, and sequencing

Tissue samples from each specimen were dried and used in previously described DNA extraction protocols (Crandall and Fitzpatrick Jr., 1996). Polymerase chain reaction (PCR, Saiki et al., 1988) products for the complete 18S rRNA (~2000 bp, Whiting et al., 1997; Whiting, 2002), partial 28S rRNA (~2500 bp, Whiting et al., 1997; Whiting, 2002) and histone H3 (333 bp, Colgar et al., 1998) nuclear genes, and the partial 16S (~460 bp, Crandall and Fitzpatrick Jr., 1996) mitochondrial gene were amplified using one or more sets of general primers from the literature. Standard PCR conditions (5 μl of 10× *Taq* buffer, 6–8 μl of 25 mM $MgCl_2$, 8 μl of 10 mM dNTPs, 5 μl each of two 10 mM primers, 1.25 U *Taq*, and ~20 μl double distilled water) were used on a Perkin-Elmer 9700 machine under the following conditions: an initial denaturation at 96 °C for 3 min followed by 40 cycles of 95 °C for 1 min, 46 °C for 1 min, and 72 °C for 1 min, followed by chain extension at 72 °C for 10 min. PCR products were visualized by agarose (1.2%) gel electrophoresis and purified using the Millipore Montage purification system. Sequences were generated in both directions on an ABI Prism 3730 capillary autosequencer using the ABI big-dye Ready-Reaction kit and following the standard cycle sequencing protocol but using 1/16th of the suggested reaction volume.

2.3. Phylogenetic analyses

Nucleotide sequences were aligned using Clustal X (Thompson et al., 1997) with the default parameters and refined by eye. Because many regions of the 16S, 18S, and 28S gene segments used for analysis are extremely divergent among the ingroup taxa and therefore difficult to align reliably, GBlocks v0.91b (Castresana, 2000) was used to eliminate poorly aligned positions and divergent regions of the Clustal X alignment (GBlocks parameters used for 16S/18S/28S: minimum number of sequences for a conserved position = 26/26/26; minimum number of sequences for a flanking position = 40/36/43; Maximum number of contiguous non-conserved positions = 8/8/8; minimum length of a block = 6/5/5; allowed gap positions = with half). Phylogenetic analyses of combined datasets can reveal hidden support for relationships in conflict among analyses of individual markers (Gatesy et al., 1999); therefore, the GBlocks-pruned

Table 1
Taxonomy, voucher identification codes, and GenBank accession numbers for gene sequences from Decapoda species included in this study

Taxon	Voucher ID	Gene			
		16S	18S	28S	H3
Decapoda Latreille, 1802					
Dendrobranchiata Bate, 1888					
Penaeoidea Rafinesque, 1815					
Penaeus semisulcatus de Haan, 1844	KC1269	DQ079731	DQ079766	DQ079809	DQ079698
Pleocyemata Burkenroad, 1963					
Stenopodidea Claus, 1872					
Stenopus hispidus (Olivier, 1811)	MLP119	DQ079734	DQ079769	DQ079812	DQ079701
Caridea Dana, 1852					
Atyoidea de Haan, 1849					
Atyoida bisulcata (Randall, 1840)	KC2138	DQ079704	DQ079738	DQ079774	DQ079661
Typhlatya pearsei Creaser, 1936	MLP85.1	DQ079735	DQ079770	DQ079813	DQ079702
Alpheoidea Rafinesque, 1815					
Lysmata debelius Bruce, 1983	MLP121	DQ079718	DQ079752	DQ079793	DQ079681
Lysmata wurdemanni (Gibbes, 1850)	MLP120	DQ079719	DQ079753	DQ079794	DQ079682
Palaemonoidea Rafinesque, 1815					
Creaseria morleyi (Creaser, 1936)	MLP102.1	DQ079710	DQ079746	DQ079784	DQ079671
Cryphiops caementarius (Molina, 1782)	JC1219	DQ079711	DQ079747	DQ079785	DQ079672
Macrobrachium potiuna (Muller, 1880)	KC2094	DQ079721	DQ079756	DQ079797	DQ079685
Macrobrachium sp.	MLP123.2	DQ079720	DQ079754	DQ079795	DQ079683
Palaemon elegans Rathke, 1837	KACpael	DQ079729	DQ079764	DQ079807	DQ079696
Palaemonetes paludosus (Gibbes, 1850)	MLP124	N	DQ079755	DQ079796	DQ079684
'Reptantia'					
Achelata Scholtz and Richter, 1995					
Palinuroidea Latreille, 1802					
Jasus edwardsii (Hutton, 1875)	KC725	DQ079716	**AF235972**	DQ079791	N
Panulirus regius De Brito Capello, 1846	KC2167	DQ079730	DQ079765	DQ079808	DQ079697
Scyllarus arctus (Linnaeus, 1758)	KC2159	DQ079732	DQ079767	DQ079810	DQ079699
Anomala					
Galatheoidea Samouelle, 1819					
Aegla abtao Schmitt, 1942	KAC-Aa4	**AY050067**	**AF439390**	**AY595965**	DQ079658
Uroptychus parvulus (Henderson, 1885)	KACurpa	**AY595926**	**AF439386**	**AY596097**	DQ079703
Munida subrugosa (White, 1847)	KACmusu	**AY050075**	**AF439382**	**AY596099**	DQ079688
Hippoidea Latreille, 1825					
Emerita brasiliensis Schimitt, 1935	KACembr	DQ079712	**AF439384**	DQ079786	DQ079673
Lomisoidea Bouvier, 1895					
Lomis hirta (Lamarck, 1810)	KAClohi	**AY595928**	**AF436013**	**AY596101**	DQ079680
Paguroidea Latreille, 1802					
Lithodes santolla (Molina, 1782)	LAClisa	**AY595927**	**AF439385**	**AY596100**	DQ079679
Astacidea Latreille, 1802					
Astacoidea Latreille, 1802					
Astacus astacus (Linnaeus, 1758)	JF134	**AF235983**	**AF235959**	DQ079773	DQ079660
Cambarellus shufeldtii (Faxon, 1884)	KC1210	**AF235986**	**AF235962**	DQ079778	DQ079665
Cambaroides japonicus (de Haan, 1841)	KC695	**AF235987**	DQ079742	DQ079779	DQ079666
Cambarus maculatus Hobbs and Pflieger, 1988	KC74	**AF235988**	**AF235964**	DQ079780	DQ079667
Orconectes virilis (Hagen, 1870)	JC897	**AF235989**	**AF235965**	DQ079804	DQ079693
Pacifastacus leniusculus (Dana, 1852)	JF64	**AF235985**	**AF235961**	DQ079806	DQ079695
Parastacoidea					
Astacopsis gouldi (Horwitz, 1991)	KC1883	**AF135969**	DQ079737	DQ079772	DQ079659
Cherax glaber Riek, 1967	KACchgl	**AF135978**	DQ079745	DQ079783	DQ079670
Nephropoidea Dana, 1852					
Acanthacaris caeca (A. Milne-Edwards, 1881)	KC1877	N	DQ079736	DQ079771	N
Homarus americanus H. Milne-Edwards, 1837	KAChoam	**AF370876**	**AF235971**	DQ079788	DQ079675
Homarus gammarus (Linnaeus, 1758)	KC2162	DQ079714	DQ079749	DQ079789	DQ079676
Nephrops norvegicus (Linnaeus, 1758)	KC2163	DQ079726	DQ079762	DQ079803	DQ079692
Nephropsis aculeata Smith, 1881	KC2117	DQ079727	DQ079761	DQ079802	DQ079691
Brachyura Latreille, 1802					
Cancroidea Latreille, 1802					
Cancer pagurus Linnaeus, 1758	KC2158	DQ079708	DQ079743	DQ079781	DQ079668
Grapsoidea MacLeay, 1838					
Pachygrapsus marmoratus (Fabricius, 1787)	KACpama	DQ079728	DQ079763	DQ079805	DQ079694

Table 1(*continued*)

Taxon	Voucher ID	Gene			
		16S	18S	28S	H3
Majoidea Samouelle, 1819					
Maja squinado (Herbst, 1788)	KAC2168	DQ079723	DQ079758	DQ079799	DQ079687
Potamoidea Ortmann, 1896					
Geothelphusa sp.	MLP125	DQ079715	DQ079750	DQ079790	DQ079677
Portunoidea Rafinesque, 1815					
Carcinus maenas (Linnaeus, 1758)	KACcama	DQ079709	DQ079744	DQ079782	DQ079669
Macropipus puber (Linnaeus, 1767)	KACmapu	DQ079722	DQ079757	DQ079798	DQ079686
Necora puber (Linnaeus, 1767)	KAC2161	DQ079724	DQ079759	DQ079800	DQ079689
Thalassinidea					
Callianassoidea Dana, 1852					
Biffarius arenosus (Poore, 1975)	BaV3	DQ079705	DQ079739	DQ079775	DQ079662
Callichirus major (Say, 1818)	KAC1864	DQ079707	DQ079741	DQ079777	DQ079664
Callianassa subterranea (Montagu, 1808)	KACcasu	DQ079706	DQ079740	DQ079776	DQ079663
Lepidophthalmus louisianensis (Schmitt, 1935)	KAC1852	DQ079717	DQ079751	DQ079792	DQ079678
Sergio mericeae Manning and Felder, 1995	KAC1865	DQ079733	DQ079768	DQ079811	DQ079700
Outgroups					
Euphausiacea Dana, 1852					
Euphausia eximia Hansen, 1911	KACeuex	DQ079713	DQ079748	DQ079787	DQ079674
Nematoscelis sp.	KACnesp	DQ079725	DQ079760	DQ079801	DQ079690

Sequences obtained from GenBank are indicated in bold. An 'N' designates gene sequences we were unable to acquire.

datasets from each gene region were concatenated into a single combined dataset consisting of 3601 bp, which is available online (http://inbio.byu.edu/faculty/kac/crandall_lab/pubs.html). Because one of our goals is to date the major decapod radiations using model-based estimation procedures, in order to be methodologically consistent, we employed only model-based methods of tree reconstruction. The combined dataset was used to reconstruct phylogenies using Maximum Likelihood (ML) heuristic searches in PAUP* v4b10 (Swofford, 2002) and Bayesian methods coupled with Markov chain Monte Carlo (BMCMC) inference as implemented in MrBayes v3.04b (Ronquist and Huelsenbeck, 2003). Model selection for ML and BMCMC analyses followed the procedure outlined by Posada and Buckley (2004) as implemented in ModelTest v3.6 (Posada and Crandall, 1998). ML searches (Felsenstein, 1981) were run using 100 random addition replicates and TBR branch swapping. Confidence in the resulting relationships was assessed using the non-parametric bootstrap procedure (Felsenstein, 1985) with 200 bootstrap replicates, using heuristic searches of one random addition with TBR branch swapping per replicate. For BMCMC techniques, four independent analyses were run with each consisting of four chains. Each Markov chain was started from a random tree and run for 3.0×10^6 cycles, sampling every 1000th generation. Model parameters were treated as unknown variables with uniform default priors and were estimated as part of the analysis. To confirm that our Bayesian analyses converged and mixed well, we monitored the fluctuating value of likelihood and all phylogenetic parameters graphically and compared means and variances of all likelihood parameters and likelihood scores from independent runs using the program Tracer v1.2 (Rambaut and Drummond, 2003). All sample points prior to reaching stationary were discarded as burn-in. The posterior probabilities (p*P*) for individual clades obtained from separate analyses were compared for congruence and then combined and summarized on a 70% majority-rule consensus tree (Huelsenbeck and Imennov, 2002; Huelsenbeck et al., 2002).

2.4. Testing alternative hypotheses

Alternative a priori phylogenetic hypotheses from the literature were tested under both likelihood and Bayesian frameworks. Likelihood topology tests were conducted using our molecular data and the Shimodaira and Hasegawa (1999, SH) test as implemented in PAUP*. Goldman et al. (2000), Buckley (2002), and Strimmer and Rambaut (2002) have pointed out that the SH test may be subject to a certain type of bias such that the number of trees included in the confidence set tends to be very large as the number of trees to be compared increases, which makes the test conservative. However, as these authors recognized and Shimodaira (2002) concluded, the SH test is still safe to use and is a good option when the number of candidate trees is not very large and more data are accumulated. Ten thousand replicates were performed for every topology test resampling the partial likelihoods for each site (RELL model). Because there are differences between the taxon sampling of the a priori hypotheses and our dataset, alternative topologies were constructed in MacClade by rearranging only the branches representing the infraor-

dinal lineages in conflict. Bayesian topology tests were performed by calculating the p*P* of the set of trees containing the a priori hypothesis, as described in Huelsenbeck et al. (2002).

2.5. Reference fossils

The decapod fossil record is continually being updated and reclassified, due to new discoveries and because many fossils are described from incomplete specimens causing uncertainty as to their phylogenetic affinities. Consequently, where possible, fossil references for this study were taken from species where descriptions were based on nearly complete specimens or where recent phylogenetic studies have placed fossil species relative to extant groups (Amati et al., 2004; Rode and Babcock, 2003; Schram and Dixon, 2003; Tshudy and Sorhannus, 2003). Additionally, the fossils selected for calibration points in this study were chosen based on the precision of the estimated date of the oldest known representative for particular clades, across several levels of divergence relative to the taxa sampling of our phylogeny. Based on these factors and the ages of potential fossils relative to their placement on the phylogeny, a set of seven fossils were used as calibrations in our analyses (Table 2). Additionally, because the Bayesian method chosen for divergence time estimation (see below) requires at least one calibration to consist of an upper limit (maximum age), we set the split between the crayfish superfamilies Astacoidea and Parastacoidea as an upper limit of 185 MYA based on the splitting of Pangea (Crandall et al., 2000).

Although fossil burrows attributed to crayfish have been described from the Permian, it is often difficult to determine this association with certainty (Babcock et al., 1998; Hasiotis, 2002). Therefore, with respect to crayfish lineages we have chosen to use only fossil records from descriptions of preserved animals (Imaizumi, 1938; Van Straelen, 1928). Furthermore, a number of marine Jurassic fossil lobster species have been assigned to the Astacidea, although their phylogenetic relationships are still being investigated (Amati et al., 2004; Schram and Dixon, 2003). Because the majority of these species are marine, they represent ancestral lineages to the crayfish. In terms of calibrations, we have chosen the oldest described marine lobster affiliated with the Astacidea, but not specifically aligned with the Nephropoidea, to calibrate the infraorder Astacidea.

The oldest fossil ascribed to the decapods is the Late Devonian *P. newberryi* Whitfield, 1880, which has been placed within the Reptantia by several authors due to astacidean-like features (Christoffersen, 1988; Felgenhauer and Abele, 1983; Schram et al., 1978), although at least one of these also cites the presence of characters with 'natantian' affinities (Felgenhauer and Abele, 1983). A recent phylogenetic study incorporating both fossil and extant taxa surprisingly places *P. newberryi* in a polytomy with the Thalassinida and 'Eurysternalia' (Achelata, Anomala, and Brachyura) (Schram and Dixon, 2003), although there has been no consensus as to its phylogenetic affiliations. Therefore, our use of this fossil to date the basal-most node of the monophyletic 'Reptantia' clade is conservative.

Table 2
Taxonomy and ages of fossils used as calibrations for divergence time estimations

Taxonomy	Species	Reference	Geologic age (MYA)	Node #
Suborder Pleocyemata				
Infraorder Caridea				
Family Palaemonidae	*Palaemon antonellae*	Garassino and Bravi (2003)	Early Cretaceous (Albian) (99–112)	C1
	Alburnia petinensis	Bravi and Garassino (1998)	Early Cretaceous (Albian) (99–112)	C1
'REPTANTIA'	*Palaeopalaemon newberryi*	Whitfield (1880)	Late Devonian (Famennian) (354–364)	C2
Infraorder Astacidea				
Family Chimaerastacidae	*Chimaerastacus pacifluvialis*	Amati et al. (2004)	Mid Triassic (Upper Ladinian) (227–234)	C3
Superfamily Astacoidea				
Family Astacidae	*Astacus licenti*	Van Straelen (1928)	Late Jurassic (144–159)	C4
	Astacus spinirostris	Imaizumi (1938)	Late Jurassic (144–159)	C4
Infraorder Anomala				
Family Aeglidae	*Protaegla miniscula*	Feldmann et al. (1998)	Early Cretaceaous (Albian) (99–112)	C5
Infraorder Brachyura				
Family Cancridae	*Notocarcinus sulcatus*	Schweitzer and Feldmann (2000)	Mid Eocene (41.3–49)	C6
	Eocarcinus praecursor	Withers (1932)	Early Jurassic (Pliensbachian) (190–195)	C7

Calibration C8 is 185 MYA, based on the splitting of Pangea.

2.6. Divergence time estimation

Decapoda divergence times were estimated using the Bayesian method of Thorne and Kishino (2002) (referred to as TK) and the likelihood heuristic rate-smoothing algorithm (AHRS) of Yang (2004). The former approach is an extension of Thorne et al. (1998) and Kishino et al. (2001) Bayesian methods and the latter builds on Yoder and Yang (2000) and Yang and Yoder (2003) likelihood methods. These extended versions can accommodate multiple fossil calibration points and multiple genes, allow for missing taxa, and in the case of AHRS facilitate automatic assignment of branches to rate groups using a rate-smoothing procedure (Sanderson, 1997, 2002). As previously shown, simultaneous analysis of gene sequences from multiple loci and multiple calibrations is expected to improve estimates of divergence times and rate estimates (Pérez-Losada et al., 2004; Thorne and Kishino, 2002; Yang and Yoder, 2003; Yang, 2004). The two approaches implemented here estimate branch lengths without assuming a molecular clock, and then estimate times and rates by minimizing the discrepancies in branch lengths and by minimizing rate changes over branches. Moreover, both methods make use of the rate-evolution model of Thorne et al. (1998) and Kishino et al. (2001), but the TK approach averages over the rates in the MCMC procedure while the AHRS approach optimizes rates together with divergence times. Another difference is that the AHRS does not need a prior for divergence times, which might be considered an advantage. There is some evidence that time estimation by the Bayes approach may be sensitive to the prior model of the divergence times (Yoder and Yang, 2004). In contrast, in the TK method it is possible to specify fossil calibrations as lower or upper bounds on node ages. The likelihood method does not deal with such constraints and uses only fixed node ages for fossil calibration. As a result, standard errors calculated for estimated divergence times are serious underestimates. The importance of accounting for uncertainties in fossil calibrations has been emphasized by Graur and Martin (2004). Nevertheless, the performance of the TK and AHRS methods in real data analysis has never been explored, as these methods are only beginning to be widely used (Yang, 2004). A recent study published by our group compared several Bayesian and likelihood approaches using 18S rRNA sequences and single calibrations (Pérez-Losada et al., 2004). Here, we have extended the comparison to the case of multiple genes and multiple calibration points.

2.6.1. Bayesian-based TK method

We used the multi-locus Bayesian method of Thorne and Kishino (2002) as implemented in the multidivtime package (http://statgen.ncsu.edu/thorne/multidivtime.html). The mean of the prior distribution for the time separating the ingroup root from the present (rttm) and the standard deviation (SD) of this prior distribution (rttmsd) were set to 6 (600 MY). Alternative values ranging from 5 to 7 were also tried but final estimates did not change much (±10 MY). After inspecting the branch lengths estimated by estbranches for each gene, the evolutionary rate of the root node was given a gamma prior distribution with mean (rtrate) and SD (rtratesd) both equal to 0.027 substitutions at the average site per 100 MY. We chose this prior to obtain a distribution for the root that was simultaneously reasonable and relatively diffuse. The rtrate and rtratesd were estimated as suggested in the multidivtime manual. Prior distributions approximated under the MCMC approach included a burn-in period of 10^6 steps, after which 5×10^5 samples were collected at every 100th cycle; posterior distributions (less diffuse) included a burn-in period of 10^5 steps, after which 5×10^5 samples were collected at every 100th cycle. Default options were chosen for all the other parameters of the prior distribution and the MCMC procedure. Convergence was monitored by checking the proportion of successes (psuc) of times and rate changes proposed along the Markov chain. Four independent chains were run from different starting points. Parameters of the evolutionary model were estimated under the F84 + Γ model, the most complex model implemented in this software. This model is less parameterized than the best-fit models selected by ModelTest (see Section 3), however, previous studies (see Yang and Yoder, 2003 and references therein) have shown that it is actually the rate variation among sites parameter that has the greatest effect on divergence time estimation. All the parameters within the model as well as the branch lengths were estimated separately for every gene.

2.6.2. Likelihood-based AHRS method

We used the likelihood heuristic rate-smoothing algorithm of Yang (2004) as implemented in PAML3.14 (Yang, 1997). Sequence data were analyzed using the same F84 + Γ model and parameters of evolution chosen for the Bayesian analysis. Likelihood analyses were performed using SmallDiff (small value used in the difference approximation of derivatives) values of 1e−6 and 0.5e−6. Only the results showing the best likelihood scores are reported here. Branches at each locus were automatically classified into four rate groups according to their estimated rates (default option). This assignment was then checked manually using UPGMA in PHYLIP-ver3.6a (Felsenstein, 2004) as described in Yang (2004). The distributions of substitution rates for branches were similar among the four categories for each gene, justifying hence the use of the automatic assignment scheme.

2.6.3. Calibrations

Calibration points for the divergence time analysis were taken from known fossils representing major decapod lineages (Table 2). Given that most fossils are dated

to an age range, the midpoint of each range was used for the divergence time estimations, using the 1999 GSA Geologic Time Scale to determine dates. Fossil calibrations for estimating decapod divergence times were accommodated differently, depending on the method used and introduced into the analysis as follows: (1) under the TK method calibrations were used as lower limits except for the Astacoidea/Parastacoidea split, which was treated as an upper limit; (2) under the AHRS method calibrations were treated as fixed ages. All minimum or fixed age calibrations were mapped to the node prior to the basal node of the clade of interest.

2.6.4. Calibration comparisons

The most important factors affecting divergence time estimation using molecular data are the number and distribution of the calibration points on the tree (Lee, 1999; Thorne and Kishino, 2002; Yang and Yoder, 2003; Yoder and Yang, 2000), although some methods seem to be more sensitive than others (Pérez-Losada et al., 2004). To explore the relative performance of the Bayesian TK and likelihood AHRS approaches at estimating divergence times relative to calibration number and distribution, we performed multiple analyses using 14 calibration schemes, and compared these results to the chronogram estimated using all the calibrations. For these comparisons, analyses using the TK method were run twice, once treating calibrations as upper and lower limits and a second time using all calibrations as fixed ages for comparison to AHRS fixed age estimates. To construct the calibration schemes, the eight calibrations (seven fossil dates plus the Pangea split) were arranged chronologically from oldest to youngest and separate analyses were run with a single fossil calibration removed in each consecutive analysis, one at a time in chronological order, until only a single fossil remained. This process was repeated twice, first starting with removing the oldest fossils so that progressively younger fossils remained and the reverse where younger fossils were removed first. For TK analyses treating calibrations as age limits, the upper limit was maintained in all analyses, resulting in a calibration scheme with one less analysis. TK BMCMC analyses were performed as described above, only using a prior distribution burn-in period of 10^6 steps, after which 10^5 samples were collected at every 100th cycle, and a posterior distribution burn-in period of 10^5 steps, after which 10^5 samples were collected at every 100th cycle.

Although divergence times were estimated under both TK and AHRS methods and using multiple combinations of calibration points to explore their relative performance, our best estimate (see below) of the diversification of the Decapoda lineages (including 95% confidence intervals; CI) was calculated using the TK Bayesian method treating the seven fossil calibrations as minimum ages and the Astacoidea–Parastacoidea split as a maximum age.

2.6.5. Fossil cross-validation

Before performing our divergence time estimation, concordance within the eight fossil calibration points was assessed using the new cross-validation method described in Near and Sanderson (2004) and Near et al. (2005). This method attempts to identify fossil calibrations that generate inconsistent, and potentially erroneous, molecular age estimates by measuring the agreement between molecular age estimates derived using any one single fossil calibration (fixed age) and all other available fossil calibration. In a first step, the sum of the squared differences between molecular and fossil age estimates, SS, was calculated for each fossil calibration and then ranked based on their magnitude. In a second step, the average squared deviation, s, for all fossils in the analysis was calculated by sequentially removing the fossils with the greatest SS and recalculating s until only two fossils remained. Finally, the significance of change on the variance of s before and after fossil calibration removal was determined using a one-tailed F test.

3. Results

3.1. Decapod phylogenetics

We obtained 35 new complete 18S, and 32 partial 16S, 43 partial 28S, and 46 partial H3 gene sequences, Accession Nos. DQ079658–DQ079813 (Table 1). For ML searches, a GTR + Γ + I model (base frequencies = 0.2593, 0.2165, 0.2737; Rmat = 0.9538, 2.7863, 2.0907, 0.9950, 4.2081; gamma shape parameter = 0.5303, proportion invariable sites = 0.3830) was chosen for the concatenated dataset; for BMCMC analyses, models GTR + Γ + I (18S, 28S, and 16S) and TVM + Γ + I (H3) were implemented in MrBayes. Tree topologies reconstructed in both ML and BMCMC methods were not conflicting (SH test $P = 0.41$), although the BMCMC phylogeny was less resolved and therefore only the ML tree is presented (Fig. 2). In both analyses, the Pleocyemata, Reptantia, and all of the major infraorders were recovered as monophyletic clades with strong nodal support in at least one framework (thick black or grey branches, Fig. 2). However, there is very little support for infraordinal relationships within the Pleocyemata. This is evident when comparing our placement of the stenopod lineage with previous morphological hypotheses; the ML tree recovered a caridean + reptant clade (a priori hypothesis Fig. 1B), but this is not a significantly different topology than Fig. 1C (stenopod + reptant clade; SH $P = 0.51$, p$P = 0.42$) or Fig. 1A using the SH test ($P = 0.18$). However, a caridean + stenopod clade arrangement (Fig. 1A) is a significantly worse hypothesis in the BMCMC analysis ($P = 0.03$; Table 3).

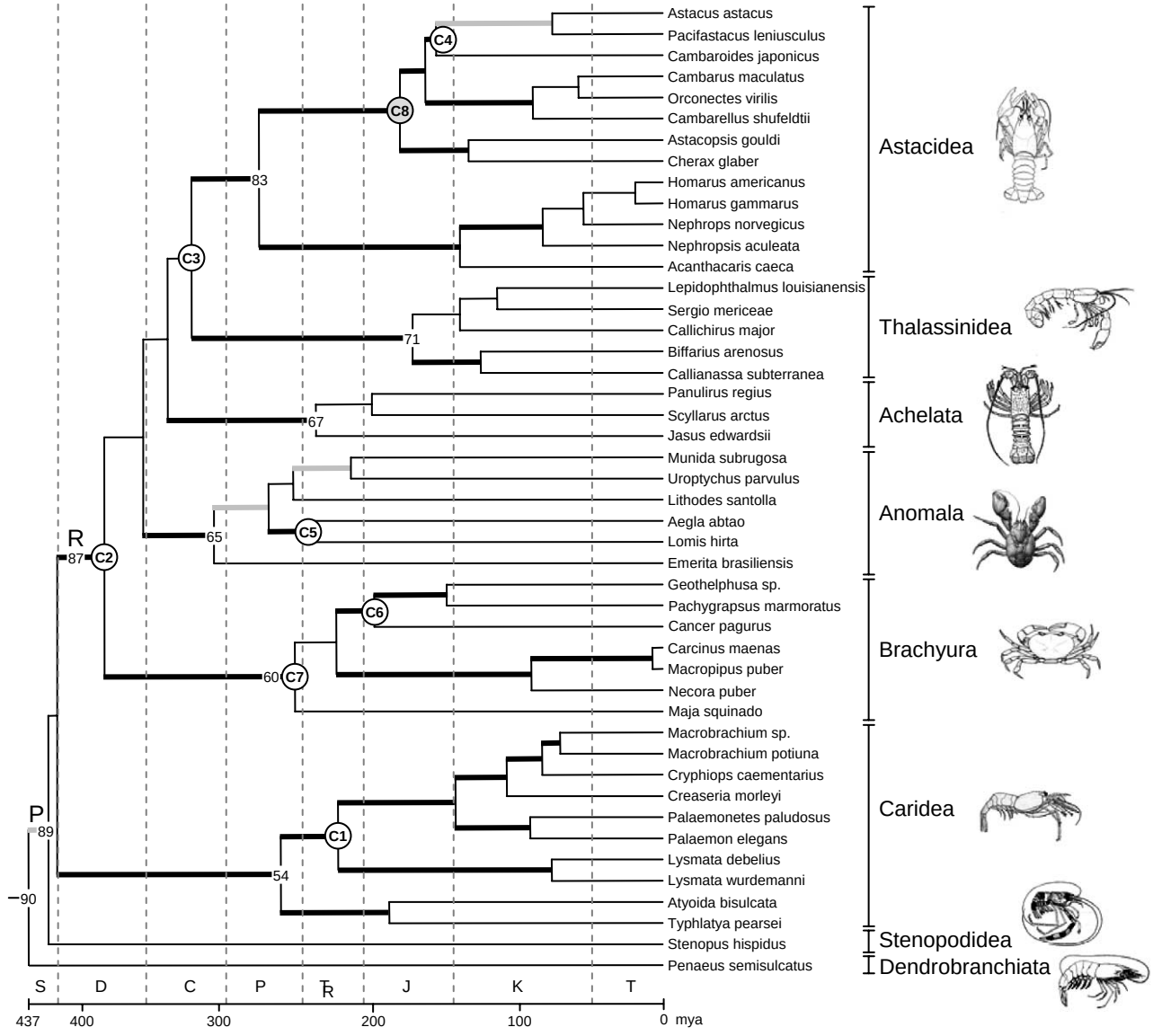

Fig. 2. Decapod divergence time chronogram estimated using topology of ML tree. On branches with both ML bootstrap values >70% and BMCMC pP > 0.95, support is indicated by a thick black line; branches strongly supported by only one tree reconstruction method are indicated by thick grey lines. Fossil calibration nodes are indicated by C1–C8, corresponding with Table 2. Node numbers from divergence time estimations are included for reference on nodes of important decapod lineages (see Table 4). The decapod infraorders are delineated, and the nodes corresponding to the suborder Pleocyemata (P) and the informal Reptantia (R) are indicated on the phylogeny. The major geologic periods are also mapped onto the phylogeny, using the following standard symbols: S, Silurian; D, Devonian; C, Carboniferous; P, Permian; Ŧ, Triassic; J, Jurassic; K, Cretaceous; T, Tertiary.

With respect to relationships within the reptant clade, both the Pleocyemata and Reptantia clades were recovered with strong support in at least one method. Second, the Astacidea is monophyletic, containing monophyletic nephropoid and astacid lineages. Third, the Thalassinidea is sister to the Astacidea, with weak pP support in BMCMC analyses. Finally, contrary to all but one of the only other molecular studies including representatives of the major reptant lineages (Fig. 1F, Crandall et al., 2000), our analyses place the Brachyura and Anomala as the basal reptant lineages. In comparisons with a priori hypotheses, this arrangement is found to be significantly better than hypotheses Figs. 1D–F using Bayesian pP (Table 3).

3.2. Decapod divergence time estimation

A likelihood ratio test significantly rejected ($P < 0.001$) the null hypothesis that all genes, separately and combined, were evolving with rate constancy across the decapods, justifying the use of non-clocklike molecular methods to estimate divergence times. Cross-validation analysis revealed appreciable deviation between molecular and fossil ages for both TK and AHRS

Table 3
Likelihood (SH) and BMCMC topology tests of previous hypotheses of decapod relationships, as shown in Figs. 1A–F

Fig. 1	SH		BMCMC	
	$\Delta-\ln L$	*P* value	*N*	p*P*
A	3.37	0.18	372	0.03
B	—	—	3013	0.26
C	0.51	0.45	4799	0.42
D	6.28	0.26	15	0.001
E	6.16	0.17	1	0.00009
F	5.50	0.26	12	0.001

For SH tests, the difference in likelihoods ($\Delta-\ln L$) and the corresponding *P* values are indicated. In BMCMC analyses, the number of trees (*N*) congruent with the previous hypothesis out of the posterior distribution of 11,400 trees is shown, with the corresponding posterior probability (p*P*) values.

methods as indicated by the SS values (3.5×10^4–12×10^4). Sequential removal of the six fossils with the highest SS values generated changes in the variance of *s*, however, none of these changes were significant (*F* test <4.0 for all the comparisons). Consequently, as a result of our cross-validation test, we did not exclude any of the eight delineated calibrations. Multiple independent Bayesian runs using the TK method produced similar mean estimates, although the 95% CI were larger than expected; however, by constraining the age of one of the backbone calibrations within the interval of its first paleontological occurrence, the analysis produced similar mean divergence time estimates, but the SD was reduced by half (data not shown). The decapod TK chronogram based on the single ML topology and treating the calibration points as minimum or maximum ages places the origin of the Dendrobranchiata and Pleocyemata decapod lineages in the early Silurian (437 MYA; Fig. 2). This implies that the stem line of the decapods emerged even earlier; however, we are unable to estimate this age given our taxon sampling. Based on this analysis, the radiation of the major decapod lineages occurred rapidly. The reptant lineage originated 385 MYA and all of the major reptant infraorders were present by the late Carboniferous, 60 MY later (Fig. 2, Table 4). The radiation of the extant taxa within each infraorder, however, occurred at different periods of time. The natant lineages have an early origin (417–423 MYA), however, the caridean superfamilies Alpheoidea, Atyoidea, and Palaemonoidea radiate in the early Permian (263 MYA). Among the Brachyuran superfamilies sampled, the Majoidea has the oldest lineage (254 MYA). The Achelata originate 341 MYA, with radiation of the extant lineages (Palinuridae and Scyllaridae) occurring as early as 239 MYA. The Thalassinidea appear 325 MYA, with the radiation of the Callianasoidea occurring at least 173 MYA.

Within the Astacidea and Anomala, we have sampled all the extant superfamilies. Therefore the divergence time estimates for the radiation of these groups are more accurate. The anomalan lineage originated 309 MYA, with the extant superfamilies radiating between 244 and 309 MYA. The Astacidea lineage originated 325 MYA, with the divergence between the astacid lineages (Astacoidea, Parastacoidea) and the Nephropoidea occurring 278 MYA. Within the astacids, the radiation of the Parastacidae (~134 MYA) occurred earlier than the Astacidae (76 MYA) or the Cambaridae (90 MYA). The Nephropodidae radiated as early as 140 MYA, with the genus *Homarus* appearing ~19 MYA.

Table 4
Comparison of divergence times for major decapod lineages using all fossil calibrations, as estimated from the TK method incorporating calibrations as minimum ages (TK-*ma*), and the TK and AHRS methods using calibrations as fixed ages (TK-*fa* and AHRS-*fa*, respectively)

Taxon (node)	Divergence time (95% CI) MYA		
	TK-*ma*	TK-*fa*	AHRS-*fa*
Decapoda (90)	437 (394–515)	411 (386–443)	423 (173–673)
Pleocyemata (89)	423 (385–499)	397 (378–422)	421 (240–602)
Caridea (54)	263 (217–322)	158 (138–181)	140 (83–197)
Stenopodidea (89)	423 (385–499)	397 (378–422)	421 (240–602)
Reptantia (87)	385 (360–450)	**359**	**359**
Achelata (67)	239 (174–310)	169 (122–219)	177 (29–325)
Anomala (65)	309 (261–372)	199 (161–245)	231 (99–363)
Astacidea (83)	278 (235–330)	211 (193–228)	216 (167–265)
Brachyura (60)	254 (203–317)	**194**	**194**
Thalassinidea (71)	173 (121–233)	116 (83–152)	107 (37–177)

Divergence times are taken from the crown node in each clade except for the Stenopodidea, where there is only a single representative included in this analysis. Because both the Reptantia and crown Brachyura nodes contained a calibration, in the fixed age analyses these estimates are constrained to be 359 and 194 MYA, respectively; these calibration times are indicated in bold. Node numbers for each clade correspond to node numbers included on the chronogram in Fig. 2.

3.3. Divergence time methods comparison

Decapod divergence times estimated under the TK approach using calibrations as minimum node ages were different from those estimated under the TK and AHRS methods using fixed age calibrations (Table 4). For four of the nodes corresponding to the Decapoda, Pleocyemata, Stenopodidea, and Reptantia taxa the time differences ranged between 2 and 26 MY across all comparisons, but for the other six nodes the differences ranged between 57 and 110 MY for the TK minimum age vs. TK fixed age comparison and 60–123 MY for TK minimum age vs. AHRS comparison. The estimates using fixed calibrations were more congruent with each other, regardless of method.

Time chronograms estimated under the Bayesian and likelihood approaches using four genes and 14 different combinations of eight calibrations illustrate that divergence time estimates can be severely affected by the number and distribution of the calibrations used across the tree (Fig. 3). For example, in Figs. 3A, C, and E, as older fossil calibrations are progressively removed from the

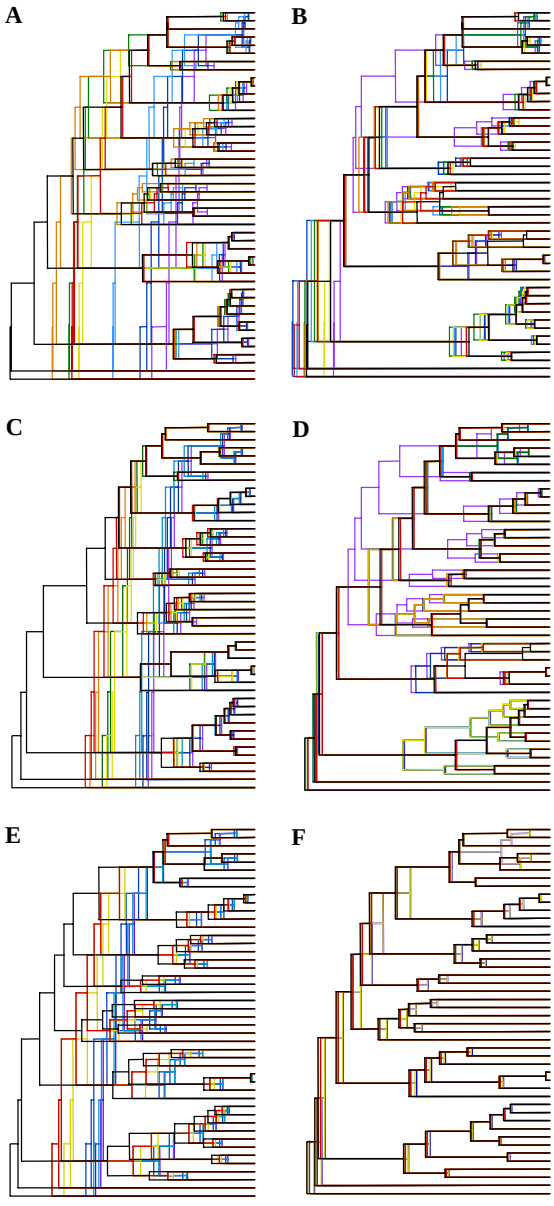

Fig. 3. Comparison of divergence time estimates from Bayesian (TK) and Likelihood (AHRS) methods under 14 different calibration schemes. In each panel, the best estimate chronograms based on all eight calibrations are black. Estimated chronograms from the successive removal of calibrations are mapped behind our best estimate in color, with these colors representing the following number of remaining fossil calibrations: red = 7, orange = 6, yellow = 5, green = 4, light blue = 3, dark blue = 2, and purple = 1. All TK minimum age analyses incorporated one upper limit, resulting in one less calibration scheme than fixed age estimates. (A) AHRS estimates comparing chronograms from the successive removal of deep (older) calibrations; (B) AHRS estimates comparing chronograms from the successive removal of shallow (younger) calibrations; (C) TK fixed age estimates comparing chronograms from the successive removal of deep calibrations; (D) TK fixed age estimates comparing chronograms from the successive removal of shallow calibrations; (E) TK minimum age estimates comparing chronograms from the successive removal of deep calibrations; and (F) TK minimum age estimates comparing chronograms from the successive removal of shallow calibrations.

analysis, the estimates of the entire backbone of the phylogeny are pulled towards younger dates, with differences as large as 152 (TK minimum age)–258 (AHRS) MY between node estimates based on eight calibrations vs. only the youngest calibration. While the opposite trend is observed when removing younger calibrations from the analysis, older calibrations produce more stable backbone estimates, and hence more stable estimates across the tree. For fixed age estimates, we observed a crown effect, where removal of calibrations from a specific lineage affected estimates within that lineage, while estimates across the rest of tree remained relatively stable. For example, in Fig. 3D, when calibration C1 from the Caridea and C6 from the Brachyura are removed, only the estimates within these lineages are significantly overestimated; however, these overestimations remain stable as calibrations are removed from other areas of the phylogeny. Finally, while neither method treating fossils as fixed ages remained stable as younger calibrations were removed, the TK divergence estimates utilizing fossils as minimum/maximum ages remained stable even when only a single deep fossil calibration point was incorporated (Fig. 3F).

4. Discussion

4.1. Decapod radiation

This study presents the first molecular phylogenetic hypothesis of the infraordinal relationships within the Decapoda. However, it is not the final answer to the long debate regarding decapod relationships; indeed, it appears to add yet another scheme to the already large set of hypotheses concerning decapod phylogenetic relationships. However, our results do support several relationships that seem to be stable based on both molecules and morphology, i.e., the monophyly of the suborder Pleocyemata and the informal 'Reptantia' (Crandall et al., 2000; Dixon et al., 2003; Schram, 2001). Furthermore, the infraorders included in our analyses are all strongly supported as monophyletic; however, this is a hypothesis that will continue to be tested as additional taxa from underrepresented decapod groups (especially from within the Caridea and Brachyura) are added to the molecular dataset. Of particular interest are several lineages not represented in our analyses due to difficulty in obtaining the necessary specimens. The taxonomy of these groups, including the polychelids, glypheoids, thaumastochelids, and entoplometopodids, have been revised several times based on recent morphological estimates of phylogeny (Amati et al., 2004; Dixon et al., 2003; Scholtz and Richter, 1995; Schram, 2001), and inclusion in molecular analyses may provide additional insights into their phylogenetic placement within the decapods (Ayhong and O'Meally, 2004).

While there is strong support for the monophyly of the infraorders, there is little support for the relationships among them, and in fact, determining these relationships is one of the biggest remaining issues in decapod systematics (Abele, 1991). While in our analyses the monophyletic Astacidea sister to the Thalassinidea contradict Scholtz and Richter's (1995) hypothesis, this general arrangement mirrors conjectures by Schram (1986) that the thoracic endoskeleton anatomy of Thalassinidea indicates a closer relationship to the astacideans than to the anomalans. Furthermore, the genetic, morphological, and ecological distinctiveness of the astacid and nephropid lineages, the consistent monophyly of these sister taxa in both molecular- and morphology-based analyses, and the estimated divergence times warrants the elevation of these lineages to separate infraorders, the Astacidea (crayfish) and the Nephropoidea (clawed lobsters); however, the erection of new classification schemes is beyond the scope of this research. Perhaps, the most controversial result of these analyses, however, is the placement of the Brachyura and Anomala as the basal reptant lineages. In fact, the unrooted topology of reptant lineages based on our molecular data is the same as that recovered in several of the most recent morphological phylogenies, at least one of which also uses euphausiids as an outgroup (see Fig. 1E; Dixon et al., 2003; Schram and Dixon, 2003). Although this reversal of reptant rooting seems troubling on the surface, the similar branching patterns between molecular and morphological hypotheses is encouraging; there only seems to be a difference in the polarization of the characters between methodologies. Additional taxon sampling will most certainly affect phylogenetic reconstructions of the poorly supported reptant infraordinal relationships, using either morphology- or molecular-based characters. Furthermore, given the extreme diversity of decapod forms, issues of character polarization are perhaps not too surprising; evaluating character states across highly modified morphologies is a worthwhile, although challenging endeavor. Furthermore, the hypothesis of reptant relationships presented here forces a re-interpretation of many of the morphological characters currently used to define reptant lineages (i.e., 'Fractosternalia,' defined by fused posterior thoracic sternites and a bi-partite secula), making many of them plesiomorphic rather than synapomorphic. In order to understand the evolution of decapod morphological diversity in the context of this phylogeny, investigation of further characters is necessitated. Further investigations, including combined molecular and morphology phylogenetic analyses, additional morphological character investigation, and studies of outgroup choice relative to character polarization, are required to understand these differences. However, such analyses are beyond the scope of this paper and our current data set, but will be pursued in detail by our group in future work.

We can use model-based phylogenetic tree topology tests (ML and BMCMC) to statistically compare our phylogenetic results with previous hypotheses. These topology tests indicate that although the particular arrangement of the carid and stenopod lineages relative to the reptant lineages is unclear, it is most likely that they are not sister to each other (Fig. 1A). With respect to the previous molecular hypotheses of reptant relationships (Figs. 1E and F) and to the morphological baseline of decapod phylogeny established by Dixon et al. (2003) (Fig. 1D), ML topology tests find no significant difference, while Bayesian methods show significant differences. These results indicate that BMCMC methods are much more sensitive to topological differences than ML methods. Furthermore, none of these studies exhibit strong nodal support for reptant relationships. As a more conservative test, however, the SH test indicates that there is no clear consensus, nor no clear hypothesis that is significantly better or worse, between molecular and morphological estimates of decapod phylogenetic relationships.

The basal position of the Brachyura in our phylogeny, although contradictory to most other morphology-based hypotheses of decapod relationships, matches the current understanding of the fossil record well. Provisionally, the Brachyura contain the second oldest known reptant fossil, *Imocaris tuberculata* (Schram and Mapes, 1984), indicating a long evolutionary history. Schram (1986) noted Brachyuran radiation events in the Cretaceous and in the Eocene when many of the modern families of crabs are found for the first time. However, our analysis indicates that many of the modern families may have had a much earlier origin. Also of interest relative to previous hypotheses of decapod crustacean radiations are the dates of astacid divergence. Our estimated divergence time of the astacid lineage in the early Permian (264 MYA) matches well with fossil crayfish and burrows associated with Permian and Early Triassic deposits (Hasiotis and Mitchell, 1993) and the hypothesis by Crandall et al. (2000) that crayfish have a Pangean origin.

Although we have estimated decapod divergence times without assuming a molecular clock and using multiple molecular markers and fossil calibration points, and these estimates appear to be concordant to a large degree with the decapod fossil record, our analyses come with a number of caveats. There are inaccuracies associated with the fossil record and with phylogeny estimation that are not taken into account (Graur and Martin, 2004). We assumed that the fossil ages are known with no error and the performed cross-validation test seems to support this idea; however, the magnitude of the deviation between fossil and molecular data estimates (85 to −100% on average) or inconsistency based on single calibration points is of concern. Presumably these deviations are canceling each other out in the multiple

calibration analysis (the variance of s was non-significant), but bias in our final time estimates due to fossil inconsistency cannot be ruled out completely. The divergence time estimation methods we have utilized are heavily dependent on topology and our molecular ML phylogeny is significantly different than most morphological hypotheses; therefore, our estimates represent only a single hypothesis of decapod evolution from a larger, incongruent set. These alternative topologies would possibly generate different estimates for the crown nodes of the infraorders, but the two main conclusions of our analyses—that the Decapoda originated in the Silurian and have experienced a fast radiation with all of the major infraorders present by the late Carboniferous—would not change. Furthermore, the monophyletic Pleocyemata and the informal 'Reptantia' are consistent in all hypotheses of decapod relationships, and therefore the divergence time estimates of these clades (423 and 385 MYA, respectively) can be used as common time points regardless of the particular arrangement of lineages. Nevertheless, future advances in divergence time estimation methodologies could take advantage of the Bayesian framework to account for uncertainties in topology estimation and fossil dating and use different priors for rates and divergence times, as those included in Aris-Brosou and Yang (2002). An extension of this Bayesian approach to include multiple genes and calibrations would be desirable.

4.2. Divergence time estimation method comparison

Our methods comparison further illustrates the potential pitfalls of divergence time estimations, where number and phylogenetic distribution of calibrations can severely affect estimates. Since fossils do not fix the ages of internal nodes but merely constrain them to be minimum ages (Smith, 1994), it seems more appropriate to constrain nodes to lie within some interval rather than fix them to a particular time (Norell, 1992). This is one of the strengths of the TK method versus the AHRS algorithm. However, because TK appropriately incorporates fossil calibration uncertainty, estimates have large confidence intervals. Where possible, this effect can be minimized by including multiple upper limits of fixed ages in the analysis. While each of the two methods compared appears to have different strengths relative to the calibrations used (young versus old), in general using a combination of both deep and shallow calibrations will provide better estimates across the entire phylogeny. Furthermore, where possible, using at least one calibration within each crown lineage will help alleviate clade-specific inaccuracies. However, given constraints in the number of fossil calibrations available, more consistent results are obtained if fossils represent at least one deep/old backbone calibration and are treated as minimum ages using the TK method. Divergence times estimated under any of the methods tested here using only shallow calibrations should be treated more cautiously, with older dates being treated as potentially severe underestimates.

5. Conclusions

Rapid diversification and radiation is characteristic of the Crustacea as a whole (Schram et al., 1978), and this is a trend readily apparent in our divergence time estimates of decapod lineages (Fig. 2). Major decapod radiation events have been proposed in the Eocene (Brachyura, Schram, 1986), the Cretaceous (Brachura, Schweitzer, 2001), and the Triassic (macrurous forms, Schram, 1986). Our molecular-based divergence time estimates are older than hypotheses based solely on the fossil record, with the radiation of the 'natant' infraorders occurring in the Devonian, the reptant infraorders in the Carboniferous, Anomalan diversification in the Permian–Triassic, and the Callianassoidea and Palaemonoidea in the Cretaceous. As decapod paleontological research is a quickly expanding field of research (Feldmann, 2003), it will be most interesting to track the knowledge of decapod fossil date ranges relative to molecular-based divergence time estimations.

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