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Pancrustacean phylogeny: hexapods are terrestrial crustaceans and maxillopods are not monophyletic

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Recent molecular analyses indicate that crustaceans and hexapods form a clade (Pancrustacea or Tetraconata), but relationships among its constituent lineages, including monophyly of crustaceans, are controversial. Our phylogenetic analysis of three protein-coding nuclear genes from 62 arthropods and lobopods (Onychophora and Tardigrada) demonstrates that Hexapoda is most closely related to the crustaceans Branchiopoda (fairy shrimp, water fleas, etc.) and Cephalocarida + Remipedia, thereby making hexapods terrestrial crustaceans and the traditionally defined Crustacea paraphyletic. Additional findings are that Malacostraca (crabs, isopods, etc.) unites with Cirripedia (barnacles, etc.) and they, in turn, with Copepoda, making the traditional crustacean class Maxillopoda paraphyletic. Ostracoda (seed shrimp)—either all or a subgroup—is associated with Branchiura (fish lice) and likely to be basal to all other pancrustaceans. A Bayesian statistical (non-clock) estimate of divergence times suggests a Precambrian origin for Pancrustacea (600 Myr ago or more), which precedes the first unambiguous arthropod fossils by over 60 Myr.

Keywords: arthropod phylogeny; Cambrian explosion; Crustacea; Hexapoda; molecular systematics; Pancrustacea

1. INTRODUCTION

Establishing phylogenetic relationships among the major arthropod groups, especially the hyper-speciose Hexapoda and the morphologically diverse Crustacea, would be a major advance toward resolving the tree of life. Recent molecular analyses indicate that hexapods and crustaceans form a clade (Pancrustacea or Tetraconata) (Friedrich & Tautz 1995; Boore *et al.* 1998; Giribet *et al.* 2001; Regier & Shultz 2001; Mallatt *et al.* 2004), but relationships among its constituent lineages are controversial (Spears & Abele 1998; Giribet *et al.* 2001; Martin & Davis 2001; Regier & Shultz 2001; Lavrov *et al.* 2004; Mallatt *et al.* 2004). Resolving these lineages would provide an improved phylogenetic context for documenting the many complex morphological transformations that have occurred during arthropod evolution. Clarifying the role of homoplasy (i.e. parallelisms due to constraints and convergences due to natural selection) would be another benefit of a robust phylogeny. Unfortunately, identifying sufficient characters to robustly resolve closely spaced Palaeozoic (or earlier) divergences has been a challenge. Sequence data from multiple, appropriately evolving, protein-coding nuclear genes have been successfully used to resolve other lineages (see, for example, Murphy *et al.* 2001) and also hold promise for arthropods. We address relationships within Pancrustacea by analysing sequence data from three such genes and strongly resolve relationships of several major groups.

2. MATERIAL AND METHODS

(a) *Taxon sampling and data generation*

Sixty-two species of Arthropoda, Tardigrada and Onychophora were sampled (see table S1 in electronic Appendix A). Specific RNA sequences were amplified by reverse transcription followed by polymerase chain reaction (PCR); gel-isolated PCR fragments were then reamplified using nested PCR, re-gel-isolated and sequenced; sequences were assembled and datasets for phylogenetic analyses constructed (see references in Regier & Shultz (2001)). Sequence data were derived from three genes: elongation factor-1α (1131 nucleotides), the largest subunit of RNA polymerase II (2025 nucleotides) and elongation factor-2 (2178 nucleotides). GenBank numbers (see table S1 in electronic Appendix A) and the aligned nucleotide dataset (see dataset in the electronic Appendices or go to www.umbi.umd.edu/users/jcrlab/Arthropoda3gn2004.doc) are available.

(b) *Phylogenetic analyses*

Nucleotides with third codon positions removed from the 3-gene concatenated sequence were analysed by maximum parsimony under equal weights and by maximum likelihood (Swofford 2002). The latter incorporated a general time reversible model with among-site-rate-variation modelled by a gamma distribution plus a separate parameter for invariable sites. Concatenated amino acids (conceptually translated in MacClade; Maddison & Maddison 2002) were analysed by maximum parsimony under equal weights (Swofford 2002), a Bayesian statistical approach (Huelsenbeck & Ronquist 2001) using the Jones, Taylor and Thornton model (Jones *et al.* 1992), and a modified-likelihood approach (Adachi & Hasegawa 1994), in which the favoured protml tree was selected from the 91 673 most-parsimonious trees (tree lengths = 6853–6861). Non-parametric bootstrap analyses (Felsenstein 1985) were performed for all approaches except protml. To calculate bootstrap values for the Bayesian analysis, we wrote a computer program in C (called BP_link, freely downloadable from http://www.umbi.umd.edu/users/jcrlab) that semi-automates this process by linking already available software packages. This allowed a direct comparison of bootstrap percentages and posterior probabilities.

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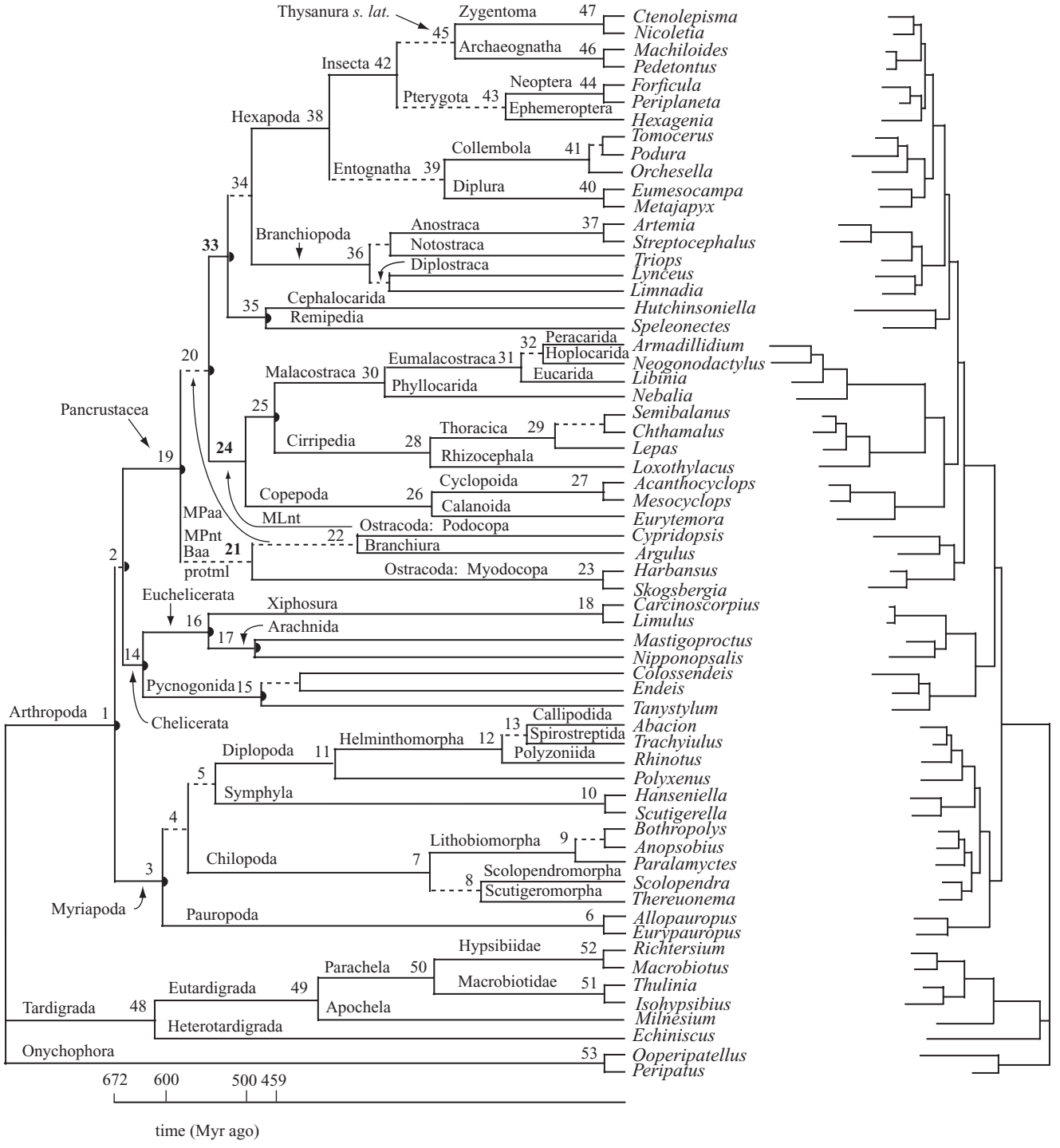

Figure 1. Maximum-likelihood, 3-gene, amino acid ('protml') tree selected from 91 673 most-favoured maximum-parsimony trees. Nodes are arbitrarily numbered on the cladogram (left) for reference to table 1. Branch lengths from the same analysis are shown in phylogram format (right). Averaged absolute divergence time estimates are for nodes identified by half-filled circles only. Note that variance for time estimates is substantial (see figure S1 for full results). Nodes that are either 'supported' or 'well supported' (defined in text) are basally connected to solid branch lines. Less well-supported nodes are connected to dashed lines. Recovery of nodes 21, 24 and 33 is of particular interest and their numbers are in bold type.

(c) *Divergence time estimates*

Divergence time estimates at 12 nodes were performed using a Markov chain Monte Carlo procedure for Bayesian analysis of amino acid sequences (Thorne & Kishino 2002) from 17 diverse arthropods, one onychophoran and one tardigrade. Evolutionary rates at adjoining nodes were assumed to be autocorrelated rather than following a strict molecular clock, and individual genes were assigned separate autocorrelation parameters. Fossil-based boundary conditions were also incorporated (Benton 1993). More details can be found in the legend to figure S1 (see electronic Appendices).

3. RESULTS AND DISCUSSION

(a) *Assessing node support*

In the current study, 40 new crustacean sequences were obtained from elongation factor-1α, elongation factor-2

Table 1. Clade recovery assessment from combined analysis of EF-1α, Pol II, and EF-2 sequences.

node[a]	taxonomic group	bootstrap percentage[b] MLnt	MPnt	MPaa	Baa	posterior probability for Baa	recovered by protml[c]	supported /well supported[d]
1	Arthropoda	52	65	99	98	100	§	✓✓
2	Pancrustacea + Chelicerata	[7]	32	[33]	38	66	§	
•	Pancrustacea + Myriapoda	46	[35]	[17]	[35]			
•	Myriapoda + Chelicerata	[< 5]	[7]	35	[< 30]			
3	Myriapoda	96	91	66	97	100	§	✓✓
4	Myriapoda − Pauropoda	[39]	[21]	72	74	100	§	
•	Myriapoda − Diplopoda	24	[21]	[< 5]	[< 30]			
5	Diplopoda + Symphyla	[26]	[5]	[18]	[24]		§	
•	Diplopoda + Chilopoda	[21]	30	45	[< 30]			
•	Symphyla + Chilopoda	[18]	[30]	[21]	49	96		
•	Pauropoda + Symphyla	44	45	[< 5]	[< 30]			
6	Pauropoda	100	100	100	100	100	§	✓✓
7	Chilopoda	100	97	87	96	100	§	✓✓
8	Chilopoda: (Scolopendromorpha + Scutigeromorpha)	71	82	66	[53]		§	
9	Chilopoda: Lithobiomorpha	72	54	77	93	100	§	✓✓
10	Symphyla	100	100	100	100	100	§	✓✓
11	Diplopoda	84	57	95	96	100	§	✓✓
12	Diplopoda: Helminthomorpha	99	95	90	96	100	§	✓✓
13	Diplopoda: Helminthomorpha: Callipodida + Spirostreptida	82	77	64	49	76	§	
14	Chelicerata	84	82	84	87	100	§	✓✓
15	Pycnogonida	100	100	100	100	100	§	✓✓
16	Euchelicerata	100	100	100	100	100	§	✓✓
17	Arachnida	80	70	94	96	100	§	✓✓
18	Xiphosura	100	100	100	100	100	§	✓✓
19	Pancrustacea	100	100	99	100	100	§	✓✓
•	Crustacea	[< 5]	[< 5]	[< 5]	[< 30]			
20	Pancrustacea − (Ostracoda + Branchiura)	[28]	28	21	[< 30]		§	
•	Pancrustacea − (Ostracoda: Myodocopa + Branchiura)	28	[9]	[21]	[< 30]			
•	Pancrustacea − (Ostracoda: Podocopa + Branchiura)	[< 5]	[< 5]	[16]	[< 30]			
•	Pancrustacea − Ostracoda: Myodocopa	[< 5]	[< 5]	19	[< 30]			
21	Ostracoda + Branchiura	[55]	66	[15]	64	99	§	
•	Ostracoda: Myodocopa + Branchiura	91	98	[19]	[< 30]			
22	Ostracoda: Podocopa + Branchiura	[9]	[< 5]	56	87	100	§	
•	Ostracoda	[< 5]	[< 5]	[< 5]	[< 30]			
23	Ostracoda: Myodocopa	100	100	100	100	100	§	✓✓

(*Continued.*)

Table 1. (*Continued.*)

node[a]	taxonomic group	bootstrap percentage[b] MLnt	MPnt	MPaa	Baa	posterior probability for Baa	recovered by protml[c]	supported /well supported[d]
24	Copepoda + Cirripedia + Malacostraca	88	66	74	69	100	§	✓✓
25	Cirripedia + Malacostraca	100	99	89	95	100	§	✓✓
26	Copepoda	100	100	100	100	100	§	✓✓
27	Copepoda: Cyclopoida	100	100	100	100	100	§	✓✓
28	Cirripedia	100	100	100	100	100	§	✓✓
29	Cirripedia: Thoracica	99	100	100	100	100	§	✓✓
•	Maxillopoda (± Ostracoda)	[< 5]	[< 5]	[< 5]	[< 30]			
30	Malacostraca	100	100	100	100	100	§	✓✓
31	Eumalacostraca	92	100	99	90	100	§	✓✓
32	Malacostraca: Eumalacostraca: Peracarida + Hoplocarida	81	87	58	53	92	§	
33	Branchiopoda + Hexapoda + Cephalocarida + Remipedia	71	43	18	48	100	§	✓
34	Branchiopoda + Hexapoda	[6]	[10]	33	[< 30]		§	
•	Hexapoda + Cephalocarida + Remipedia	71	45	[14]	[< 30]			
•	Branchiopoda + Cephalocarida + Remipedia	[< 5]	[6]	[< 5]	<30	70		
35	Cephalocarida + Remipedia	78	72	29	67	100	§	✓
36	Branchiopoda	95	85	98	98	100	§	✓✓
37	Branchiopoda: Anostraca	100	100	100	100	100	§	✓✓
38	Hexapoda	80	62	75	94	100	§	✓✓
39	Diplura + Collembola (= Entognatha)	[25]	[26]	53	59	100	§	
•	Diplura + Insecta	66	31	[36]	[38]			
40	Diplura	78	46	92	96	100	§	✓✓
41	Collembola	100	100	100	100	100	§	✓✓
42	Insecta (= Ectognatha)	69	62	70	76	100	§	✓
43	Pterygota	[23]	[28]	29	[< 30]		§	
44	Neoptera	85	[53]	75	92	100	§	✓✓
45	Thysansura *s. lat.*	73	65	31	33	72	§	
46	Archaeognatha	100	100	100	100	100	§	✓✓
47	Zygentoma (= Thysanura *s. str.*)	96	98	82	89	100	§	✓✓
48	Tardigrada	100	100	100	100	100	§	✓✓
49	Eutardigrada	100	100	100	100	100	§	✓✓
50	Parachela	100	100	100	100	100	§	✓✓
51	Macrobiotidae	100	100	99	100	100	§	✓✓
52	Hypsibiidae	100	100	100	100	100	§	✓✓
53	Onychophora	100	100	100	100	100	§	✓✓

[a] Node numbers refer to groups in figure 1; '•' identifies groups not present in figure 1.

[b] Bootstrap percentages are listed for four phylogenetic procedures: MLnt, maximum-likelihood analysis of nucleotide codon positions 1 + 2; MPnt, maximum-parsimony analysis of nucleotide codon positions 1 + 2; MPaa, maximum-parsimony analysis of amino acids; Baa, Bayesian analysis of amino acids. Brackets (with or without bootstrap percentages enclosed) indicate that that clade was not recovered in the favoured topology or in the strict consensus when there were multiple favoured topologies.

[c] § indicates that that taxonomic group was recovered in the tree of highest lnL for this protein likelihood method (protml). No bootstrap analysis was performed.

[d] ✓ indicates that that taxonomic group is 'supported', and ✓✓ that it is 'well supported' (see text for definitions).

and RNA polymerase II (total, *ca*. 5334 nucleotides (nt) or *ca*. 1778 inferred amino acids (aa)) and combined with published sequences, including those from the outgroups Onychophora and Tardigrada (Regier *et al.* 2004*b*). A total of 62 taxa (table S1) were analysed using maximum parsimony (MP), maximum likelihood (ML), Bayesian (B) statistical methods, and protein-based parsimony + likelihood (protml). Rapidly evolving third-codon positions were not included in analyses of nucleotides, and a χ^2-test could not reject base compositional homogeneity for the remaining nucleotides ($p = 0.671$). To assess the degree of clade support we evaluated: (i) sensitivity to five method-character combinations (i.e. MLnt, MPnt, MPaa, Baa and protml); (ii) posterior probabilities from Baa analysis; and (iii) non-parametric bootstrap support from MLnt, MPnt, MPaa and Baa. To facilitate discussion, we will call 'well supported' those nodes that are recovered by all five method-character combinations, that have bootstrap support of greater than 85% in at least one combination, and that have a posterior probability (in the Baa analysis) of 100%. The criteria for 'supported' nodes are the same except that their highest bootstrap support is 70–85%. Individual gene analyses supported no clades that were not also supported by combined data analyses (unpublished observations).

The protml tree of highest likelihood is shown in figure 1, and the recovery, posterior probabilities and bootstrap values of numbered nodes are provided in table 1. Thirty-seven of the numbered, internal nodes are well supported and three are supported (identified in the last column of table 1 with cross-reference to the corresponding node numbers shown in figure 1). For the Bayesian amino acid (Baa) analysis, 43 groups had posterior probabilities of 100%. Out of these 43, 38 had bootstrap values above 70% (compare columns 7 and 6 in table 1), and three out of the five nodes with lower bootstrap values were still either supported or well supported (nodes 24, 33, 35 but not 21, 39), indicating that, with our dataset and model parameters, posterior probabilities are in large agreement with our other criteria for assessing node support (Erixon *et al.* 2003).

(b) *Monophyly of Pancrustacea, Myriapoda and Chelicerata, but uncertain interrelationships*

Our results reaffirm that extant arthropods are arranged in three well-supported monophyletic lineages—Pancrustacea, Myriapoda and Chelicerata. Relationships within these groups are not affected by removal of the panarthropod outgroups (unpublished observations). Until recently, myriapod monophyly was challenged primarily by the possibility that hexapods originated from within the group, a view now largely, but not completely (Kraus 2001), abandoned in favour of the Pancrustacea concept. Monophyly of Chelicerata was questioned by one study (Giribet *et al.* 2001) that placed Pycnogonida (sea spiders) as the sister to all other arthropods, but other studies (Regier & Shultz 2001; Vilpoux & Waloszek 2003), including this one, resolve pycnogonids as well-supported, basally divergent chelicerates. Relationships among the three major arthropod lineages are uncertain in the present analyses, with some favouring Mandibulata (= Pancrustacea + Myriapoda) and others favouring Paradoxopoda (= Chelicerata + Myriapoda). Uncertainty is present in others studies as well (Mandibulata: Edgecombe *et al.* 2000; Giribet *et al.* 2001; Paradoxopoda: Friedrich & Tautz 1995; Hwang *et al.* 2001; Mallatt *et al.* 2004).

(c) *Major splits within Pancrustacea*

As in other recent analyses, we recover Pancrustacea as a well-supported monophyletic group, but our results are unique in also identifying three pancrustacean lineages (clades 21, 24, 33 in figure 1). Clade 24 is well supported and encompasses three diverse crustacean groups—Malacostraca, Cirripedia and Copepoda—with the Malacostraca + Cirripedia subclade (node 25) receiving up to 100% bootstrap support (table 1). The traditional but controversial clade Maxillopoda (= Thecostraca (includes Cirripedia), Copepoda, Mystacocarida, Branchiura and sometimes Ostracoda) is here recovered as poly- or paraphyletic. Past debates on the phylogenetic status of Maxillopoda generally focused on the group's extreme morphological diversity or on competing sets of diagnostic characters (Martin & Davis 2001) but rarely in modern phylogenetic terms. Falsification of Maxillopoda requires that a subset of maxillopodans be recovered as a sister clade to one or more non-maxillopodan lineages, and this is provided by our analysis.

Clade 33, which is supported, includes four classes within two well-supported clades (Hexapoda (Regier *et al.* 2004*a*) and Branchiopoda) and one supported clade (Cephalocarida + Remipedia; table 1), although relationships among the lineages remain ambiguous. It is noteworthy that extant members occupy either non-marine environments—Hexapoda on land and in freshwater and Branchiopoda generally in freshwater (Schram 1986)—or marine environments so unusual that the taxa remained undiscovered until the latter half of the twentieth century—Remipedia in anchialine caves (Yager 1981; Schram 1986) and Cephalocarida in benthic flocculent suspensions (Sanders 1955; Schram 1986). It is possible that early members of the clade had a proclivity for near-shore or marginal marine habitats or were competitively excluded by other 'crustaceans', perhaps together with myriapods and most chelicerates.

These findings reinforce the value of developmental genetic studies that use *Artemia* brine shrimp (Branchiopoda) as a model for understanding morphological evolution in hexapods (e.g. Averof & Cohen 1997). The advent of the Pancrustacea hypothesis inspired studies of the evolution of hexapod morphology from a primitive 'crustacean' condition. However, interpretations of comparative analyses are problematic when relevant phylogenetic relationships are unknown, in this case, the sister group to Hexapoda. Many morphological characters deemed consistent with the molecule-based Pancrustacea concept were based on malacostracans (Dohle 2001), implying that these crustaceans would be most informative of hexapod evolution. However, our results indicate that hexapods are more closely related to a subset of non-malacostracan lineages, which includes the Branchiopoda.

The three non-hexapod classes in clade 33—Branchiopoda, Cephalocarida, Remipedia—have each been regarded as the 'most primitive' and most basal crustaceans by a subset of workers (see Martin & Davis 2001), a debate driven, in part, by carcinology's somewhat anachronistic search for the 'Ur-crustacean'. By contrast, our analyses

show that these lineages are phylogenetically derived, whether they retain primitive morphologies or not. One parsimony-based study using 18S rDNA also united Cephalocarida and Remipedia and placed them in a non-basal position within Crustacea, but the authors seemed to regard this as an artefact of long-branch attraction (Spears & Abele 1998). However, our result supports a literal interpretation of their findings, as ours is based on different genes and generated by probabilistic-model-based likelihood and Bayesian methods that are less prone to long-branch anomalies than parsimony.

Our recovery of clades 24 and 33 and their subclades differs from that found with 18S + 28S ribosomal nucleotides (Mallatt *et al.* 2004). However, in that case a maximum-likelihood reanalysis of the data (in which only approximately one-third as many pancrustceans were sampled) reveals that bootstrap values are 65% or less for all inter-class relationships (unpublished observations), so we consider the current results based on protein-coding nuclear genes to be more compelling and not in strong conflict with the ribosomal analysis.

Our results also differ from those published in a recent study on mitochondrial gene order (Lavrov *et al.* 2004). Here, too, there is reason to be circumspect. In particular, the single character (a tRNA rearrangment) that defines a clade consisting of Cirripedia, Cephalocarida, Branchiura and Pentastomida is actually missing in Cirripedia. Instead, the cirripede is included because it shares a separate (and also homoplasious) tRNA rearrangement with the cephalocarid, which in turn is missing the diagnostic transposition defining Pancrustacea. Although the authors' parsimony-based argument is valid, the fact that this conclusion is based on only two homoplasious characters should raise concern. Indeed, the ease of interpretation and certainty with which mitochondrial gene order was originally hoped to provide phylogenetic information are now being challenged, particularly in arthropods (see Negrisolo *et al.* 2004). As for other characters, those based on gene order are undoubtedly appropriate only at certain taxonomic levels (Hickerson & Cunningham 2000).

Clade 21 includes Ostracoda and Branchiura (the latter being most closely related to the unrepresented Pentastomida (tongue worms); Abele *et al.* 1989; Lavrov *et al.* 2004) as a group (Oligostraca; Zrzavy *et al.* 1998) that here forms the sister group to all other pancrustaceans. Although not supported (recovered by only three out of five approaches), it is consistent with analyses of nuclear ribosomal nucleotides (Spears & Abele 1998; Mallatt *et al.* 2004) and morphology (Zrzavy *et al.* 1998). Its members have oligomeric bodies and share aspects of ovariole structure. The early divergence of Oligostraca is compatible with Cambrian-age fossils of ostracod-like arthropods (Schram 1986) and purported pentastomids (Walossek & Müller 1994). Furthermore, our two molecular alternatives (MPaa, MLnt; see figure 1) either place Ostracoda and Branchiura as a paraphyletic grade at the base of Pancrustacea or else move Ostracoda: Podocopa to the base of clade 24, keeping Ostracoda: Myodocopa + Branchiura at the base of Pancrustacea. Thus, our data consistently indicate that Ostracoda and Branchiura belong at or near the base of Pancrustacea, although their precise phylogenetic positions remain uncertain. We also note that Ostracoda is invariably, but inconsistently, split by Branchiura, i.e. Branchiura is grouped with Ostracoda: Myodocopa by nucleotides and Ostracoda: Podocopa by amino acids (see Oakley & Cunningham 2002), suggesting inadequate modelling of character transformations of these sparsely sampled and ancient groups.

(d) *Absolute divergence times*

Using the above phylogenetic results, clade divergence time estimations were performed for a subset of 19 panarthropod taxa (figure S1). Abbreviated results (i.e. averaged time estimates without ranges or standard deviations) are mapped onto figure 1 (see the 12 nodes with filled half-circles and also the time-scale below). According to these results (and at the level of two standard deviations), Pancrustacea separated from both Chelicerata and Myriapoda no later than 601 Myr ago (in broad agreement with Pisani *et al.* (2004)), and possibly several hundred million years earlier, i.e. well before the first unambiguous arthropod fossils (Benton 1993; but see Maas & Waloszek 2001) and the 'Cambrian explosion' (Regier & Shultz 1998). The basal divergences within Pancrustacea, Chelicerata and Myriapoda occurred no later than 530, 578 and 544 Myr ago, respectively, and possibly several hundred million years earlier. Within Pancrustacea, the six traditional crustacean classes had all appeared by 388 Myr ago and no earlier than 522 Myr ago.

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