Molecular Evolution of the Arthropod Hemocyanin Superfamily

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Arthropod hemocyanins are members of a protein superfamily that also comprises the arthropod phenoloxidases (tyrosinases), crustacean pseudohemocyanins (cryptocyanins), and insect storage hexamerins. The evolution of these proteins was inferred by neighbor-joining, maximum-parsimony, and maximum-likelihood methods. Monte Carlo shuffling approaches provided evidence against a discernible relationship of the arthropod hemocyanin superfamily and molluscan hemocyanins or nonarthropodan tyrosinases. Within the arthropod hemocyanin superfamily, the phenoloxidase probably emerged early in the (eu-)arthropod stemline and thus form the most likely outgroup. The respiratory hemocyanins evolved from these enzymes before the radiation of the extant euarthropodan subphyla. Due to different functional constraints, replacement rates greatly vary between the clades. Divergence times were thus estimated assuming local molecular clocks using several substitution models. The results were consistent and indicated the separation of the cheliceratan and crustacean hemocyanins close to 600 MYA. The different subunit types of the multihexameric cheliceratan hemocyanin have a rather conservative structure and diversified in the arachnidan stemline between 550 and 450 MYA. By contrast, the separation of the crustacean (malacostracan) hemocyanin subunits probably occurred only about 200 MYA. The nonrespiratory pseudohemocyanins evolved within the Decapoda about 215 MYA. The insect hemocyanins and storage hexamerins emerged independently from the crustacean hemocyanins. The time of divergence of the insect proteins from the malacostracan hemocyanins was estimated to be about 430–440 MYA, providing support for the notion that the Hexapoda evolved from the same crustacean lineage as the Malacostraca.

Introduction

In the body fluids of many arthropod species, oxygen is delivered by large copper-containing proteins that are termed hemocyanins (for review, see Markl 1986; van Holde and Miller 1995). The principal structure of a hexamer of six similar or identical subunits in the 75-kDa range is conserved within all arthropod hemocyanins, although in many cases the hexamers associate to create quaternary structures with up to 8×6 subunits (Markl and Decker 1992). Hemocyanins have been studied thoroughly only in the Chelicerata and the malacostracan Crustacea, but they have been identified in all arthropod subphyla, including the Myriapoda (Mangum et al. 1985; Jaenicke et al. 1999) and Hexapoda (Sánchez et al. 1998). In recent years, other proteins have been discovered within the arthropod phylum that display significant structural and sequence similarities to the hemocyanins but serve very different functions (e.g., Beintema et al. 1994; Burmester and Scheller 1996). Thus, the arthropod hemocyanin superfamily also includes (1) the phenoloxidases (EC 1.14.18.1), which are copper-containing tyrosinases that play a key role in the sclerotization of the arthropod cuticle, in wound healing, and in humoral immune defense (e.g., Söderhäll and Cerenius 1998) and are synthesized as zymogens (prophenoloxidases; PPOs) that are activated by cleavage of a N-terminal peptide; (2) the insect hexamerins, which neither contain copper nor bind oxygen and are regarded mainly as storage proteins associated with molting cycles or nutritional conditions (Telfer and Kunkel 1991; Burmester 1999*a*); (3) the crustacean pseudohemocyanins or cryptocyanins, which closely resemble the hemocyanins of this taxon but do not have the copper-binding sites and are also thought to act as storage proteins (Burmester 1999*b*; Terwilliger, Dangott, and Ryan 1999); and (4) the dipteran hexamerin receptors, which are, surprisingly, related to their own ligands (Burmester and Scheller 1996).

The phylogeny of the hemocyanin superfamily, as well as the evolutionary changes associated with the emergence of the different proteins, cannot be understood without considering the phylogeny of the Arthropoda. The origin and the evolutionary relationships of this taxon are one of the most extensively debated issues in evolutionary biology (e.g., Fortey and Thomas 1997) (fig. 1). As already pointed out by Mangum et al. (1985), the exclusive, but not universal, appearance of the hemocyanins in the Euarthropoda provides an additional argument in favor of the monophyly of this taxon. In this paper, the evolutionary history of the hemocyanin superfamily is inferred and correlated with the recent advances in the understanding of arthropod phylogeny.

Materials and Methods

Sequence Data and Multiple Alignment

Complete nucleotide sequences of 20 insect and 2 crustacean PPOs, 12 hemocyanin subunits, 3 crustacean nonrespiratory pseudohemocyanins, and 50 insect hexamerins are available from the EMBL/GenBank DNA databases. The SwissProt database contains additional amino acid sequences of six hemocyanins. The hexamerin receptors were ignored in this study because only parts of the sequences can be successfully aligned (Burmester and Scheller 1996). Below, the EMBL/GenBank accession numbers of the nucleotide sequences are given where available; asterisks indicate SwissProt accession numbers.

Key words: Chelicerata, Crustacea, hemocyanin, hexamerin, Hexapoda, phenoloxidase.

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Mol. Biol. Evol. 18(2):184–195. 2001

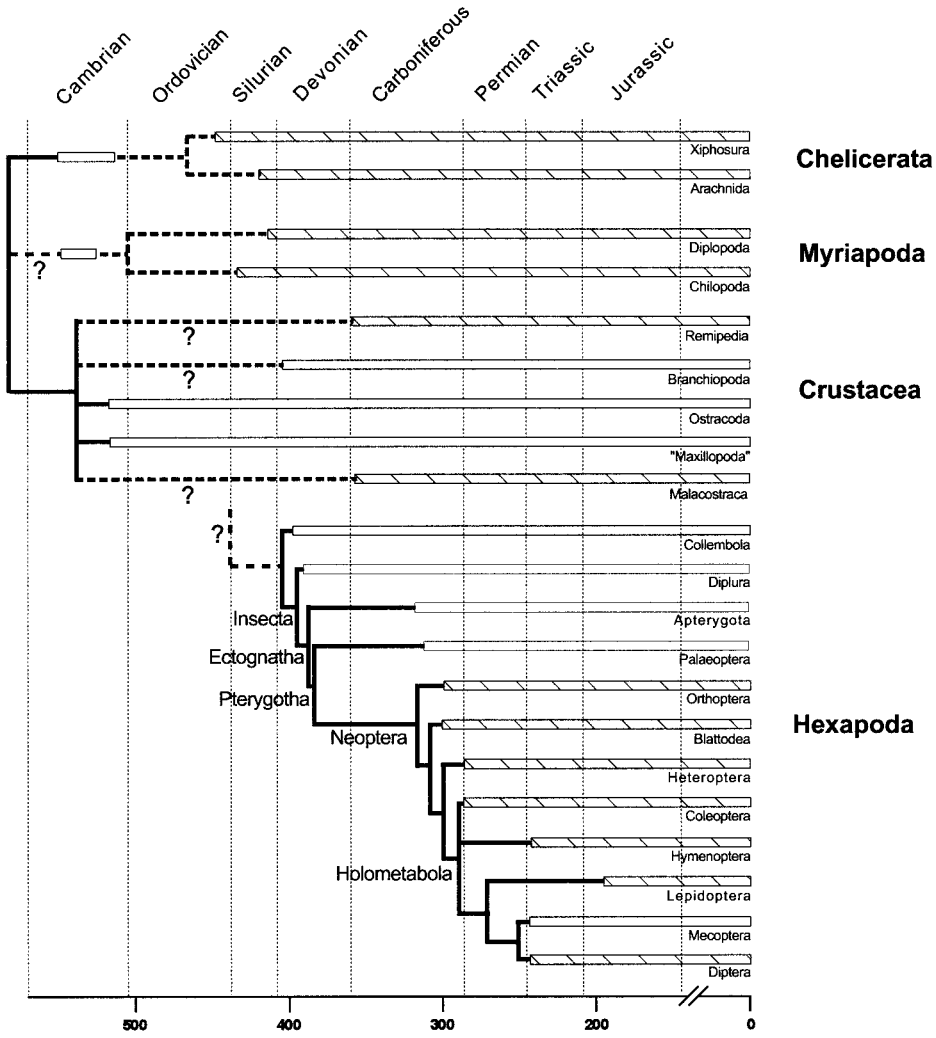

FIG. 1.—Evolution and phylogenetic relationships of the main extant Arthropoda in archaeological context, with special emphasis on the insect orders. The divergence times and the archaeological records were combined from different sources (Kukalová-Peck 1991; Briggs, Weedon, and White 1993). The taxa in which either hexamerins or hemocyanins have been described are cross-hatched.

I. A. Crustacean prophenoloxidases: PPOs of *Penaeus monodon* (PmoPPO; AF099741) and *Pacifastacus leniusculus* (PlePPO; X83494).
   B. Insect prophenoloxidases:
      1. Coleoptera: PPO of *Tenebrio molitor* (TmoPPO; AB020738).
      2. Lepidoptera: *Bombyx mori* prophenoloxidases 1 (BmoPPO1; D49370), 2 (BmoPPO2; D49371), and 3 (BmoPPO3; E12578); *Manduca sexta* prophenoloxidase (MsePPO; L42556) and prophenoloxidase 1 (MsePPO1; AF003253); *Hyphantria cunea* prophenoloxidases 1 (HcuPPO1; U86875) and 2 (HcuPPO2; AF020391).
      3. Diptera: *Drosophila melanogaster* prophenoloxidases A1 (DmePPOA1; D45835), A2 (DmePPO2; AC006074), and A3 (DmePPO3; AC008352); *Sarcophaga bullata* prophenoloxidases 1 (SbuPPO1; AF161260) and 2 (SbuPPO2; AF161261); *Anopheles gambiae* prophenoloxidases 1 (AgaPPO1; AF004915), 2 (AgaPPO2; AF004916), 3 (AgaPPO3; L76038, AF031626), 4 (AgaPPO4; AJ010193), 5 (AgaPPO5; AJ010194), and 6 (AgaPPO6; AJ010195); prophenoloxidase of *Anopheles stephensi* (AstPPO; AF062034).
4. II. Chelicerate hemocyanins: *Androctonus australis* hemocyanin Aa6 (AauHc6; P80476*); *Eurypelma californicum* hemocyanin subunits a (EcaHcA; X16893), b (EcaHcB; AJ290429), c (EcaHcC; AJ277489), d (EcaHcD; AJ290430), e (EcaHcE; X16894), f (EcaHcF; AJ277491), and g (EcaHcG; AJ277492); *Limulus polyphemus* hemocyanin II (LpoHc2; P04253*); *Tachypleus tridentatus* hemocyanin α (TtrHcA; Linzen et al. 1985).
5. III. Crustacean hemocyanins: *Cancer magister* hemocyanin subunit 6 (CmaHc6; U48881); *Panulirus interruptus* hemocyanin subunits a (PinHcA; P04254*), b (PinHcB; P10787*), and c (PinHcC; S21221*); *Palinurus vulgaris* hemocyanin (PvuHc; P80888*); *Homarus americanus* hemocyanin A (HamHcA; AJ272095); *Penaeus vannamei* hemocyanin (PvaHc; X82502).

6. IV. Crustacean pseudohemocyanins (cryptocyanins): *C. magister* cryptocyanin (CmaCC1; AF091261); *H. americanus* pseudohemocyanins 1 (HamPHc1; AJ132141) and 2 (HamPHc2; AJ132142).
7. V. Insect hemocyanin: *Schistocerca americana* hemocyanin (SamEHP; AF038569).
8. VI. Insect hexamerins:
9. A. Hemimetabola.
    1. Orthoptera: *Locusta migratoria* juvenile hormone–binding hexamerin (LmiJHBP; U74469).
    2. Blattodea: *Periplaneta americana* hexamerin (PamHex; L40818), *Blaberus discoidalis* hexamerin (BdiHex; U31328).
    3. Heteroptera: *Riptortus clavatus* cyanoproteins a (RclCyanA; D87272) and b (RclCyanB; D87273).
4. B. Holometabola.
    1. Coleoptera: *Leptinotarsa decemlineata* diapause protein 1 (LdeDP1; X76080; X86074); *Tenebrio molitor* ENP86 (TmoENP86; AB021700).
    2. Hymenoptera: *Camponotus festinatus* hexamerin 2 (CfeHex2; AJ251271); *Bracon hebetor* hexamerin (BheHex; I25974).
    3. Lepidoptera: *Galleria mellonella* LHP82 (GmeLHP82; L21997) and arylphorin (GmeAryl; M73793); *Bombyx mori* sex-specific storage proteins 1 (BmoSSP1; X12978) and 2 (BmoSSP2; P20613); *Manduca sexta* arylphorins α (MseAryla; P1429) and β (MseArylb; M24370; M24371) and methionine-rich storage protein (MsMRSP; L07609); *Hyalophora cecropia* arylphorin (HceAryl; AF032396), riboflavin-binding hexamerin (HceRbH; AF032397), methionine-rich hexamerin F (HceMtHF; AF032398), and methionine-rich hexamerin S (HceMtHS; AF032399); *Hyphantria cunea* storage proteins 1 (HcuSP1; U60988) and 2 (HcuSP2; AF157013); *Trichoplusia ni* acidic juvenile-hormone-suppressible protein (TniAJHSP1; P22327; Jones, Taylor, and Thornton 1990) and basic juvenile-hormone-suppressible proteins 1 (TniBJHSP1; L03280) and 2 (TniBJHSP2; L03281); *Choristoneura fumiferana* diapause-associated proteins 1 (CfuMtH1; AF007767) and 2 (CfuMtH2; AF007768); *Spodoptera litura* arylphorin (SliAryl; AJ249471), methionine-rich storage protein (SliMRSP; AJ249470), and moderately methionine-rich storage proteins A (SliMMRSP-A; AJ249469) and B (SliMMRSP-B; AJ249468).
    4. Diptera: *Drosophila melanogaster* larval serum proteins 1α (DmeLSP1a; AE003489), 1β (DmeLSP1b; U63556), 1γ (DmeLSP1g; AE003467), and 2 (DmeLSP2; X97770); *Calliphora vicina* arylphorins (CviLSP1a; M76480; CviLSP1b; M76479) and larval serum protein 2 (CviLSP2; U89789); *Anopheles gambiae* hexamerins 1 (AgaHEX1; U51225), A1 (AgaHEXA1; AF020870), A2 (AgaHEXA2; AF020871), and A3 (AgaHEXA3; AF020872); *Anopheles arabiensis* hexamerin A (AarHEXA; AF020873); *Anopheles quadriannulatus* hexamerin A (AquHEXA; AF020874); *Anopheles merus* hexamerins A1 (AmerHEXA1; AF020875) and A2 (AmerHEXA2; AF020876); *Anopheles melas* hexamerins A1 (AmelHEXA1; AF020877) and A2 (AmelHEXA2; AF020878); *Aedes aegypti* hexamerins 1γ (AaeHEX1g; U86079) and 2α (AaeHex2a; U86080).

Sequence Alignments

A multiple alignment of the amino acid sequences was constructed by CLUSTAL X (Thompson et al. 1997). Further manipulations were carried out with GeneDoc, version 2.5 (Nicholas and Nicholas 1997). The alignment was corrected under the assumption of secondary-structure conservation, considering the crystallographic structures of *L. polyphemus* (Hazes et al. 1993) and *P. interruptus* (Gaykema et al. 1984; Volbeda and Hol 1989) hemocyanins. Long gap regions, as well as some highly divergent regions, were removed from the final data set. The alignment of the amino acid sequences was imposed on that of the available 86 nucleotide sequences. The final alignments comprised 608 amino acid or 1,824 nucleotide positions and are available at the EMBL database (ftp://ftp.ebi.ac.uk in the directory pub/databases/embl/align/) under accession numbers DS43916 (DNA) and DS43917 (protein) or on request from the author.

Sequence Similarities

Two different Monte Carlo shuffling approaches were applied to estimate the significance of the observed amino acid sequence similarities. The *Z* score (observed value − mean value/standard deviation) was obtained by invoking the ''-ran'' option of the GAP program of the GCG package, version 8.0 (Genetics Computer Group, Wisconsin) with 100 shuffles. The *Z* score gives the significance estimate in terms of standard deviations above the mean. In a second approach, which took into account database sizes, the PRSS3 program (FASTA package; Pearson 2000) was used to estimate probability scores (assuming a gap creation penalty of −12 and a gap length penalty of −2 with 1,000 shuffles).

Molecular Phylogenetic Inference

The program packages PHYLIP, version 3.6 (Felsenstein 1999), and TREE-PUZZLE, version 4.02 (Strimmer and von Haeseler 1996), were used for phylogenetic analyses. Distances between pairs of protein sequences were calculated according to the following substitution models: Dayhoff's PAM001 matrix (Dayhoff, Schwartz, and Orcutt 1978), the JTT model (Jones, Taylor, and Thornton 1992), and Kimura's (1983) corrected percent differences. Distances were corrected for gamma distribution of evolutionary rates. Indels between pairs of sequences were regarded as missing data. Nucleotide distances were calculated according to the HKY model (Hasegawa, Kishino, and Yano 1985). Because saturation of silent sites was assumed, only the

Table 1
Group-Specific Evolution Rates as Inferred from Different Substitution Models

Method	Chelicerate Hemocyanins	Crustacean Hemocyanins	Crustacean PHc	Insect Hemocyanin	Insect Hexamerins
PAM	0.58 ± 0.03	1.29 ± 0.07	1.97 ± 0.11	0.92 ± 0.07	2.70 ± 0.16
Kimura	0.56 ± 0.02	1.27 ± 0.07	2.04 ± 0.12	0.87 ± 0.07	2.37 ± 0.15
JTT	0.57 ± 0.03	1.28 ± 0.08	1.95 ± 0.12	0.94 ± 0.07	2.64 ± 0.16

NOTE.—Rates are given in amino acid replacements per site per 10^9 years.

first and second codon positions were used for phylogenetic inference. Tree constructions were performed by the neighbor-joining method (Saitou and Nei 1987). Additional trees were constructed using the maximum-parsimony methods implemented in the PROTPARS and DNAPARS programs of the PHYLIP package. The reliability of the trees was tested by bootstrap analysis (Felsenstein 1985) with 100 or 500 replications (SEQBOOT program). Maximum-likelihood analyses were performed using TREE-PUZZLE, version 4.02, with 1,000 puzzling steps assuming rate heterogeneity with eight gamma categories.

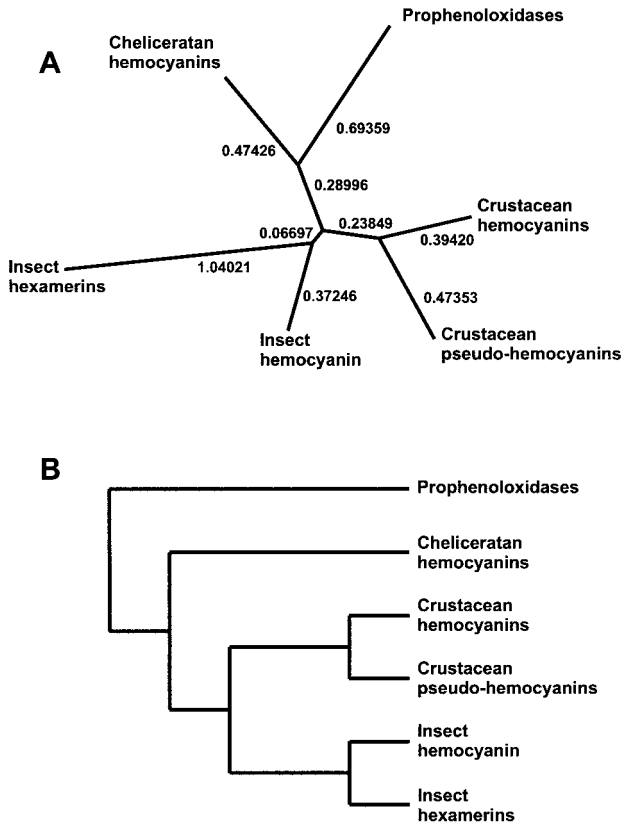

FIG. 2.—Molecular relationship among the members of the arthropod hemocyanin superfamily. Pooled molecular distances between prophenoloxidases (PPOs), chelicerate hemocyanins, crustacean hemocyanins and pseudohemocyanins, and insect hexamerins were calculated according to Dayhoff's PAM001 matrix, and a simplified phylogenetic tree was calculated by the neighbor-joining method (*A*). This tree was rooted under the assumption that the PPOs were the outgroup (*B*). In *B,* the branch lengths are arbitrary.

Calibrations and Time Estimations

The distance matrices were calculated according to the above methods and were imported into the Microsoft EXCEL 97 spread sheet program. A relative-rate test was successively applied to single proteins or groups of proteins according to the topology of the tree (cf. Burmester et al. 1998). Rate constancy of different subtrees was also evaluated by the likelihood ratio test implemented in TREE-PUZZLE, version 4.02. To estimate the divergence times of the different protein families, the replacement rates were calculated separately for each protein family under the assumptions that (1) the Merestomata and Arachnida separated about 450 MYA (Dunlop and Selden 1997), (2) the brachyuran and palinuran Decapoda separated about 150 MYA (Briggs, Weedon, and White 1993), and (3) the Orthoptera separated from the other neopteran insects 320 MYA (Kukulovà-Peck 1991), and the brachyceran nematoceran Diptera separated about 210 MYA (Fraser et al. 1996) (fig. 1). Replacement rates in the text are given as inferred from the PAM distances of the alignment used for phylogenetic inference (see also table 1). This data set does not include some highly variable regions; thus, the replacement rates of the complete sequences are about 5% higher. The lengths of the branches that lead from the basalmost internal nodes to the node that connects the protein families were extrapolated from the branch-specific rates. These calculations were performed independently for both branches that diverge at this node. The confidence limits were estimated using the observed standard deviation of the inferred replacement rates or based on the maximum-likelihood standard deviations.

Results and Discussion

Although hemocyanins, pseudohemocyanins, PPOs, and hexamerins form a functionally highly diversified protein superfamily, most sequences and structural core elements are strikingly conserved, allowing one to trace the evolutionary history of these proteins. In all analyses (neighbor joining, maximum parsimony, and maximum likelihood), four well-supported clades were recovered that comprised (1) PPOs, (2) chelicerate hemocyanins, (3) crustacean hemocyanins, and pseudohemocyanins (cryptocyanins), and (4) hexamerins of the insects (fig. 2*A*). The position of the single insect hemocyanin is less well supported, but it is very likely in the sister group position to the insect hexamerins (see below). However, phylogenetic methods do not resolve

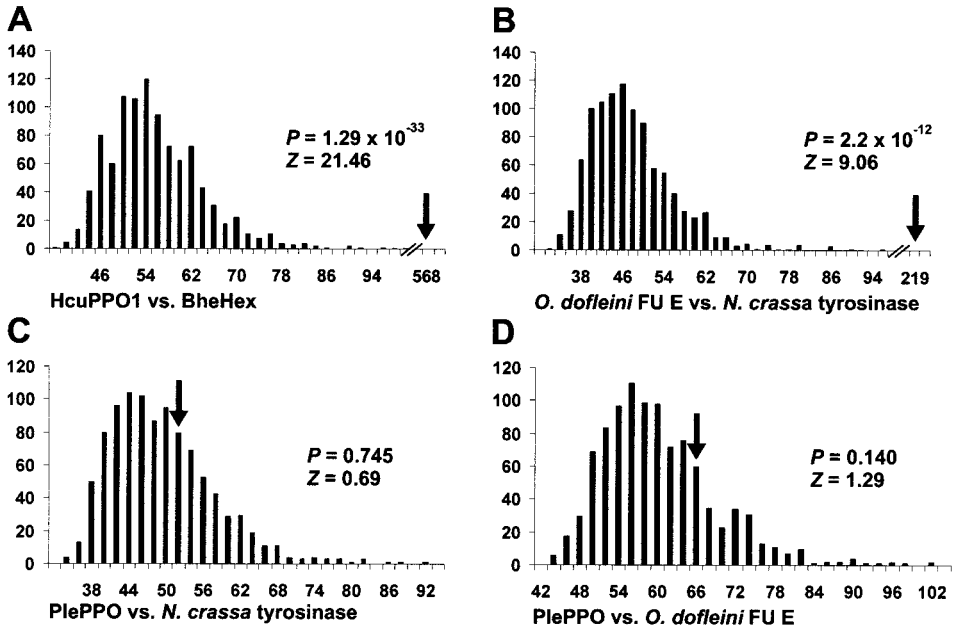

FIG. 3.—Statistical analysis of protein sequence similarities. *A,* Comparison of HcuPPO1 and BheHex. *B, Neurospora crassa* tyrosinase (GenBank accession number M33271) and *Octopus dofleini* functional unit E (GenBank accession number AF020548). *C,* PlePPO and *N. crassa* tyrosinase. *D,* PlePPO and *O. dofleini* functional unit E. The distribution of the observed similarities between the input sequence and 1,000 random sequences as obtained by PRSS3 from the FASTA package are presented; the actual sequence similarity is indicated by an arrow. The *Z* value was obtained using GAP from the GCG program package.

the direction of evolution. In cases where the evolutionary changes are similar in different clades, methods like midpoint rooting may be used (Swofford et al. 1996). While relative-rate tests show that, with a few exceptions, the substitution rates are rather constant within the different clades, there is considerable variance between them (table 1). Thus, independent evidence is required to define the most ancient branching event.

Do Arthropod and Molluscan Hemocyanins Share a Common Ancestry?

Copper-containing oxygen-carriers have also been found in the Mollusca. These hemocyanins are clearly related to the copper-bearing tyrosinases of many nonarthropod eukaryotic and prokaryotic taxa (e.g., Miller et al. 1998). The common characteristic of all hemocyanins and copper-based tyrosinases is the presence of two distinct copper-binding sites, CuA and CuB, each of which contains two or three histidine residues (''type 3'' copper-binding centers). Some authors have used either the molluscan hemocyanins or nonarthropod tyrosinases to root a tree of the arthropod hemocyanin superfamily (e.g., Kawabata et al. 1995; Durstewitz and Terwilliger 1997; Terwilliger, Dangott, and Ryan 1999). This implies a common origin of the arthropodan and other copper proteins, but also the presence of significant sequence similarities; otherwise, the resulting tree will be misleading (e.g., Wheeler 1990). To evaluate the sequence similarities, selected pairs of sequences from the arthropod hemocyanin family and molluscan hemocyanins or nonarthropod tyrosinases were compared using two different Monte Carlo shuffling approaches (fig. 3). Within the arthropod hemocyanin superfamily, the observed similarity scores were highly significant, even with the sequences that exhibited the largest PAM distance (HcuPPO1 and BheHex) ($P = 1.29 \times 10^{-33}$; $Z = 21.46$) (fig. 3*A*). However, the similarity scores were not significant when two members from different superfamilies were compared (figs. 3*C* and *D*). PlePPO was chosen here for comparison because of the short branch in neighbor-joining analysis; none of the other pairs of sequences tested reached the significance level. Although there is per se no definite limit that allows the discrimination between ''significant'' similarities and those arising by chance, the observed scores were not better than expected for any random sequence of the same length and amino acid composition. Thus, the molluscan hemocyanins and related tyrosinases cannot be used as outgroups to root the arthropod hemocyanin tree. It is noteworthy that copper-bearing respiratory proteins evolved twice independently of functional tyrosinases. Nevertheless, it should be pointed out that such a negative result does not formally exclude an ancient relationship of these protein families which is hidden today by a large number of amino acid replacements that have accumulated since a possible divergence in early metazoan evolution.

Which Is the Most Ancient Branch?—Outgroup Selection

Because no natural outgroup is available that roots the tree, alternative considerations are required to define the most ancient branch of the hemocyanin superfamily. An outgroup position of the insect hexamerins (as proposed by Hughes 1999) must be regarded as highly unlikely because of (1) general arthropod phylogeny (rel-

atively recent appearance of the insects), (2) the improbability of independent loss of hexamerins in the other Arthropoda, and (3) the improbability of copper-binding site creation in PPO and hemocyanins. Hughes' (1999) misinterpretation is clearly due to inappropriate midpoint rooting of clades with different evolution rates (table 1). Insect and crustacean hemocyanins can be excluded as possible outgroups as well, because this, as above, would require an unreasonable early origin of the insect hexamerins, which have eventually been lost in clades other than the Hexapoda. As already pointed out (Burmester and Scheller 1996; Sánchez et al. 1998), the universal occurrence of a PPO in the Arthropoda which is a key enzyme in the melanin-forming pathway for the construction of the arthropod exoskeleton suggests its emergence before the radiation of the main arthropod subphyla. PPO sequences are available only from the Hexapoda and the Crustacea, but there is biochemical evidence that similar enzymes probably exist in the Chelicerata (Nellaiappan and Sugumaran 1996) and the Myriapoda (e.g., Xylander 1996). Any other topology of the tree except that displayed in figure 2*B* would therefore require either the PPOs or the hemocyanins to have emerged several times independently of each other. Such an assumption is rather unlikely, especially in view of the structural and functional similarities of the chelicerate and crustacean hemocyanins (e.g., Linzen et al. 1985; Hazes et al. 1993), which clearly distinguish them from the PPOs (Burmester and Scheller 1996). Taken together, an ancient origin of the PPOs should be considered reasonably well supported, and these proteins are assumed to be the outgroup in the following (fig. 2*B*).

Evolutionary Relationships Within the Hemocyanin Superfamily

The final alignment of all available sequences from the arthropod hemocyanin superfamily comprises 93 complete amino acid sequences. To minimize the influence of possible alignment errors, long indels and highly heterologous regions were removed. In figure 4, a 50% majority-rule consensus tree resulting from a neighbor-joining analysis based on the PAM distances is displayed. The general topology of this tree is similar to those previously published (Beintema et al. 1994; Burmester and Scheller 1996; Sánchez et al. 1998) but essentially differs from other recent analyses (Durstewitz and Terwilliger 1997; Hughes 1999; Terwilliger, Dangott, and Ryan 1999). As discussed above, the latter trees are probably biased by the choice of a heterologous (unrelated) or incorrect outgroup. To assess the reliability of the present tree, several additional phylogenetic methods, as well as analysis of the available nucleotide sequences, were applied. In table 2, the bootstrap confidence values of 10 selected nodes (fig. 4) deriving from these approaches are displayed. As pointed out above, in all analyses the monophyly and the general relationships of the major protein families are strongly supported, with the exception of the insect hemocyanin.

The tyrosinase reaction of the PPOs probably reflects the most ancient function of this protein superfamily. As already pointed out, the presence of such enzymes is essential for the process of cuticle tanning. One can speculate that the emergence of the PPOs is closely linked to the formation of the arthropod exoskeleton. The occurrence of the hemocyanins and PPOs in Cheliceara, Crustacea, Myriapoda, and Hexapoda strongly suggests that these proteins diverged before the radiation of the euarthropodan subphyla (as assumed in fig. 4). This event might be correlated with an animal's increase in size and the formation of a hard cuticle, making simple oxygen diffusion inefficient. Thus, specific oxygen transport proteins were required, and the oxygen-converting PPOs stood ready as the evolutionary tool that, after gene duplication, evolved into the oxygen-transporting hemocyanins.

The hemocyanins of the Crustacea and Chelicerata individually form two well-separated clades (fig. 1 and table 1, nodes C and F). The chelicerate hemocyanin subunits are basal, consistent with the assumption that the Chelicerata is a rather distinct arthropod taxon (Dunlop and Selden 1997). There is strong statistical support for the hypothesis that crustacean pseudohemocyanins (cryptocyanins) are included within the clade of the true crustacean hemocyanins (Burmester 1999*b*; Terwilliger, Dangott, and Ryan 1999), but their arrangement relative to the known crustacean hemocyanin subunits is not well resolved in the different analyses (table 1, node G). Most likely they are in sister group position to all known crustacean hemocyanins, although an association with the γ-type subunits (see below) cannot be excluded with sufficient confidence. The insect hexamerins are closely related to the crustacean proteins, with the hexamerins forming a well-supported monophylum. Although the statistical (bootstrap) support is not very strong (node H, table 1), the association of the insect hemocyanin with the hexamerins was supported in all but two analyses, implying an evolution of the hexamerins within the hexapod lineage.

Diversity of PPOs in Holometabolous Insects

Although PPOs have been described by biochemical means in all euarthropodan subphyla, sequences are only available from the malacostracan Crustacea and the holometabolous insects. The clades leading to PPOs of these subphyla are well separated (fig. 4 and table 2, node B). Although the resolution of the arrangement within the insect PPOs is generally poor, there is consistent support for the existence of three different PPO classes among the insects (adjacent to node B, fig. 4). While two of them contain PPO sequences only from the Diptera or Lepidoptera, respectively, the third (in which monophyly is, however, supported by only 64%) comprises PPOs from Diptera, Lepidoptera, and Coleoptera. Although up to six different PPO genes have been described in insects, which may exhibit differential expression patterns (Müller et al. 1999), the physiological relevance of the existence of multiple PPOs is not yet clear. A timescale of PPO evolution cannot be read-

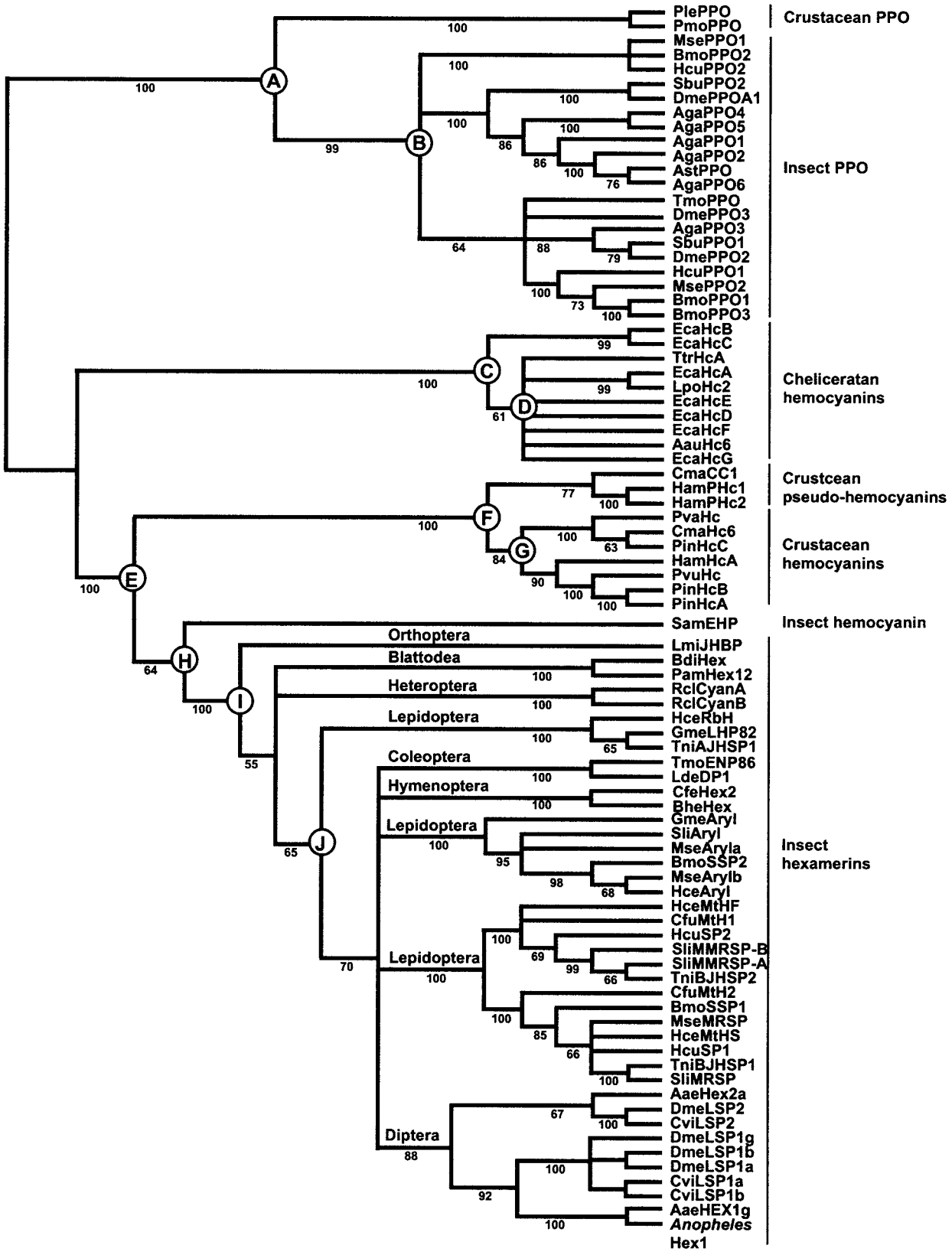

FIG. 4.—Majority-role consensus tree deriving from a neighbor-joining tree of the alignment of the amino acid sequences. The prophenoloxidases were regarded as the outgroup. Bootstrap confidence limit probabilities were estimated from 100 replications and are given below the branches. Nodes supported by <50% bootstrap values are collapsed. The 10 hexamerin-1 proteins from the Anopheles genus are represented by a single branch. See table 2 for an extended analysis of the robustness of nodes A–J.

Table 2
Support Values in Percentages of Selected Nodes of the Tree in Figure 4

Method	A	B	C	D	E	F	G	H	I	J
NJ (aa)	100	99	100	61	100	100	84	64	100	65
MP (aa)	100	99	88	56	100	100	68	92	96	58
ML (aa)	99	99	98	46	98	72	81	ns	95	46
NJ (DNA)	100	100	100	79	100	100	86	80	100	68
MP (DNA)	100	96	100	55	100	100	ns	88	100	39
ML (DNA)	99	97	100	99	100	100	80	ns	97	52

NOTE.—Neighbor-joining (NJ) and maximum-parsimony (MP) values were calculated by using 100 or 500 bootstrap replicates of the alignments of the amino acids (aa) or the nucleotides (DNA), respectively, whereas maximum-likelihood (ML) values were inferred from TREE-PUZZLE with 1,000 quartet puzzling steps. ns = node not supported in this analysis.

ily inferred because the orthology of the different PPOs is not resolved. It is, nevertheless, noteworthy that the internal arrangement of the single known coleopteran PPO (TmoPPO) within one of the branches (fig. 4) suggests that different PPO types had already diverged well before the radiation of the Mecopteria (i.e., Lepidoptera, Diptera, Trichoptera, Siphonaptera, and Mecoptera) about 270 MYA (fig. 1).

A Timescale for Arthropod Hemocyanin Evolution

While the rates of evolutionary changes are, with a few exceptions among the hexamerins (Burmester et al. 1998), rather uniform within the different protein families, there is considerable variation between them (fig. 2*A* and table 1). Therefore, no general molecular clock can be employed to infer the divergence times. However, likelihood ratio tests and relative-rate tests suggest that local clocks probably still apply and may be used to infer a timescale of the molecular evolution of the different members of the hemocyanin superfamily, as well as an at least tentative timescale for arthropod phylogeny.

Ancient Divergence of the Chelicerate Hemocyanin Subunits

The hemocyanins of the Chelicerata are very often multimers of hexamers that are formed by different subunits that occupy distinct positions in the native oligomer (Markl and Decker 1992). For example, the 24mer (4×6) hemocyanin of the tarantula *E. californicum* is composed of seven different subunits (EcaHcA–EcaHcG in fig. 4). In addition, the sequences of two hemocyanin subunits from the Xiphosura *L. polyphemus* and *T. tridentatus* (LpoHcA and TtrHcII) and one from the scorpion *A. australis* (AauHc6) are known. While the chelicerate hemocyanin subunits form a well-supported common clade, the phylogenetic resolution of the relationships between them is poor. This indicates a lack of phylogenetic signal that may be interpreted as the result of a rapid diversification from a single primordial chelicerate hemocyanin. However, the association of EcaHcB and EcaHcC is strongly supported (99% bootstrap value in neighbor-joining analysis). These subunits are located in the interhexamer contact zones and play an important role in the formation of the tarantula 4×6 hemocyanin. Thus, the ancestor of the tarantula hemocyanin was likely a hexamer composed of a-, b/c-, d-, e-, f-, and g-type subunits, with each occupying a distinct position in the hemocyanin structure and the arachnidan 4×6 hemocyanin evolving after gene duplication and formation of distinct b and c subunits.

There is also good statistical (99%) and immunological evidence (Kempter et al. 1985) for a close relationship of subunit A of *E. californicum* (EcaHcA) and subunit II of the horseshoe crab Limulus (LpoHcII). Xiphosura (*L. polyphemus*) and Arachnida (*E. californicum*) diverged about 450 MYA (Dunlop and Selden 1997). Assuming this date and the orthology of LpoHcII and EcaHcA, a substitution rate of $5.82 \pm 0.31 \times 10^{-10}$ per site per year was inferred (table 1). Then, the earliest time of divergence of the chelicerate hemocyanin subunits took place about 564 ± 23 MYA, probably in the stemline of the Chelicerata. The date of the EcaHcB/EcaHcC split was estimated to have occurred about 418 ± 18 MYA. Immunological data of the arachnidan hemocyanins suggest that these subunit types split before Araneae and Scorpiones diverged (Kempter et al. 1985), probably in the Silurian period (Dunlop and Selden 1997). Therefore, the estimated time is consistent with the available fossil record and indicates that the cheliceratan hemocyanins evolved in an at least approximate clocklike manner. The other *E. californicum* subunit types diversified about 476 ± 65 MYA. Therefore, the 24mer hemocyanin built by seven different subunits appears to be the original quaternary structure of the arachnidan hemocyanins, and other subunit compositions of hemocyanins in several nonorthognathan Araneae should be interpreted as secondary rearrangements (cf. Markl 1986).

Hemocyanin Subunit Diversity in the Crustacea

Hemocyanins have been found in most Malacostraca (fig. 1) but are apparently absent in other crustacean taxa (Mangum 1983), with the possible exception of the Remipedia (Yager 1991). Nevertheless, their universal appearance in the other arthropod subphyla indicates that a respiratory hemocyanin was present within the crustacean stemline. Markl (1986) noted three different hemocyanin subunit types in the higher Decapoda. The seven known crustacean subunits form two distinct clades, representing the α (HamHcA, PinHcA, PinHcB, PvuHc) and γ (PinHcC, PvaHc, CamHc6) types (fig. 4), while β-type sequences are still unknown. According to the fossil record, the brachyuran and palinuran Deca-

Table 3
Estimated Divergence Times in MYA

Method	Chelicerate versus Crustacean Hemocyanins[a]	Radiation of Crustacean PHc and hemocyanins[b]	Hexapoda versus Crustacea[c]	Insect Hemocyanin versus Hexamerins[c]
PAM (aa)	622 ± 31	212 ± 23	445 ± 8	389 ± 4
Kimura (aa)	598 ± 28	209 ± 29	446 ± 8	400 ± 5
JTT (aa)	597 ± 26	214 ± 17	437 ± 7	383 ± 4
Maximum-likelihood (aa)	634 ± 43	225 ± 14	459 ± 21	388 ± 11
HKY (DNA)	518 ± 40	216 ± 3	396 ± 6	374 ± 4
Mean	593 ± 43	215 ± 6	437 ± 24	387 ± 9

[a] Calibration dates: Arachnida-Xiphosura 450 MYA (Dunlop and Selden 1997).
[b] Calibration dates: Brachyura-Palinura 150 MYA, Palinura-Astacura 180 MYA (Briggs, Weedon, and White 1993).
[c] Calibration dates: Orthoptera-Neoptera 320 MYA, Brachycera-Nematocera 210 MYA (Kukulovà-Peck 1991; Fraser et al. 1996).

poda diverged at the end of the Jurassic period, about 150 MYA, while the split of the astacura and palinuran Decapoda was about 180 MYA (Briggs, Weedon, and White 1993). The rate of amino acid substitution was calculated to be 1.29×10^{-9} per site per year, about two times as fast as that of the chelicerate hemocyanins. This calculation indicates a divergence of the α and β subunit types 200 ± 13 MYA. This suggests the radiation of the known crustacean hemocyanin subunits in the stemline of the Decapoda, which is in agreement with the immunological data (Markl 1986). The pseudohemocyanins (cryptocyanins) are included in the clade of the crustacean hemocyanins (Burmester 1999*b*; Terwilliger, Dangott, and Ryan 1999). Consistent with their proposed role as storage proteins, the pseudohemocyanins have a rather high replacement rate of 1.97×10^{-9} per site per year. According to the molecular-clock calculations, crustacean hemocyanins and pseudohemocyanins diverged about 215 MYA (table 3).

Origin of the Insect Hexamerins—Another Change in Function

There is strong statistical evidence that the insect hexamerins are monophyletic (table 2, node I). Their association with the single insect hemocyanin implies that the hexamerins most likely emerged after the Hexapoda branched off from the Crustacea. The selective advantage of the evolution of hexamerins may have been the uncoupling of the storage protein function (hexamerins) from oxygen transport (hemocyanins). The pattern of hexamerin evolution generally follows the accepted scheme of the phylogeny of insect orders, with the exception of the lepidopteran riboflavin-binding proteins (HceRbH, GmeLHP82, TniAJHSP1), which appear to be in a basal position within the hexamerins of the other holometabolan insects (fig. 4). Based on the assumption that the Orthoptera (represented by Lmi-JHBP) diverged from the other Neoptera about 320 MYA (Kukulovà-Peck 1991) and the brachyceran diverged from the nematoceran Diptera about 210 MYA (Fraser et al. 1996), nearly identical amino acid replacement rates of $2.70 \pm 0.16 \times 10^{-9}$ and $2.60 \pm 0.09 \times 10^{-9}$ per site per year, respectively, were inferred (table 1). These rates are significantly higher than those within the hemocyanins and are likely due to fewer restrictions in hexamerin evolution, which is not bound to respiratory functions with complex allosteric regulations (Burmester and Scheller 1996). Extrapolating from the Orthoptera-Neoptera node to the branching point of all hexamerins from the insect hemocyanin (i.e., the hexamerin stemline), a mean divergence time of 387 ± 9 MYA was calculated (fig. 5 and table 3), supporting the notion that the hexamerins evolved within the Hexapoda. A rather slow amino acid replacement rate of $9.24 \pm 0.71 \times 10^{-10}$ per site per year was estimated for the insect hemocyanin.

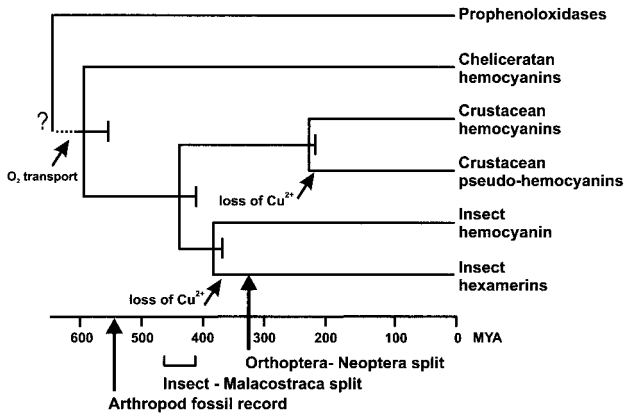

FIG. 5.—A timescale of the evolution in the hemocyanin protein superfamily. A linearized tree was obtained on the basis of corrected protein distance data. The divergence times were estimated as described in the text; the bars represent the standard errors. At the bottom of the figure, the following are indicated: *a*, the Orthoptera-Neoptera split (320 MYA), used for calibration of the hexamerin branch; *b*, the first conclusive arthropod fossil record; and *c*, the inferred divergence time of the Malacostraca and the Hexapoda.

Implications for Arthropod Phylogeny

The evolutionary analysis of the hemocyanin superfamily strongly supports the generally accepted (Chelicerata,(Crustacea, Insecta)) phylogeny. Unfortunately, hemocyanin sequences are still missing from the Myriapoda and crucial crustacean taxa. Nevertheless, the available hemocyanin data provide additional information on arthropod phylogeny. If one accepts a sister group position of the Crustacea and the Insecta (e.g., Friedrich and Tautz 1995; Boore, Lavrov, and Brown 1998), the Crustacea are very probably paraphyletic in terms of the insects (Zrzavý, Hypša, and Vlášková 1997; García-Machado et al. 1999; Shultz and Regier 2000;

Wilson et al. 2000), because the diversification of the different crustacean classes had already commenced in the Cambrian period (Briggs, Weedon, and White 1993), but the insects did not appear before the early Devonian (Kukulavà-Peck 1991) (fig. 1). There is, nevertheless, the theoretical possibility that the insects derived from a yet-unknown pre-Devonian taxon which had already diverged from the other Crustacea in the Cambrian period. However, under the assumption that the replacement rate in the common insect hemocyanin + hexamerin stemline was the same as that in the insect hemocyanin, the time of divergence of insects and crustaceans was calculated to be 437 ± 24 MYA (table 3 and fig. 5). If this protein evolved with the faster crustacean rate, the estimated time of divergence is even more recent (about 420 MYA). In a second approach, the time of the Insecta-Crustacea split was extrapolated from the crustacean hemocyanins. Then, a crustacean-insect divergence time of about 450 ± 17 MYA was inferred, in good agreement with that based on the insect proteins. A Cambrian divergence of the Crustacea and the Insecta at ≥520 MYA, as proposed by the Tracheata hypothesis (e.g., Brusca and Brusca 1990), is rejected with $P < 0.01$. These calculations provide additional indication that within the possibly paraphyletic Crustacea, the insects and the Malacostraca are in fact closely related and may share a last common ancestor that is more recent than that of the other crustacean classes (cf. fig. 1). Interestingly, a close Malacostraca-Insect relationship is also supported by long-ignored morphological evidence (e.g., Börner 1909; Crampton 1922), by eye morphology (Dohle 1997), and by comparative studies of embryonic development (Averof and Akam 1995), as well as by recent molecular phylogenetic studies (García-Machado et al. 1999; Shultz and Regier 2000; Wilson et al. 2000). Using the same methods, the time of the last common ancestor of the cheliceratan and crustacean hemocyanins was inferred as close to 600 MYA, a date which is consistent with the view of a rather early divergence of Crustacea and Chelicerata in the evolution of the Arthropoda (e.g., Dunlop and Selden 1997).

Conclusions

The phylogenetic analyses of the hemocyanin superfamily demonstrate a remarkably good resolution of the trees at the higher taxonomic level of the subphyla. This notion is not trivial, because most of the other molecular studies on arthropod phylogeny hardly ever reach a similar statistical confidence (e.g., Turbeville et al. 1991; Friedrich and Tautz 1995; Regier and Shultz 1997; Spears and Abele 1997; Shultz and Regier 2000). Although molecular-clock assumptions like those of the present paper are unquestionably problematic and may be biased by factors beyond the experimental control, there is consistent support for the hypothesis that local clocks apply within the subgroups of the cheliceratan and crustacean hemocyanins, as well as within the insect hexamerins. These clocks can be used to approximate divergence times within the different subgroups, as well as among them. The time estimates may either be in general agreement with the commonly accepted scheme of arthropod phylogeny or support alternative views.

Acknowledgments

I thank J. Markl and H. Decker for continuous support, M. Friedrich for sending references, K. Kusche and R. Voit for providing sequence data before publication, J. Felsenstein for a prerelease version of PHYLIP, version 3.6, J. R. Harris for critical reading of the paper and correcting the language, and the anonymous referees for their helpful comments. This work was supported by the Deutsche Forschungsgemeinschaft (Bu956/3).

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RICHARD THOMAS, reviewing editor

Accepted October 12, 2000