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Hemocyanin Suggests a Close Relationship of Remipedia and Hexapoda

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The Remipedia are enigmatic crustaceans from anchialine cave systems, first described only 30 years ago, whose phylogenetic affinities are as yet unresolved. Here we report the sequence of hemocyanin from *Speleonectes tulumensis* Yager, 1987 (Remipedia, Speleonectidae). This is the first proof of the presence of this type of respiratory protein in a crustacean taxon other than Malacostraca. *Speleonectes tulumensis* hemocyanin consists of multiple distinct (at least three) subunits (StuHc1–3; Hc, hemocyanin). Surprisingly, the sequences are most similar to hexapod hemocyanins. Phylogenetic analyses showed that the *S. tulumensis* hemocyanin subunits StuHc1 and StuHc3 associate with the type 1 hexapod hemocyanin subunits, whereas StuHc2 associates with the type 2 subunits of hexapods. Together, remipede and hexapod hemocyanins are in the sister-group position to the hemocyanins of malacostracan crustaceans. Hemocyanins provide no indication of a close relationship of Myriapoda and Hexapoda but support Pancrustacea (Crustacea + Hexapoda). Our results also suggest that Crustacea are paraphyletic and that Hexapoda may have evolved from a Remipedia-like ancestor. Thus, Remipedia occupy a key position for the understanding of the evolution of hexapods, which are and have been one of the world's most speciose lineage of animals.

Introduction

In 1979, Yager discovered in an anchialine cave in the Bahamas the first specimens of Remipedia, which represented a new class of crustaceans with currently about 20 described extant species (Yager 1981; Koenemann, Schram, Hönemann, and Iliffe 2007). Living remipedes harbor a number of unique features, which include the loss of eyes, biramous antennulae, three pairs of postmandibular mouthparts adapted to a predatory feeding mode and to grooming, and the lack of tagmatization of the trunk (Schram and Lewis 1989; Koenemann, Schram, Iliffe, et al. 2007; van der Ham and Felgenhauer 2008). The phylogenetic affinities of Remipedia are still controversial. On the basis of the homonomous segmentation of their trunk, which supposedly represents the ancestral ground pattern in the evolution of Arthropoda, it has been suggested that Remipedia occupy a basal position within Crustacea or even Mandibulata (Schram 1986; Wills 1997; Giribet et al. 2001). Molecular phylogenetic analyses of ribosomal RNA and mitochondrial genomes, as well as limb morphology, have indicated an association of Remipedia with various crustacean classes assigned to the "Maxillopoda" (Ito 1989; Spears and Abele 1997; Lim and Hwang 2006). However, these results may be due to long branch attraction phenomena (Spears and Abele 1997; Telford et al. 2008; von Reumont et al. 2009). In fact, the pattern of mitochondrial gene arrangement convincingly excluded Remipedia from the maxillopod assemblage, which in that study comprised Branchiura, Cephalocarida, Cirripedia, and Pentastomida (Lavrov et al. 2004). Other molecular phylogenetic analyses have found Remipedia at various positions within Crustacea, albeit with poor support (Regier and Shultz 2001; Regier et al. 2005; Hassanin 2006; Carapelli et al. 2007). A recent study of arthropod brain morphology suggested that Remipedia belong to a monophylum of Malacostraca and Hexapoda within paraphyletic crustaceans (Fanenbruck et al. 2004; Fanenbruck and Harzsch 2005). The discovery of free-living lecithotrophic remipede larvae and analysis of early larval development of Remipedia tentatively support their assumed relationship to malacostracan crustaceans (Koenemann, Schram, Bloechl, et al. 2007; Koenemann et al. 2009).

Oxygen transport in the hemolymph of various arthropod and mollusk taxa is facilitated by copper proteins referred to as hemocyanins (Markl and Decker 1992; van Holde and Miller 1995; Burmester 2002). Arthropod and mollusk hemocyanins are not related but evolved independently from distinct copper-containing enzymes (Burmester 2001, 2002). Hemocyanins of Arthropoda are large hexameric or oligohexameric proteins composed of similar or identical subunits in the size range of 75 kDa. Each subunit can bind to an O_2 molecule by the virtue of two copper ions that are coordinated by six histidine residues. Hemocyanins have been thoroughly studied in Chelicerata and Crustacea, occur in Myriapoda (Jaenicke et al. 1999; Kusche and Burmester 2001), and have recently been identified in Onychophora (Kusche et al. 2002) and Hexapoda (Hagner-Holler et al. 2004; Pick et al. 2008, 2009). Within Crustacea, hemocyanins have been thought to be confined to Malacostraca (Mangum 1985; Markl and Decker 1992). Hemocyanin sequences have been shown to be informative for the inference of phylogenies within Arthropoda (Burmester 2001; Kusche and Burmester 2001; Kusche et al. 2002, 2003). Thus, lineage-specific presence and phylogenetic analyses could be useful in assessing the position of Remipedia.

Here, we report the identification and molecular cloning of hemocyanin cDNA from the remipede *Speleonectes tulumensis* and show that molecular phylogenetic analyses of these hemocyanin sequences provide evidence for a close relationship of Remipedia and Hexapoda.

Materials and Methods

Sample Preparation

Speleonectes tulumensis was collected on Yucatan Peninsula, Mexico. Animals were cut into small pieces and the tissue was preserved in RNAlater (Qiagen, Hilden,

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Germany). Total RNA and protein were extracted using TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. The protein and RNA samples were immediately used or kept frozen at −20 °C until use.

Cloning of Hemocyanin cDNA

Three micrograms of total RNA was converted into cDNA by Superscript III reverse transcriptase employing an oligo-dT primer according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The resulting cDNA was used for standard polymerase chain reaction (PCR), using degenerate primers (forward primer: 5′-ATGGAYTTYC CNTTYTGGTGGA-3′; reverse primer 5′-GTNGCGGTY TCRAARTGYTCCAT-3′) that had been derived from conserved coding regions of arthropod hemocyanins (amino acid sequences: MDFPFWW and MEHFETAT) (Burmester 2001; Hagner-Holler et al. 2004; Pick et al. 2009). Fragments of the expected size were cloned into the pGem-T Easy/JM109 *Escherichia coli* system (Promega, Mannheim, Germany) and 14 independent clones were sequenced by a commercial service (Genterprise, Mainz, Germany). Rapid amplification of cDNA ends (RACE) experiments (5′ and 3′ RACE) were carried out by an RNA ligase–mediated rapid amplification method employing the GeneRacer Kit with SuperScript III reverse transcriptase. Sets of gene-specific primers were constructed according to the partial sequences (supplementary table 1, Supplementary Material online). The cDNA fragments were cloned and sequenced as described. Sequences were assembled using ContigExpress (Vector NTI Advance 10.3; Invitrogen) and GeneDoc 2.7 (Nicholas, Nicholas, and Deerfield 1997).

Western Blotting

Protein extracts were denatured in sample buffer (31.25 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate [SDS], 2.5% β-mercaptoethanol, and 5% glycerol) at 95 °C for 5 min and loaded onto a 10% polyacrylamide gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to standard procedures. Semidry electro-transfer of proteins onto nitrocellulose membranes (Hartenstein, Würzburg, Germany) was carried out for 2 h at 0.8 mA/cm^2. Nonspecific binding sites were blocked for 1 h with 2% nonfat dry milk in tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.4; 140 mM NaCl). The membranes were incubated for 2 h at room temperature with polyclonal antibodies that had been raised against various insect and crustacean hemocyanins (anti-*Homarus americanus*, anti-*Panulirus interruptus*, anti-*Cancer pagurus*, and anti-*Thermobia domestica* hemocyanin antibodies) diluted 1:5,000 in 2% milk/TBS. The nitrocellulose filters were washed three times with TBS for 15 min and incubated for 1 h with a goat antirabbit antibody coupled with alkaline phosphatase (Dianova, Hamburg, Germany), diluted 1:10,000 in TBS. After a final washing step, detection was carried out with nitro-blue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

Sequence and Phylogenetic Analyses

Tools provided with the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (http://www.expasy.org) were used for the analyses of DNA and amino acid sequences. Signal peptides were predicted using SignalP 1.1 (Nielsen et al. 1997). Deduced amino acid sequences of *S. tulumensis* hemocyanin were included in a previously published multiple alignment of arthropod hemocyanins, insect hexamerins, and crustacean pseudohemocyanins (Pick et al. 2009) employing MAFFT with the L-INS-i method and the BLOSUM 62 matrix (Katoh et al. 2005). A list of sequences used in this study is provided in supplementary table 1, Supplementary Material online.

After the exclusion of N- and C-terminal extensions, the final multiple sequence alignment contained 800 positions and 96 sequences. The appropriate model of amino acid sequence evolution (WAG + Gamma model; Whelan and Goldman 2001) was selected by ProtTest (Abascal et al. 2005) using the Akaike Information Criterion. Bayesian phylogenetic analysis was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). We assumed the WAG model with a gamma distribution of substitution rates. We used uninformative priors on all trees. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed with one cold and three heated chains that were run for 8 million generations. Starting trees were random and trees were sampled every 100th generation. Two independent runs were performed in parallel and were continued until runs had converged (average standard deviations of split frequencies were stationary and lower than 0.005). The program Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer/) was used to examine log-likelihood plots and MCMC summaries for all parameters. Posterior probabilities were estimated on the final 60,000 trees (burn-in = 20,000). RAxML 7.0.4, assuming the WAG evolutionary model with gamma distributions, was used for maximum likelihood (ML) analyses, and the resulting tree was tested by bootstrapping with 1,000 replicates. Tree-Puzzle 5.2 (Schmidt et al. 2002) was used to test alternative tree topologies. Hypothesis testing was performed by four methods: A Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa 1999), a two-sided Kishino–Hasegawa (2sKH) test (Kishino and Hasegawa 1989), a one-sided KH (1sKH) test based on pairwise SH tests (Goldman et al. 2000), and an Expected Likelihood Weight (ELW) test (Strimmer and Rambaut 2002). SH, 1sKH, and ELW tests performed 1,000 resamplings using the RELL method.

Results and Discussion

Identification of Hemocyanin in *S. tulumensis*

A set of degenerate oligonucleotide primers was designed according to conserved regions arthropod hemocyanin sequences (Burmester 2001; Pick et al. 2009). Reverse transcription-PCR using total RNA extracted from an adult *S. tulumensis* resulted in the amplification of fragments in the range of 550 bp. After cloning and sequencing, three distinct hemocyanin cDNA sequences were identified, which were termed StuHc1, StuHc2, and StuHc3, respectively (Hc, hemocyanin). The missing 5′ and 3′ regions of

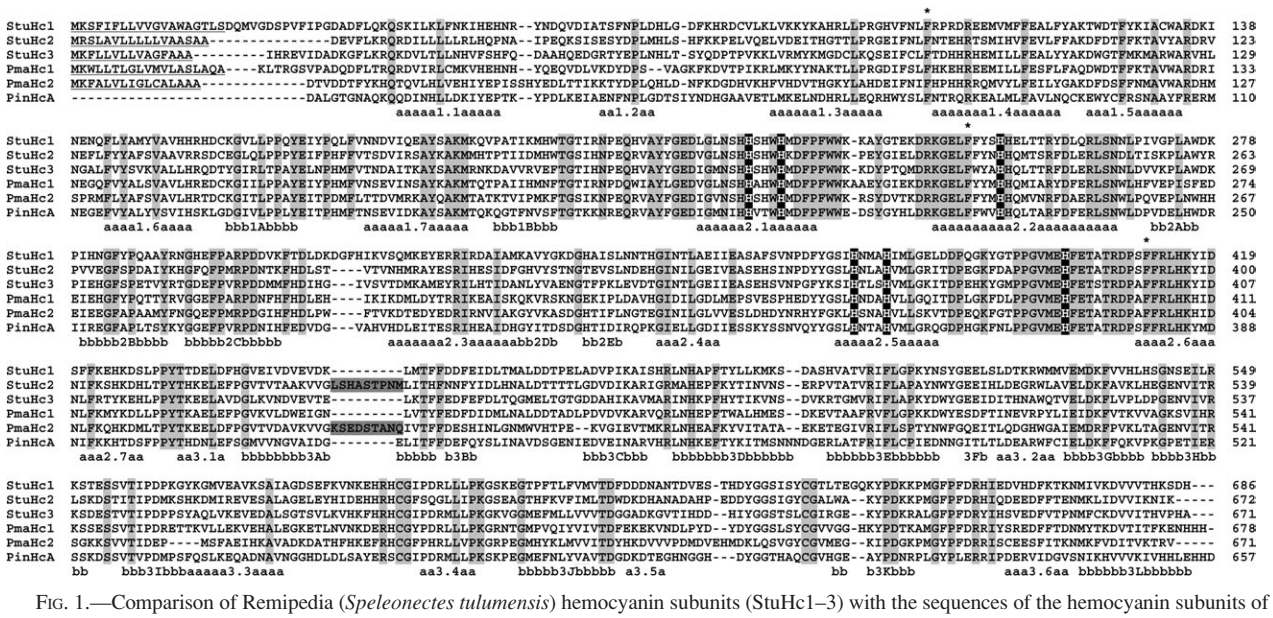

FIG. 1.—Comparison of Remipedia (*Speleonectes tulumensis*) hemocyanin subunits (StuHc1–3) with the sequences of the hemocyanin subunits of the stonefly *Perla marginata* (PmaHc1, PmaHc2) and the hemocyanin subunit a from the spiny lobster *Panulirus interruptus* (PinHcA). The copper-binding histidines are shaded in black, other conserved residues in light gray. The asterisks in the upper row denote the phenylananines that stabilize the O_2 binding. In the lower row, the secondary structure of PinHcA is given (Gaykema et al. 1984). The conserved insertion of nine amino acids in beta-sheet 3A of StuHc2 and hexapod hemocyanin subunit 2 are shaded in dark gray.

the hemocyanin cDNAs were obtained by 5′ and 3′-RACE. The full-length cDNA sequences measure 2,422; 2,531; and 2,506 bp (supplementary fig. 1, Supplementary Material online). The deduced amino acid sequences comprise 686, 672, and 671 amino acids, of 51.6–55.6% identity (fig. 1).

Sequence comparisons with other hemocyanins showed that the residues required for reversible oxygen binding and subunit cooperativity (Hazes et al. 1993; Burmester 2002) are present in all three sequences of *S. tulumensis*, suggesting that these proteins are able to carry out respiratory functions. The conserved residues include the six copper-binding site histidines in the central second domain, as well as three phenylalanines that stabilize the binding of O_2 in the first and second domains (fig. 1). The phenylalanine in the first domain of the subunits is assumed to be a key residue in the regulation of oxygen binding in most hemocyanins (Hazes et al. 1993).

All *S. tulumensis* hemocyanins harbor the typical N-terminal leader sequences required for trans-membrane export via the endoplasmic reticulum (cf. fig. 1 and supplementary fig. 1, Supplementary Material online), resulting in native polypeptides of 656–667 amino acids with inferred molecular masses of 75.7–77.1 kDa. The occurrence of hemocyanin proteins in *S. tulumensis* was further established by Western blotting employing various polyclonal antibodies and antisera that had been raised against insect or crustacean hemocyanins. Two distinct bands in the range of 75–80 kDa were recognized by most antibodies (fig. 2), which are in excellent agreement with the molecular masses predicted from the translated cDNA sequences: StuHc1 is interpreted to form the upper band, and StuHc2 and StuHc3 are together the lower band. In summary, Remipedia do have functional hemocyanin sequences that occur in three subunits and are expressed in the adult organism.

Respiratory Proteins in Remipedia and Other Crustacea

Among the crustacean groups, hemocyanins have only been identified in Malacostraca (Mangum 1985; Markl and Decker 1992; Burmester 2002). In various non-malacostracan crustaceans (Branchiopoda, Ostracoda, Copepoda, and Cirripedia), hemoglobins usually serve as oxygen carriers (Mangum 1985). Sequence data corroborate these findings because extensive Blast searches on genomic sequences or expressed sequence tags in EMBL/GenBank and in the database of the “Deep Metazoan Phylogeny” project did not recover any hemocyanin from a non-malacostracan crustacean. There have been only two reports of the possible presence of hemocyanins in Cirripedia and Remipedia. It is, however, uncertain whether the putative hemocyanin of the parasitic cirriped *Sacculina carcini* is an endogenous

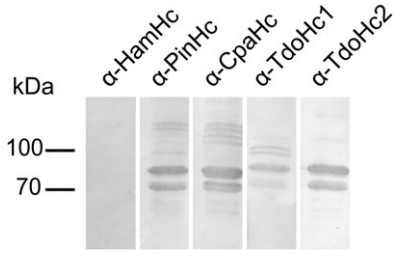

FIG. 2.—Identification of hemocyanin protein in *Speleonectes tulumensis*. About 20 μg of total protein extract from *S. tulumensis* was applied per lane and separated by SDS-PAGE. Hemocyanin subunits were detected by polyclonal antibodies raised against various insect and crustacean hemocyanins, as indicated in the upper row. The antibodies were α-HamHc, anti-*Homarus americanus* hemocyanin; α-PalHc, anti-*Panulirus interruptus* hemocyanin; α-CpaHc, anti-*Cancer pagurus* hemocyanin; α-TdoHc1, anti-*Thermobia domestica* hemocyanin 1; α-TdoHc2, anti-*T. domestica* hemocyanin 2. The positions of the molecular mass standards are given on the left side.

protein or actually derives from the decapod host, *Carcinus maenas* (Herberts and de Frescheville 1981). Yager (1991) observed large crystal structures in remipede hemocytes that resemble those of the *Limulus polyphemus* hemocyanin. In a more recent report, van der Ham and Felgenhauer (2007) also speculated about the presence of a hemocyanin-like protein in *Speleonectes* sp. Here, we demonstrate that hemocyanin actually occurs in a remipede species, which is the first unambiguous report of such respiratory proteins in a non-malacostracan crustacean.

Remipedia dwell in a high-saline and oxygen-poor environment (usually $<$1mg/ml O_2; Koenemann et al. 2006) below the halocline interface between the seawater and the overlying well-oxygenated freshwater that is typical for anchialine cave systems (Pohlmann et al. 1997). Remipedia have adapted to this hypoxic environment, and a respiratory protein that augments the delivery of oxygen within the circulatory system is certainly advantageous. Hemocyanin has been identified in Onychophora and thus was present in the last common ancestor of Arthropoda (Burmester 2002; Kusche et al. 2002). It is extremely unlikely that hemocyanin was reinvented in Remipedia. It must be assumed that this respiratory protein was present in the last common ancestor of Malacostraca and Remipedia and was the principal oxygen carrier of stemline crustaceans. Thus, the occurrence of hemocyanin is a plesiomorphic character of Crustacea. As mentioned above, most non-malacostracan crustaceans use hemoglobin not hemocyanin for oxygen transport (Mangum 1985). However, this hemoglobin is a secondary invention (apomorphic character), which most likely derived from an intracellular globin of unknown function. It is currently uncertain whether hemoglobin emerged only once in the Crustacea, which would provide evidence that at least part of the "Entomostraca" sensu Walossek (1999), that is, Branchiopoda and Maxillopoda, may represent a monophylum.

Hexapod Affinities of Remipede Hemocyanins

Surprisingly, Blast searches and pairwise sequence comparisons revealed that the hemocyanin subunits of *S. tulumensis* display the highest sequence identity with insect hemocyanin subunits. For example, StuHc1 and StuHc3 share 60.4% and 53.4%, respectively, of the amino acids with the hemocyanin 1 of the firebrat *T. domestica*, whereas StuHc2 and the hemocyanin 2 of the cockroach *Blaptica dubia* are 57.1% identical. At least 10% lower identity scores were observed when the *S. tulumensis* hemocyanins were compared with those of malacostracan crustaceans or other arthropod hemocyanins. This evidence is a first rough indicator of a close relationship of hexapod and remipede hemocyanins.

The amino acid sequences of Remipedia hemocyanins were included in an alignment of arthropod hemocyanins and related proteins (Burmester and Scheller 1996; Burmester 2002; Hagner-Holler et al. 2004; Pick et al. 2009) (supplementary fig. 2, Supplementary Material online). Phylogenetic analyses employing ML and Bayesian methods resulted in essentially identical trees (fig. 3; supplementary figs. 3 and 4, Supplementary Material online). *Speleonectes tulumensis* hemocyanins and hexapod proteins form a monophyletic clade that excludes other crustacean (malacostracan) sequences (ML bootstrap support: 48%; Bayesian posterior probability: 0.96). The other crustacean hemocyanins and pseudohemocyanins were monophyletic (100%; 1.0), but in none of the trees did the hemocyanins of the Remipedia join this clade. The relative position of the myriapod hemocyanins remains uncertain and received poor support. These proteins were either associated with the chelicerate hemocyanins (thereby supporting the "Paradoxopoda" hypothesis; Mallatt et al. 2004) or are in sister-group position to the crustacean plus hexapod proteins (supporting the traditional "Mandibulata"). In none of the trees did the myriapod hemocyanins join hexapod hemocyanins and hexamerins.

Speleonectes tulumensis hemocyanin subunits 1 and 3 (StuHc1 and StuHc3) form a common branch. This is reasonably well supported in Bayesian analyses (0.95) but only in 46% of the ML bootstrapped trees (fig. 3). A common clade of StuHc1 and StuHc3 and hexapod hemocyanin type 1 subunits plus hexamerins was recovered with 1.00 posterior probability in the Bayesian tree and supported by an ML bootstrap value of only 54%. The association of StuHc2 with the hexapod hemocyanin subunits 2 receives high support throughout (97%; 1.00) and is further corroborated by a unique and conserved insertion of nine amino acids in beta-sheet 3A (amino acid position 416–420 in StuHc2; fig. 1). Such insertion is not present in any other hemocyanin.

Tree topologies were further evaluated by hypothesis testing (table 1). The multiple ratio tests recovered the tree topology presented in figure 3 as best result and significantly reject StuHc1, StuHc2, and StuHc3 from the clade of other crustacean (malacostracan) hemocyanins. Hence, there is substantial molecular phylogenetic and structural evidence that the remipede hemocyanin subunits are orthologs of hexapod subunits. Thus, the lineage leading to the remipede and hexapod hemocyanins split into two distinct subunit types before these taxa diverged.

Are Remipedia and Hexapoda Sister Groups?

The origin of Hexapoda is notoriously disputed. Based on a large number of morphological characters, it has long been assumed that Hexapoda evolved from a myriapod-like ancestor and that these taxa form the subphylum "Tracheata" or "Atelocerata" (e.g., Brusca and Brusca 2003). However, this view has been challenged by molecular phylogenetic approaches that have provided evidence that Hexapoda are somehow, in fact, allied with Crustacea (Friedrich and Tautz 1995; Boore et al. 1998; Hwang et al. 2001; Kusche and Burmester 2001; Mallatt et al. 2004). Such topology was recovered in our analyses as well (fig. 3). A relationship of Hexapoda and Crustacea also received support in some comparative morphological and developmental biology studies (Giribet and Ribera 2000; Richter 2002), and thus Hexapoda and Crustacea have been joined in a common taxon named either "Tetraconata" (Richter 2002) or "Pancrustacea" (Zrzavý and Štys 1997).

In many analyses, Crustacea form a paraphyletic assemblage with respect to Hexapoda, but it has remained

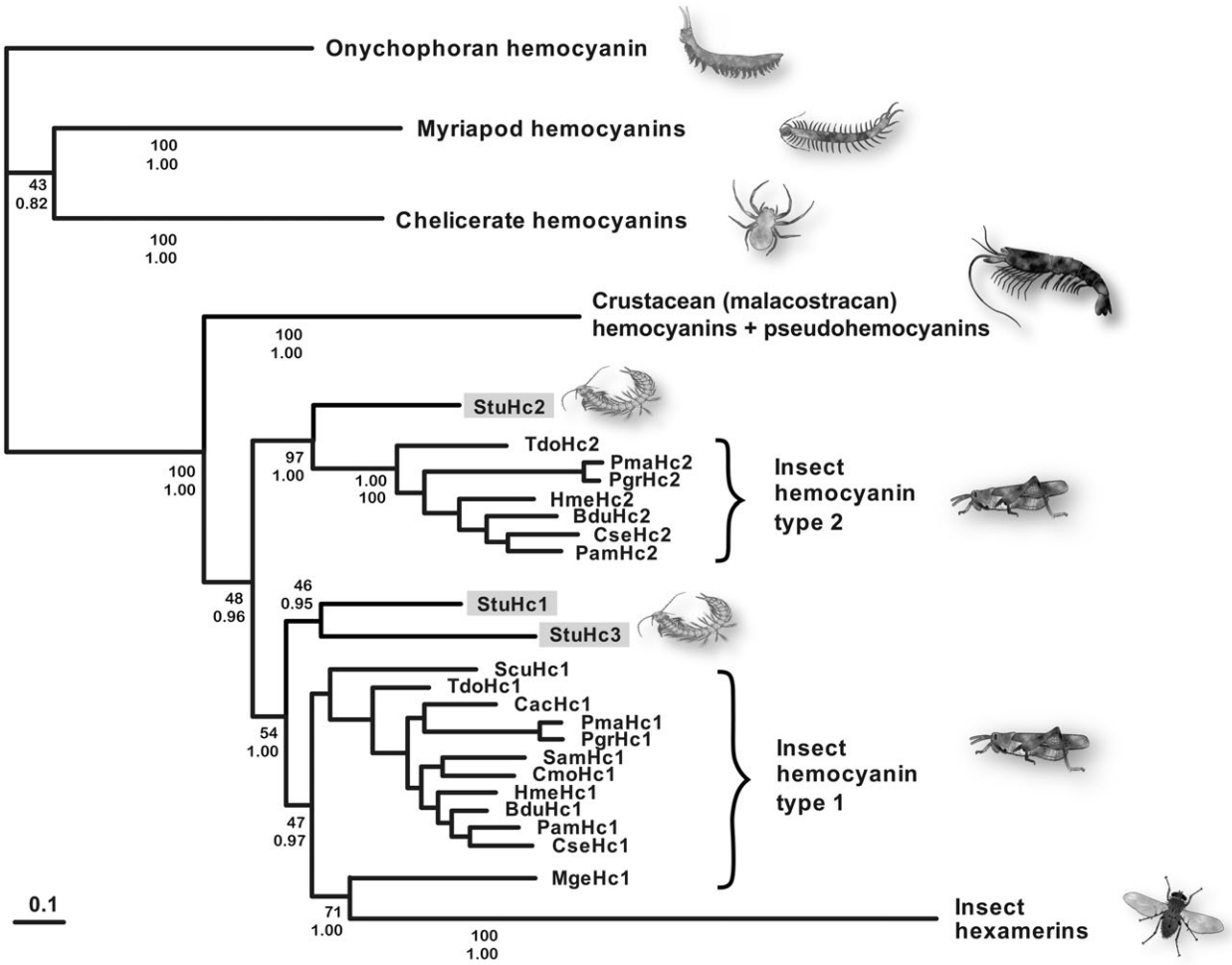

FIG. 3.—Simplified Bayesian phylogenetic tree of arthropod hemocyanins and hexamerins. The numbers at the nodes show ML nonparametric bootstrap support (above) and Bayesian posterior probabilities (below). The complete trees are shown in supplementary figures 3 and 4, Supplementary Material online.

uncertain which crustacean taxon is the sister group of the Hexapoda. In most studies, only two crustacean classes were considered: Malacostraca and Branchiopoda. Analyses employing mitochondrial genomes suggest a close relationship of Malacostraca and Hexapoda with exclusion of Branchiopoda (Garcia-Machado et al. 1999; Wilson et al. 2000; Hwang et al. 2001). Such topology is also supported by some morphological evidence such as brain structure (Harzsch 2002) and by comparative studies of embryonic development (Averof and Akam 1995). On the other hand, a clade consisting of Branchipoda and Hexapoda tends to be supported by rRNA genes and multigene analyses (Regier et al. 2005, 2008; Mallatt and Giribet 2006; Roeding et al. 2007; Dunn et al. 2008).

Here, we provide molecular evidence that actually the Remipedia may be the closest living relatives of the Hexapoda. This relationship is suggested in our study 1) by the presence of both hexapod-type hemocyanin subunits in

Table 1
–Statistical Hypothesis Tests

Tree[a]	Log *L*	Difference	SE	SH[b]	1sKH[b]	2sKH[b]	ELW[b]
1	−74,424.60	0.00	Best	1.0000 +	1.0000 +	Best	0.9983 +
2	−74,509.58	84.98	21.5520	0.0000 −	0.0000 −	—	0.0000 −
3	−74,469.85	45.25	12.0780	0.0410 −	0.0020 −	—	0.0017 −
4	−74,460.23	35.63	12.6676	0.0120 −	0.0000 −	—	0.0000 −

[a] Tree #1: Optimal tree as presented in figure 3: StuHc1 and 3 associated with type 1 hexapod hemocyanins and StuHc2 associated with type 2 hexapod hemocyanins. Tree #2: StuHc1, 2, and 3 in sister-group position to the other crustacean (malacostracan) hemocyanins. Tree #3: StuHc1 and 3 in sister-group position to the other crustacean (malacostracan) hemocyanins. Tree #4: StuHc2 in sister-group position to the other crustacean (malacostracan) hemocyanins.

[b] Plus signs (+) denote the confidence sets, minus signs (−) denote rejection of a tree. All tests used 5% significance level.

Remipedia (but absence of any other subunit type), 2) by a unique sequence motif insertion that is shared by StuHc2 and hexapod-type 2 subunits, and 3) by the orthology of remipede and hexapod hemocyanin subunits (fig. 3), which is also supported by statistical tests of alternative tree topologies. A sister-group relationship of Remipedia and Hexapoda was tentatively retrieved in another molecular tree using elongation factor 2 sequences (Regier and Shultz 2001) and was also suggested by Telford et al. (2008) on the basis of unpublished material. In a large-scale phylogenomic study by Regier et al. (2008), a clade consisting of Remipedia and Hexapoda was consistently recovered, which in that study also included the Cephalocarida (which are not considered here).

Interestingly, a recent morphological study supports a close relationship of Remipedia and Hexapoda: Based on the structure of the arthropod brain, Fanenbruck and coworkers (Fanenbruck et al. 2004) proposed a common clade of Malacostraca, Remipedia, and Hexapoda (although they did not include Myriapoda in their analyses) with the exclusion of Branchiopoda and Maxillopoda. It was noted that the arrangement of nerves, axonal tracts, neuropil compartments and cell clusters in the brains of these taxa are similar but distinct from that of any other crustacean class. This hypothesis is now reinforced by the fact that all three taxa employ hemocyanin as respiratory protein, whereas other crustacean taxa use hemoglobins (Mangum 1985; Markl and Decker 1992; Burmester 2002). Therefore, Pancrustacea may be divided into two clades consisting of the possibly monophyletic Entomostraca (which have lost hemocyanins) on one hand (Walossek 1999), and Malacostraca, Remipedia, and Hexapoda (taxon N.N.) on the other (Fanenbruck et al. 2004). However, it should be noted that we cannot reject the hypothesis of multiple independent losses of hemocyanin and independent evolution of hemoglobin in various "entomostracan" lineages. If this is the case and the cited morphological evidence is ignored, the minimal topology recovered in our analyses is (Malacostraca, (Remipedia, Hexapoda)). Then an entomostracan, possibly branchiopod, ancestor of Hexapoda cannot be excluded (Regier et al. 2005; Mallatt and Giribet 2006; Roeding et al. 2007; Dunn et al. 2008).

Conclusions: Remipedia-Like Ancestor of Hexapoda?

Although recent hypotheses focused on a branchiopod-like crustacean as possible ancestor of hexapods (Glenner et al. 2006), our findings suggest that alternatively a remipede ancestor could be proposed. Extant and fossil Remipedia may provide evidence for the *Bauplan* of the hexapod stem lineage. The oldest known hexapod fossil is the collembolan *Rhyniella praecursor* from the Devonian Rhynie *Lagerstätte* in Scotland. This species harbors various derived features, thus offering little information on early morphology of Hexapoda. Interestingly, Haas et al. (2003) noted that *Tesnusocaris goldichi*, a remipedian fossil from the Carboniferous (Emerson and Schram 1991) may be a stem-lineage hexapod. This proposal was based on the unbranched structure of the first antenna and the long and filiform caudal appendages. It may be further speculated that the enigmatic fossil *Devonohexapodus bocksbergensis* (Haas et al. 2003), which was described as a marine hexapod from the lower Devonian, may represent a transitional form between Remipedia and Hexapoda. Hexapod-like structures include leg-like palps of maxillae, absence of a second pair of antennae, and three pairs of longer uniramous thoracopods with six podomeres, but the homonomous trunk with its 38 trunk segments and the "abdominal" leglets actually provide an overall Remipedia-like appearance. In summary, it is possible that both fossil and living Remipedia occupy a key position for unraveling the evolution of Pancrustacea and thus for the understanding of morphological and functional innovations eventually resulting in the emergence of Hexapoda. However, additional molecular and morphological studies are required to unravel the position of Remipedia and to stably resolve their relationship to Hexapoda.

Supplementary Material

Supplementary table 1 and supplementary figures 1–4 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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