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Molecular Phylogeny of Arthropods and the Significance of the Cambrian "Explosion" for Molecular Systematics[1]

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SYNOPSIS. **Accurate phylogenetic reconstruction requires character systems that have evolved fast enough to have kept pace with cladogenesis but slowly enough to have conveyed the resulting phylogenetic signal to the present. Because stratigraphic evidence suggests that basal arthropod lineages arose rapidly during an ancient (Cambrian) phylogenetic radiation, the discovery of molecular sequences capable of resolving arthropod phylogeny may be a significant challenge for molecular systematists. This challenge is exemplified by our attempt to resolve arthropod phylogeny using the amino acid sequence of elongation factor-1α (EF-1α). Our fossil-based assessment of evolutionary rates indicates that EF-1α should be capable of resolving Cambrian-age divergences. However, phylogenetic analysis using EF-1α fails to establish relationships among most higher-level groups, although it does recover more recently derived clades. Here we propose two models to explain this incongruity. The Rapid Radiation Model maintains that fossil-based estimates of arthropod diversification are essentially accurate and that diversification occurred so rapidly during the Cambrian that few phylogenetically significant changes occurred in the slowly evolving EF-1α sequence. The Enhanced Preservation Model maintains that fossil-based estimates of Cambrian-age divergences reflect enhanced preservation of pre-existing lineages and that arthropod diversification occurred before the Cambrian. This model attributes lack of resolution to degradation of phylogenetic signal within EF-1α by subsequent evolution. Current evidence is more consistent with the Enhanced Preservation Model, which implies that fossil-based methods can be very misleading when attempting to gauge the phylogenetic information content of molecular sequences for Cambrian- and Precambrian-age divergences.**

INTRODUCTION

The phylogenetic relationships of the major arthropod lineages is a long-standing and contentious issue. This may stem from a level of taxonomic and morphological diversity within the phylum so high as to be beyond the ability of any one systematist to digest and synthesize. Indeed, arthropod research has long been divided into taxonomically insular traditions (*i.e.*, arachnology, carcinology, entomology, myriapodology) which may have impeded organized interdisciplinary research on arthropods and given inordinate influence to the opinions of a few synthetic workers, such as Snodgrass (1938) and Manton (1977). We have recently published a synopsis of the current status of arthropod phylogeny, including hypotheses supported by morphological and molecular characters (Regier and Shultz, 1997). We concluded that molecule-based phylogenetic analysis is probably the most efficient method for establishing relationships among extant arthropod subphyla and classes, as the properties of quantitative phylogenetic methods and molecular characters transcend traditional taxonomic boundaries. This report summarizes recent progress in the molecular systematics of arthropods and highlights the challenges that this ancient and diverse phylum represents for molecular systematists.

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PROGRESS IN THE MOLECULAR SYSTEMATICS OF ARTHROPODS

Molecular studies of arthropod relationships have relied heavily upon interpretation of 18S ribosomal RNA sequences (Field *et al.*, 1988; Patterson, 1989; Lake, 1990; Wheeler *et al.*, 1993; Spears and Abele, 1997), although more recently 28S sequences have been added (Friedrich and Tautz, 1995). These studies provide no incontestable resolution for basal arthropod relationships, although monophyly of hexapods and crustaceans exclusive of myriapods is strongly supported in the combined 18S + 28S study of Friedrich and Tautz (1995). In this same study, the monophyly of malacostracan and branchiopod crustaceans was questioned. Ballard *et al.* (1992) and Wheeler *et al.* (1993) presented arthropod phylogenies derived from analyses of 12S mitochondrial ribosomal RNA and from the nuclear protein-encoding gene ubiquitin, respectively, but both genes displayed very high levels of homoplasy. Boore *et al.* (1995) have proposed that rearrangements in mitochondrial gene order may be useful in phylogenetic analysis. Their study supports Mandibulata as a monophyletic group based on a single rearrangement involving a leu-tRNA. Unfortunately, the resolving power of this approach is limited, given the small number of potential characters and the huge diversity of the phylum. If analysis of molecular sequences is to prove decisive in arthropod phylogeny, additional informative sequences or other genomic data must be explored.

ASSESSING PROTEIN-ENCODING NUCLEAR GENES FOR ARTHROPOD SYSTEMATICS

Molecule-based reconstruction of ancient phylogenetic events, such as those that gave rise to the major arthropod lineages, requires the discovery and analysis of slowly evolving nucleotide or amino acid sequences. Methods used in screening molecular sequences for their ability to resolve relationships within a particular clade include concordance studies, which assess the ability of a gene to recover well-established phylogenetic relationships within clades of similar age (*e.g.*, Friedlander *et al.*, 1994), and the construction of fossil-based pairwise difference curves, which estimate the rate of potentially informative character change during the geological interval when a clade underwent phylogenetic diversification (*e.g.*, Mindell and Honeycutt, 1990; Graybeal, 1994). Results from such approaches indicate that the amino acid sequence of a nuclear gene, elongation factor-1α (EF-1α), evolves slowly enough to resolve very ancient phylogenetic events. EF-1α has been used successfully to reconstruct known relationships within vertebrates and arthropods (Friedlander *et al.*, 1994). The general view that metazoan EF-1α and its nonmetazoan orthologs evolve very slowly has also inspired use of this gene by investigators interested in the phylogenetic relationships among protostomes (Kojima, 1998; Kojima *et al.*, 1993; McHugh, 1997) and eukaryotes (Hasegawa *et al.*, 1993).

In an attempt to assess the specific utility of EF-1α amino acids for resolving deep divergences within arthropods, we constructed a pairwise difference curve by plotting pairwise sequence differences and fossil-based estimates of phylogenetic divergence (Benton, 1993) for selected pairs of lineages (Fig. 1). Typically, the more recent portion of such a curve has a positive slope, reflecting the accumulation of state differences between sister lineages. Older regions of the curve show a positive but decreasing slope as the number of one-time changes per site diminishes between sister taxa and more sites experience multiple changes. This apparent deceleration in the rate of change occurs because new changes occur at sites that have already changed, thus allowing sister lineages to have the same character state due to chance rather than to shared phylogenetic history. Eventually, the phylogenetic divergences between sister lineages are so ancient that most or all sites have experienced multiple changes and the observed sequence difference fluctuates stochastically about an asymptote. The asymptotic difference is a function of the number of sites free to vary and the number of states that each site can express, such that sequences composed entirely of variable sites with four or 20 equally likely states will eventually equili-

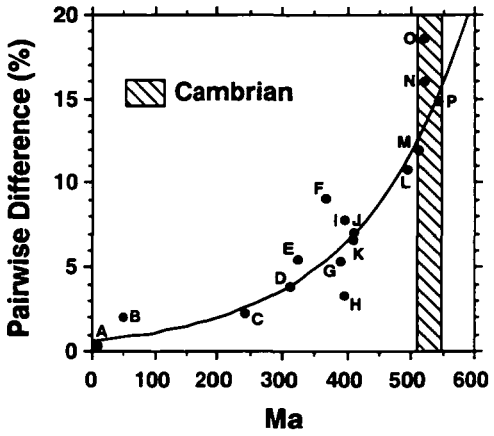

FIG. 1. Pairwise sequence differences plotted against fossil-based divergence times for amino acids of elongation factor-1α (EF-1α). The curve was constructed by plotting mean pairwise sequence differences between pairs of lineages against a minimum estimate of divergence time, as represented by the age of the oldest fossil known for either lineage. The curve indicates rapid accumulation of sequence difference during the Cambrian, suggesting that EF-1α should be informative during this time. Points were determined from comparisons of the following taxa: A, heliothine moths; B, noctuid *vs.* sphingid moths (Hexapoda: Lepidoptera); C, araneomorph *vs.* mygalomorph spiders (Arachnida); D, julid *vs.* spirobolid millipedes; E, pterygote *vs.* thysanuran insects; F, decapod *vs.* isopod crustaceans; G, pterygote *vs.* microcoryphian insects; H, spiders *vs.* whipscorpions; I, entognathous *vs.* ectognathous hexapods; J, mites *vs.* tetrapulmonate arachnids; K, millipedes *vs.* centipedes; L, arachnids *vs.* xiphosurans; M, phyllocarid *vs.* eumalacostracan crustaceans; N, barnacles *vs.* non-malacostracan arthropods; O, euchelicerates *vs.* pycnogonids; P, ostracods *vs.* non-malacostracan arthropods. Stratigraphic ranges were obtained from Briggs *et al.* (1993), Labandeira (1994), Ross and Briggs (1993), Selden (1993), Shear and Kukalová-Peck (1990) and Whatley *et al.* (1993), and most dates were obtained from Harland *et al.* (1990).

brate at mean pairwise differences of 75% or 95%, respectively. In short, the rate at which differences accumulate in a region of the curve corresponds roughly to the amount of phylogenetic signal present in that region. A literal reading of the EF-1α pairwise difference curve indicates that the rate of change was high throughout the Paleozoic including the Cambrian, but the curve shows no evidence of approaching an asymptote. These observations suggest that EF-1α should be a good candidate for resolving basal relationships within arthropods and other protostomes, most of which are thought to have originated and diversified during a period of rapid first-time amino-acid replacement.

In an attempt to provide more decisive support for arthropod relationships, we generated and analyzed sequences from EF-1α (Regier and Shultz, 1997). Twenty-one species were sampled, including multiple representatives from most major arthropod lineages (chelicerates, crustaceans, hexapods and myriapods), as well as annelids and mollusks. Phylogenetic analysis of 364 amino acid characters recovered many well-established clades (*e.g.*, Diplopoda, Hexapoda, Insecta, Chelicerata, Arachnida, Branchiopoda, Malacostraca) with strong to modest support. The study also produced two novel hypotheses (Fig. 2). Specifically, branchiopod crustaceans were reconstructed as being more closely related to hexapods and myriapods than to malacostracan crustaceans, and malacostracan crustaceans were reconstructed as the sister group to the other arthropods. To assess the results derived from analysis of EF-1α, we sequenced a second nuclear protein-encoding gene, the largest subunit of RNA polymerase II (POLII). Using POLII, we analyzed a subset of 11 taxa that included branchiopods, hexapods, myriapods and chelicerates, but for technical reasons were unable to include malacostracans and non-arthropod outgroups. Either alone or in combination with EF-1α, Pol II (194 amino acids) strongly supported branchiopods as sister group to hexapods to the exclusion of myriapods (Fig. 3). These results argue against monophyly of Atelocerata (hexapods plus myriapods), a group supported by virtually all previous morphological studies (*e.g.*, Snodgrass, 1938; Manton, 1977; Boudreaux, 1979; Weygoldt, 1986) but not by molecular studies (Friedrich and Tautz, 1995; Boore *et al.*, 1995).

We do not regard the evidence provided by EF-1α against crustacean monophyly as decisive for three reasons. First, conclusions about phylogenetic relationships among major arthropod lineages require a well-established root, which would be best achieved by a much more thorough sampling of non-arthropods. Second, diversity

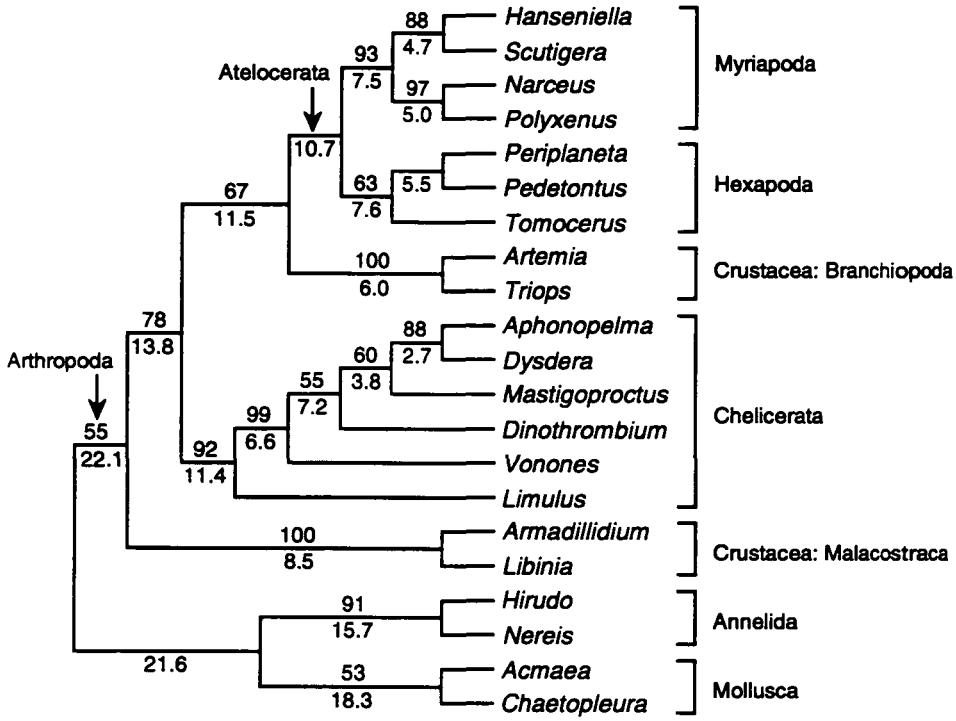

FIG. 2. Cladogram showing relationships among 17 arthropod and 4 outgroup species as indicated by maximum-parsimony analysis of 364 amino acids from elongation factor-1α (Regier and Shultz, 1997). Numbers above each internode are bootstrap percentages based on 1,000 bootstrap replicates, and numbers below internodes are percent mean pairwise sequence differences between sister lineages. Bootstrap percentages below 50% are not shown. Tree length, 537; CI, 0.63; RI, 0.63.

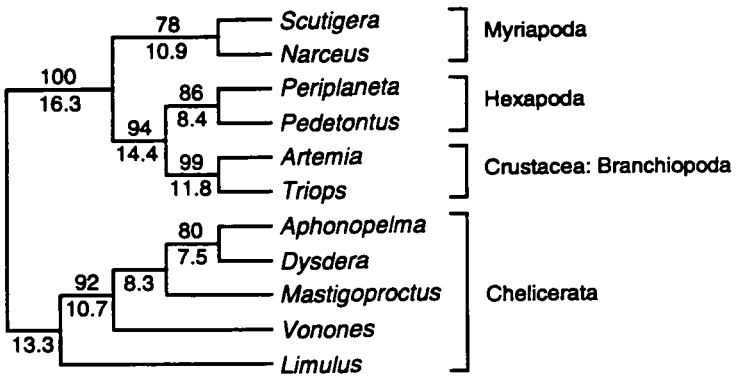

FIG. 3. Cladogram showing relationships among 11 arthropods as indicated by maximum-parsimony analysis of 364 amino acids of elongation factor-1α and 194 amino acids of the largest subunit of RNA polymerase II (Regier and Shultz, 1997). Numbers above each internode are bootstrap percentages based on 1,000 bootstrap replicates, and numbers below internodes are percent mean pairwise sequence differences between sister lineages. Bootstrap percentages below 50% are not shown. Tree length, 472; CI, 0.77; RI, 0.67.

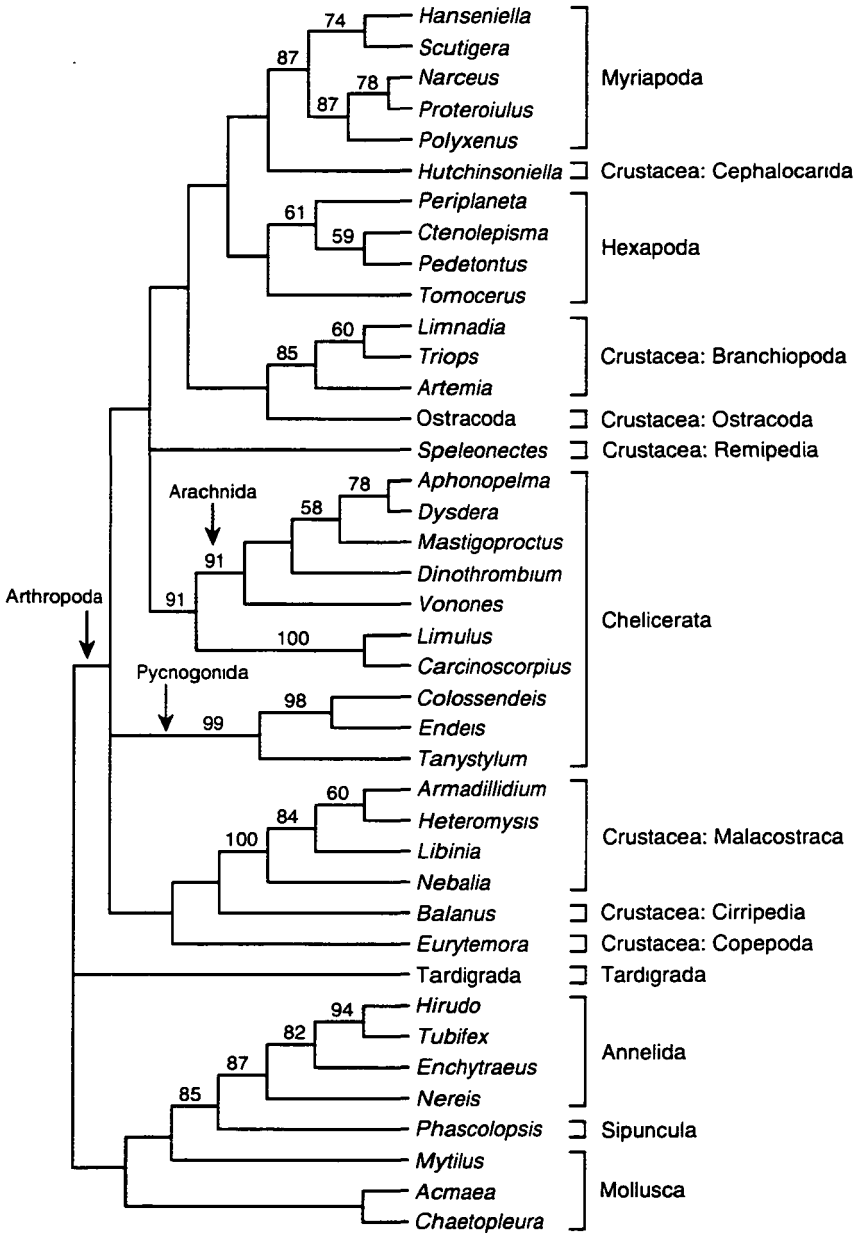

FIG. 4. Strict consensus of two cladograms showing relationships among 31 arthropod and 9 outgroup species as indicated by maximum-parsimony analysis of 360 amino acids from elongation factor-1α. Genus names are used except for the unidentified specimens of Ostracoda and Tardigrada. Numbers above each internode are bootstrap percentages based on 1,000 bootstrap replicates. Bootstrap percentages below 50% are not shown. Note lack of resolution and low empirical support for basal divergences in arthropods. Tree length, 537; CI, 0.63; RI, 0.63. GenBank Accession numbers: *Acmaea testudinalis* (U90061), *Aphonopelma chalcodes* (U90045), *Armadillidium vulgare* (U90046), *Artemia salina* (X03349), *Balanus balanoides* (AF063404), *Carcinoscorpius rotundicauda* (AF063407), *Chaetopleura apiculata* (U90062), *Colossendeis* sp. (AF063406), *Ctenolepisma lineata* (AF063405), *Dinothrombium pandorae* (U90048), *Dysdera crocata* (U90047), *Enchytraeus* sp. (AF063409), *Endeis leavis* (AF063409), *Eurytemora affinis* (AF063408), *Hanseniella* sp. (U90049), *Heteromysis formosa* (AF063410), *Hirudo medicinalis* (U90063), *Hutchinsoniella macracantha* (AF063411), *Libinia emarginata* (U90050), *Limnadia lenticularis* (AF063412), *Limulus polyphemus* (U90051), *Mastigoproctus giganteus* (U90052), *Mytilus edulis* (AF063420), *Narceus americanus* (U90053), *Nebalia hessleri* (AF063413), *Nereis*

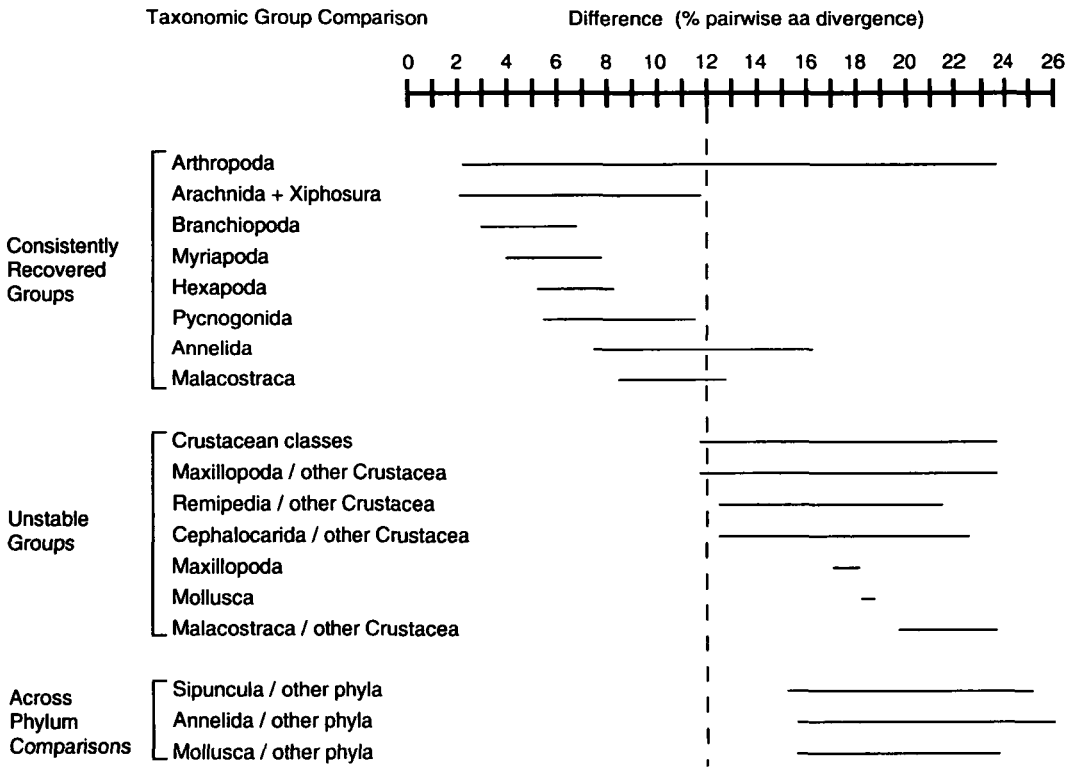

FIG. 5. Group recovery and pairwise sequence difference for amino acids of elongation factor-1α (EF-1α). Note that those groups consistently recovered by maximum-parsimony analysis generally have internal pairwise difference values of less than about 12%. Unstable or poorly resolved groups generally have internal pairwise difference values greater than 12%, which suggests that phylogenetic signal is relatively low or absent in EF-1α once sequence differences exceed this value. This result is inconsistent with the fossil-based pairwise divergence curve (Fig. 1), which indicates that phylogenetic signal should persist in EF-1α through the Cambrian.

within Crustacea has not been adequately sampled, especially for groups previously proposed as basally divergent (*e.g.*, Cephalocarida, Phyllocarida, Remipedia). Third, malacostracans are very divergent from all other arthropods, that is, the internode on the EF-1α gene tree connecting malacostracans to other arthropods is very long. This raises the possibility that malacostracan synapomorphies with branchiopods may have been overwritten and/or that malacostracan sequences are attracted to other long-branch taxa, especially the annelids and mollusks.

Increased taxon sampling provides a means of addressing all three concerns. Accordingly, we have expanded our sampling of EF-1α sequences from all major clades for a total of 40 taxa. Now, all recognized classes of Crustacea are represented, including Cephalocarida and Remipedia. Furthermore, a representative of Phyllocarida *(Ne-*

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virens (U90064), Ostracoda (AF063414), *Pedetontus saltator* (U90056), *Periplaneta americana* (U90054), *Phascolopsis gouldii* (AF063421), *Polyxenus fasciculatus* (U90055), *Proteroiulus fuscus* (AF063415), *Scutigera coleoptrata* (U90057), *Speleonectes tulumensis* (AF063416), *Tanystylum orbiculare* (AF063417), Tardigrada (AF063419), *Tomocerus* sp. (U90059), *Triops longicaudatus* (U90058), *Tubifex tubifex* (AF063422), *Vonones ornata* (U90060). Species names for Ostracoda and Tardigrada will be placed in GenBank accession after positive identification.

balia), the apparent sister group to eumalacostracans (Spears and Abele, 1997), as well as an additional eumalacostracan, have been added to reduce the basal branch length of Malacostraca. We have also added more outgroups including Tardigrada, a phylum often considered closely related to arthropods (Giribet *et al.*, 1996; Dewel and Dewel, 1997).

Several conclusions follow from phylogenetic analysis of these sequences (Fig. 4; unpublished observations). First, there is no single, completely resolved topology favored by either maximum likelihood or parsimony analyses, as illustrated by the strict consensus of parsimony trees (Fig. 4). Second, groups that were moderately to strongly recovered in the analysis of 21 taxa continue to be recovered with expanded taxon sampling. Third, monophyly of pycnogonids is strongly supported, and this clade either groups with or is close to other chelicerates. Fourth, malacostracans no longer consistently form the basal group within arthropods, but instead are found above the Pycnogonida (Chelicerata) in some resolutions. The distance of the Malacostraca to other arthropods remains high (Fig. 5; unpublished data). Fifth, crustaceans remain para- or polyphyletic, although constrained monophyly adds only about 1% in tree length. Sixth, maxillopod crustaceans, which are represented by an ostracod, a cirripede, and a copepod, are not monophyletic, a result consistent with the paucity of morphological and molecular characters uniting this group (Abele *et al.*, 1992; Wilson, 1992; Spears and Abele, 1997). Seventh, the tardigrade is consistently, although not strongly, recovered as sister group to arthropods. Eighth, bootstrap support for the deepest divergences within arthropods is low, paralleling the earlier result for 21 taxa. In conclusion, expanded taxon sampling provides evidence for the continued stability of some groups, particularly those that are more recently derived. Conversely, expanded sampling of Crustacea actually destabilizes relationships within this morphologically diverse subphylum. Other inter-subphylum-level relationships remain weakly supported and unstable.

The absence of strong support for presumed Cambrian-age divergences is unexpected given that fossil-based estimates indicate that potential phylogenetic information accumulated rapidly during the Cambrian and shows no evidence of approaching an asymptote (Fig. 1). Interestingly, recovery of taxonomic groups is correlated with pairwise sequence differences across those groups (Fig. 5). Specifically, groups are recovered if they include at least some pairwise differences less than about 12% (see upper set in Fig. 5); otherwise, they are not recovered (see middle set in Fig. 5). For comparison, pairwise distances across selected phyla are also shown (see lower set in Fig. 5). The possible significance of these observations is discussed in the next section.

SIGNIFICANCE OF THE CAMBRIAN "EXPLOSION" FOR MOLECULAR SYSTEMATICS

Whether organismal heterogeneity is measured in terms of phylogenetic diversity or morphological disparity (Gould, 1989, 1991; Fortey *et al.*, 1996), it is generally recognized that the most inclusive groups of metazoan organisms, including arthropods, made their first unambiguous appearance during the Cambrian (Benton, 1993). However, the abrupt appearance of such fossils implies only the origin of biological and/or geological factors that enhance fossilization and does not, in itself, indicate Cambrian accelerations in either the formation of new protostome lineages or major body plans. Indeed, a phylogenetically and morphologically diverse soft-bodied, minute, or environmentally restricted arthropod/protostome fauna may have existed long before the Cambrian and only acquired paleontological "visibility" with the evolution of armor, large body size, or extensive ecological or geographical ranges (Conway Morris, 1994*a, b*; Davidson *et al.*, 1995; Erwin, 1991, 1994; Wray *et al.*, 1997; Ayala *et al.*, 1998). This proposal is consistent with observations that the earliest Cambrian fossils represent phylogenetically diverse lineages (Benton, 1993; Wills *et al.*, 1995), that candidate Precambrian arthropods and protostomes are already known (Conway Morris, 1994*a*; Waggoner,

1996), and that many extant but presumably ancient lineages with characteristics of the hypothetical "hidden" Precambrian fauna (*e.g.*, many meiofaunal metazoans) are paleontologically invisible throughout most or all of their probable stratigraphic ranges (Benton, 1993). However, in the absence of appropriate *Lagerstätten*, such as described by Bengston and Zhao (1997) and Xiao *et al.* (1998), or of innovations in paleontological analysis or technology, fossil evidence alone seems to hold little immediate potential for determining whether the Cambrian "explosion" reflects enhanced preservation associated with the origin of new protostome lineages and/or body plans or merely enhanced preservation of pre-existing lineages and body plans.

These alternative scenarios for the Cambrian "explosion" suggest two explanations for the failure of EF-1α to resolve relationships among major arthropod/protostome lineages, as well as for the incongruity of this result with expectations derived from the fossil-based pairwise distance curve (Fig. 1). First, it is possible that the temporal component of the curve is accurate but that intervals between Cambrian-age phylogenetic divergences were too brief to permit a significant number of informative replacements to accumulate in the slowly evolving EF-1α amino acid sequence. We term this possibility the Rapid Radiation Model (Fig. 6). Alternatively, it is possible that the temporal component of the pairwise difference curve is wrong and that lineages with Precambrian origins only seem to originate during the Cambrian due to enhanced fossilization during this period. In this case, the range of pairwise differences among the Precambrian lineages would appear to be concentrated within the Cambrian, thus yielding a pairwise distance curve that gives the false impression of rapid accumulation of potentially informative replacements in EF-1α (Fig. 1). We term this the Enhanced Preservation Model (Fig. 7). These two explanations should be regarded as ends of a spectrum of possibilities rather than mutually exclusive conditions.

Current evidence is more consistent with the Enhanced Preservation Model than the Rapid Radiation Model. Specifically, analysis of pairwise sequence differences outside Arthropoda indicates that EF-1α is at or near its asymptotic difference at the level of the Cambrian. Pairwise sequence differences across the protostome phyla Arthropoda, Annelida, Sipuncula and Mollusca average about 20% and range from 15% to 26%. This range encompasses the average pairwise difference between protostomes and a sponge (21%) and between protostomes and a plant (26%), divergences that are clearly Precambrian in age. This observation is consistent with presence of a diverse but unrecorded Precambrian arthropod fauna, which would explain why EF-1α does not resolve apparent Cambrian-age clades. Specifically, it is reasonable to hypothesize that EF-1α is at or near its asymptotic difference (saturation) at the level of the Cambrian and Precambrian, as represented by curves G or H (Fig. 7), and that this was not indicated by the fossil-based pairwise difference curve due to inadequate preservation of the Precambrian fauna. In contrast, curve A (Fig. 7), which is pre-

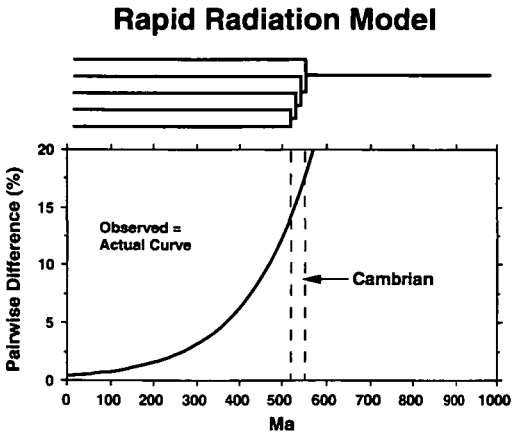

FIG. 6. Rapid radiation model for explaining the inconsistency between results of phylogenetic analysis of elongation factor-1α (EF-1α) (Fig. 4) and expectations based on a pairwise difference curve (Fig. 1). This model assumes that known fossils accurately reflect phylogenetic divergence times and that the pairwise difference curve reflects actual rates of amino-acid evolution in EF-1α. Under this model, failure of EF-1α to resolve Cambrian-age divergences is attributed to high rates of cladogenesis during the Cambrian; that is, intervals between divergences were too brief to allow a significant number of phylogenetically informative amino-acid replacements to accumulate.

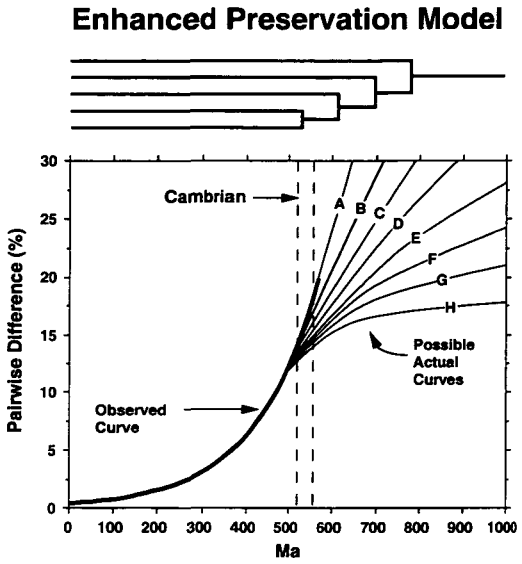

FIG. 7. Enhanced preservation model for explaining the inconsistency between results of phylogenetic analysis of elongation factor-1α (Fig. 4) and expectations based on a pairwise difference curve (Fig. 1). This model assumes that fossilization was enhanced during the Cambrian, such that lineages with a long Precambrian history appeared in the stratigraphic record for the first time. Thus, the pairwise difference curve underestimates the divergence times of arthropod/protostome lineages. Under this model, failure of EF-1α to resolve arthropod phylogeny is attributed to degradation of phylogenetic signal by multiple changes per amino-acid position.

dicted by the Rapid Radiation Model (Fig. 6), shows no evidence of approaching a near-20% asymptotic difference within arthropods, despite the fact that such an asymptote is expected given the results of the interphylum comparisons.

Validity of the Enhanced Preservation Model would have two important practical implications. First, while EF-1α does not appear to provide strong resolution of Cambrian-age phylogenetic events, it would still be useful for more recent events, especially those that occurred between the Carboniferous and Silurian (about 250 to 400 Ma) (Harland et al., 1990) (Fig. 7). This range appears to encompass basal radiations within terrestrial arthropods, namely, Arachnida (300 to 410 Ma) (Selden, 1993), Myriapoda (300 to 410 Ma) (Ross and Briggs, 1993) and Hexapoda (310 to 400 Ma) (Labandeira, 1994; Shear and Kukalová-Peck, 1990). Indeed, monophyly of these clades is strongly supported by EF-1α (Figs. 4, 5). Second, the Enhanced Preservation Model suggests that fossil-based pairwise difference curves may not be useful in identifying molecular sequences for resolving Cambrian- and Precambrian-age divergences. Rather, the phylogenetic information contained with a particular molecule may have to be estimated through comparisons with results from other molecules. In this regard, EF-1α could serve as a useful benchmark.

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