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ORIGINAL ARTICLE

# Occurrence of Hemocyanin in Ostracod Crustaceans

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**Abstract** Hemocyanin is a copper-containing protein that transports $O_2$ in the hemolymph of many arthropod species. Within the crustaceans, hemocyanin appeared to be restricted to Malacostraca but has recently been identified in Remipedia. Here, we report the occurrence of hemocyanin in ostracods, indicating that this respiratory protein is more widespread within crustaceans than previously thought. By analyses of expressed sequence tags and by RT-PCR, we obtained four full length and nine partial hemocyanin sequences from six of ten investigated ostracod species. Hemocyanin was identified in Myodocopida (*Actinoseta jonesi*, *Cypridininae* sp., *Euphilomedes morini*, *Skogsbergia lerneri*, *Vargula tsujii*) and Platycopida (*Cytherelloidea californica*) but not in Podocopida. We found no evidence for the presence of hemoglobin in any of these ostracod species. Like in other arthropods, we identified multiple hemocyanin subunits (up to six) to occur in a single ostracod species. Bayesian phylogenetic analyses showed that ostracod hemocyanin subunit diversity evolved independently from that of other crustaceans. Ostracod hemocyanin subunits were found paraphyletic, with myodocopid and platycopid subunits forming distinct clades within those of the crustaceans. This pattern suggests that ostracod hemocyanins originated from distinct subunits in the pancrustacean stemline.

**Keywords** Crustacea · Ostracoda · Respiratory protein · Hemocyanin · Phylogeny

**Abbreviations**

| | |
|---|---|
| BPP | Bayesian posterior probability |
| Hc | Hemocyanin |
| MYA | Million years ago |

## Introduction

Hemocyanins (Hcs) transport $O_2$ in the hemolymph of many molluscan and arthropod species and are present in all arthropod subphyla—chelicerates, myriapods, and pancrustaceans (hexapods and paraphyletic crustaceans)—as well as onychophorans (Burmester 2001; Kusche and Burmester 2001; Burmester 2002; Kusche et al. 2002; Pick et al. 2009; Ertas et al. 2009; Scherbaum et al. 2010). Within crustaceans, knowledge of Hc was restricted to Malacostraca (Markl and Decker 1992; Burmester 2001). Only recently has it become evident that Hc also occurs in Remipedia (Ertas et al. 2009). Other crustacean classes, such as Branchiopoda, Cephalocarida, Thecostraca, and Copepoda, appear to either employ hemoglobin for oxygen transport or are devoid of any respiratory protein (Mangum 1985).

A typical arthropod Hc subunit consists of about 620–660 amino acids with a molecular mass of about 75 kDa (van Holde and Miller 1995; Burmester 2002). The Hc subunits assemble into homo- or heterohexamers of about 450 kDa, which may associate into oligomeric structures up to 8 × 6-mers (Markl et al. 1986; Markl and Decker 1992; van Holde and Miller 1995; Burmester 2002). In each subunit, $O_2$ is bound to two copper ions that are coordinated by six histidine residues (Gaykema et al. 1984; Linzen et al. 1985).

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The class Ostracoda is a large, fossil-rich crustacean group with more than 65,000 species, at least 8,000 of which are extant (Vannier and Abe 1995; Horne et al. 2002). The root of crown group ostracods dates back ~500 million years (Tinn and Oakley 2008). Ostracods are subdivided into two subclasses, the Myodocopa and the Podocopa (Bowman and Abele 1982). The myodocopans, comprising the orders Myodocopida and Halocyprida, are all marine, with mostly weakly calcified valves. The podocopans, represented by the mainly marine Podocopida and the rare Platycopida, in contrast, possess well-calcified exoskeletons, the reason for their excellent fossil record (Horne et al. 2002). Ostracods are mostly small, covered by a chitinous, usually calcified bivalved carapace with a diameter of about 0.3–3 mm, with few exceptions up to 32 mm (Vannier and Abe 1995). The carapace envelops the whole body as a domiciliary cavity and gives a bivalve-like appearance. Many ostracods, especially smaller Podocopa, lack a heart, circulatory system, and gills (Keyser 1990; Vannier and Abe 1995; Corbari et al. 2004a, b). The myodocopids possess a heart and circulatory system (Abe and Vannier 1995). Ostracods colonized a broad variety of aquatic habitats, from marine via estuarine to nonmarine (Smith and Horne 2002; Ikeya et al. 2005; Martens et al. 2008).

The monophyly of ostracods and their positions within the (pan-) crustaceans are often debated (Horne et al. 2005; Ikeya et al. 2005; Oakley et al. 2013). Recent molecular work has provided evidence for ostracod monophyly (Oakley et al. 2013) and indicates that Ostracoda are phylogenetically related to Pentastomida and Branchiura within an ancient clade named Ichthyostraca. Ichthyostraca form the sister group to all other Pancrustacea (Regier et al. 2010; Oakley et al. 2013).

At first glance, the rather small size of ostracods suggests that a respiratory protein is unnecessary. However, the occurrence of hemoglobin was described in some podocopids (Fox 1957). In contrast, the presence of Hc has not been reported previously in ostracods. Here, we demonstrate by Western blotting, database mining, and RT-PCR that Hc in fact occurs in a broad range of ostracod species. Notably, ostracod Hc shows an ancient subunit diversity that evolved independently from that of Malacostraca and Remipedia.

## Materials and Methods

### Analysis of Ostracod Expressed Sequence Tags

We used various crustacean Hc and hemoglobin sequences to search the available Expressed Sequence Tags (ESTs) from nine ostracod species using BLAST (Altschul et al. 1990): *Actinoseta jonesi* (Myodocopa, Myodocopida, Cylindroleberoidea), *Cypridininae* sp. (Myodocopa, Myodocopida, Cypridinoidea), *Cytherelloidea californica* (Podocopa, Platycopida, Cytherellidae), *Euphilomedes morini* (Myodocopa, Myodocopida, Sarsielloidea), *Heterocypris* sp. (Podocopa, Podocopida, Cypridocopina), *Puriana* sp. (Podocopa, Podocopida, Cytherocopina), *Skogsbergia lerneri* (Myodocopa, Myodocopida, Cypridinoidea), *Vargula tsujii* (Myodocopa, Myodocopida, Cyprinidoidea), and *Vestalenula* sp. (Podocopa, Podocopida, Darwinulocopina). The ESTs were available from EMBL/GenBank (*Cypridininae* sp.) or from DRYAD (doi:10.5061/dryad.tb40v; all others). We assembled the Hc subunit sequences by the aid of Vector NTI 10.3.0 (Invitrogen; Darmstadt, Germany) and GeneDoc 2.7 (Nicholas et al. 1997).

### Animals

Three ostracod species were used for RT-PCR: Adult individuals with mixed sexes of *Cypridopsis vidua* (Podocopa, Podocopida, Cypridocopina) were obtained from a local supplier and identified by Dietmar Keyser (Hamburg). Adult females of *E. morini* (Myodocopa, Myodocopida) were caught at the pier of Santa Barbara, CA, USA using an Ekman grab (Wildco, Buffalo, NY, USA) to sample sediment from beside Stearn's Wharf Pier in Santa Barbara, CA (34° 24′ 38.41″N, 119° 41′ 14.5″W). Depending on the tide, the water at this location was 5–7 meters deep. Adult individuals of mixed sexes of *S. lerneri* (Myodocopa, Myodocopida) were caught in late summer near the coast of Florida, USA (Duck Key Viaduct, 24° 46.9′N 80° 54.58′W).

### Cloning of Hemocyanin cDNA

We transferred several individuals of *S. lerneri*, *E. morini*, or *C. vidua*, respectively, into 0.5 ml peqGOLD TriFast buffer (Peqlab, Erlangen, Germany) that had been frozen in liquid $N_2$ and crushed the mixture thoroughly with a mortar and pestle. After 5 min incubation at room temperature, the debris was removed by centrifugation (5 min 12,000×*g*, 4 °C). The total RNA was extracted according to the manufacturer's instructions. cDNA was generated with the SuperScript III reverse transcriptase (Life Technologies, Darmstadt, Germany) as stated on the manual using oligo dT primer. For the following PCR reactions, the AccuPrime™ Taq (Life Technologies) polymerase was used. A degenerated oligonucleotide primer pair was designed according to conserved regions of pancrustacean Hc subunits: 5′-GTNGCGGTYTCRAART GYTCCAT-3′ and 5′-ATGGAYTTYCCNTTYTGGTG GAA-3′. The PCR products of about 530 base pairs were cloned using the pGEM-T/*E. coli* JM109 system (Promega) and sequenced by a commercial service (GATC, Konstanz,

Germany). Full-length Hc subunits sequences were obtained by 5′ and 3′ RACE using the GeneRacer™ Kit (Life Technologies) following the manufacturer's instructions. The gene-specific primers for each subunit are listed in Supplementary Table 1. PCR products of the expected size were cloned and sequenced as described.

### Sequence and Phylogenetic Analyses

We used GeneDoc 2.7 (Nicholas et al. 1997) to assemble the partial Hc sequences. The tools provided at the ExPASy server (Swiss Institute of Bioinformatics; http://www.expasy.org/tools/dna.html) were used to predict the open reading frames (ORFs) and to translate them into amino acids. Putative signal peptides were identified with the program SignalP 4.1 (Petersen et al. 2011) using the standard options. The molecular masses of proteins were estimated employing the Compute pI/Mw tool at ExPASy (http://web.expasy.org/compute_pi).

We constructed a multiple amino acid sequence alignment that included, in addition to 13 ostracod Hc subunits, 110 sequences of Hc subunits from one onychophoran, five chelicerate, three myriapod, twelve hexapod, and 29 crustacean species as well as five malacostracan pseudohemocyanins (cryptocyanins), employing MAFFT with the L-INS-i-method (Katoh et al. 2005) (Supplementary Table 2; Supplementary Fig. 1). Phylogenetic analyses were performed using either MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001) or RAxML 7.2.8 (Stamatakis, 2006), assuming the WAG model (Whelan and Goldman 2001) with a $\Gamma$ distribution of substitution rates. The WAG model was selected with ProtTest (Abascal et al. 2005), using the Akaike information criterion. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed with one cold and three heated chains that were run for 2,000,000 generations. Prior probabilities for all trees were equal, and starting trees were random. The trees were sampled every 100$^{th}$ generation. Two independent runs were performed in parallel and were continued until the average standard deviation of split frequencies was lower than 0.005. The program Tracer 1.6 (http://tree.bio.ed.ac.uk/software/tracer/) was used to examine log-likelihood plots. Posterior probabilities were estimated on the final 15,000 trees (burnin = 5000). The tree was visualized with FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). The onychophoran Hc subunit was considered as outgroup (Kusche et al. 2002).

### Gel Electrophoresis and Western Blotting

For SDS-PAGE, several individuals of *S. lerneri* (about 160 mg) that had been stored in 300 µl 0.05 M Tris–HCl, pH 6.8, 1.0 % SDS, bromophenole blue, were crushed, sonicated twice for 20 s on ice, and centrifuged for 30 s at 2000×*g*. The proteins were separated under standard conditions on 10 % SDS-PAGE gels, applying 30 µl sample per lane. The gels were either stained with 0.1 % Coomassie Brilliant Blue in 10 % acetic acid and 25 % isopropanol or used for Western blotting.

For Western blotting, the proteins were transferred onto nitrocellulose membranes. Non-specific binding sites were blocked with 5 % non-fat dry milk in TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) for 2 h. Polyclonal antisera against various hemocyanins from the decapod crustaceans *Astacus astacus*, *Palinurus elephas*, or *Panulirus interruptus* (Markl 1986) or the hexapod *Blaptica dubia* Hc subunit Hc1 and Hc2 (Pick et al. 2009) were employed. The antisera were diluted 1:2000 in blocking solution and incubated with the membranes at 4 °C overnight. After three successive washing steps with 0.1 % Tween 20 in TBS, the second antibody (goat-anti-rabbit alkaline phosphatase conjugated IgG; Dianova, Hamburg, Germany) was applied 1:10,000 in TBS for 1 h at room temperature. After additional washing steps, bound antibodies were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris–HCl, pH 9.5, and 100 mM NaCl in the dark.

## Results

### Identification of Hemocyanin in Ostracod ESTs

The ESTs from nine ostracod species were available (von Reumont et al. 2012; Oakley et al. 2013). Employing tBLASTn, we identified Hc sequences in the ESTs of *A. jonesi*, *Cypridininae* sp., *E. morini*, *S. lerneri*, *V. tsujii* (all Myodocopa: Myodocopida), and *C. californica* (Podocopa: Platycopida) (Table 1). No Hc subunit sequences were found in the ESTs of *C. vidua*, *Heterocypris* sp., *Puriana* sp., and *Vestalenula* sp. (all Podocopa:Podocopida). The quality of the sequence data of *A. jonesi* and *E. morini* does not allow assembly of long sequences. At least three distinct Hc subunits were found in *Cypridininae* sp., one of which could be assembled into a full-length sequence (CypHc1; Table 2). The ESTs of *C. californica* gave rise to two full-length Hc subunit sequences (CcaHc1 and CcaHc2; Table 2). A full-length sequence could also be assembled from the ESTs of *V. tsujii* (VtsHc1; Table 2).

### cDNA Sequences of Ostracod Hemocyanin Subunits

We obtained by RT-PCR 22 clones from *E. morini* RNA and 29 clones from *S. lerneri* RNA, which correspond to the conserved Hc middle region. No RT-PCR product was obtained from *C. vidua*. The cDNA sequences of *E. morini* correspond to three distinct Hc subunits. The cDNA clones

**Table 1** Ostracod species analyzed in this study

| Species | Taxonomic position | Hc subunits | Method | Remarks |
|---|---|---|---|---|
| *Actinoseta jonesi* | Myodocopa, Myodocopida | At least 1 | EST data | Quality does not allow assembly |
| *Cypridininae* sp. | Myodocopa, Myodocopida | At least 3 | EST data | 1 Full-length sequence |
| *Euphilomedes morini* | Myodocopa, Myodocopida | At least 3 | EST data + RT-PCR | 3 Partial sequences |
| *Skogsbergia lerneri* | Myodocopa, Myodocopida | At least 6 | EST data + RT-PCR | 1 Full length + 5 partial sequences |
| *Vargula tsujii* | Myodocopa, Myodocopida | At least 1 | EST data | 1 Full-length sequence |
| *Cytherelloidea californica* | Podocopa, Platycopida | At least 2 | EST data | 2 Full-length sequences |
| *Cypridopsis vidua* | Podocopa, Podocopida | Not found | RT-PCR | – |
| *Heterocypris* sp. | Podocopa, Podocopida | Not found | EST data | – |
| *Puriana* sp. | Podocopa, Podocopida | Not found | EST data | – |
| *Vestalenula* sp. | Podocopa, Podocopida | Not found | EST data | – |

**Table 2** Characteristics of Hc subunits from *C. californica* (Cca), *Cypridininae* sp. (Cyp), *E. morini* (Emo), *S. lerneri* (Sle), and *V. tsujii* (Vts)

| Hemocyanin subunit | Accession number | Length (bp), incl. UTR, without polyA-tail | CDS (bp) | Amino acids | Signal peptide | Mass (kDa) | Putative glycosylation sites |
|---|---|---|---|---|---|---|---|
| CcaHc1 | HG938288 | 2318 | 2022 | 674 | 18 | 75.2 | 2 |
| CcaHc2 | HG938289 | 2116 | 2022 | 676 | 16 | 76.5 | 2 |
| CypHc1 | HG938291 | 2392 | 1983 | 661 | 16 | 75.7 | 2 |
| EmoHc1 | HG938298 | >1415 | >1335 | >445 | – | – | ≥2 |
| EmoHc2 | HG938299 | >1656 | >1353 | >451 | – | – | ≥1 |
| EmoHc3 | HG938300 | >1647 | >1347 | >449 | – | – | ≥2 |
| SleHc1 | HG938292 | >2123 | >1914 | >637 | – | – | 1 |
| SleHc2 | HG938293 | >1963 | >1347 | >449 | – | – | ≥1 |
| SleHc3 | HG938294 | >2120 | >1347 | >449 | – | – | ≥1 |
| SleHc4 | HG938295 | >1561 | >1353 | >451 | – | – | ≥1 |
| SleHc5 | HG938296 | >1492 | >1359 | >453 | – | – | ≥1 |
| SleHc6 | HG938297 | >1490 | >1365 | >455 | – | – | ≥1 |
| VtsHc1 | HG938290 | 2124 | 2007 | 669 | 16 | 76.1 | 1 |

from *S. lerneri* could be assigned to six distinct Hc subunits.

The coding sequences of *E. morini* and *S. lerneri* Hcs were completed at the 3′ ends by RACE, while attempts to obtain the 5′ ends were not successful. The assembled Hc subunit sequences of *E. morini* (EmoHc1, EmoHc2, EmoHc3) covered between 1,008 and 1,353 bp (336–451 amino acids) (Table 2). The Hc subunit sequences of *S. lerneri* (SleHc1 to 6) were between 1,347 and 1,365 bp (449–455 amino acids). The 5′ region of SleHc1 could be extended by EST data, although sequence comparisons suggested that ~100 bp of the 5′ end of the ORF was still missing (Fig. 1).

## Analyses of Ostracod Hemocyanin Subunit Sequences

We obtained a total of 13 Hc subunit sequences from five ostracod species (no hemocyanin sequences were assembled from the low quality ESTs of *A. jonesi*). The cDNAs of CcaHc1, CcaHc2, CypHc1, and VtsHc1 cover the full ORFs and measure between 2,116 and 2,318 bp and have ORFs of 2,010–2,125 bp, which translate into proteins of 661–674 amino acids (Table 2). This is in the range of a typical crustacean Hc subunit. In each ostracod Hc subunit, we identified putative signal peptides between 16 and 18 amino acids in length, which facilitate the export into the hemolymph (Fig. 1). Sequence analyses show the presence of the conserved amino acids required for $O_2$ binding, including the six copper-binding histidines. The other nine sequences of ostracod Hc subunits do not cover the full ORFs, with parts missing at the 5′ and 3′ ends (Table 2). A multiple sequence alignment that also covers the incomplete Hc subunit sequences is given in Supplementary Fig. 2.

Sequence comparisons show that the sequences are quite distinct: The two Hc subunits of the platycopid ostracod *C. californica* (CcaHc1 and CcaHc2) share 60.5 % identical amino acids (73.9 % amino acid similarity). The number of identical amino acids shared between CcaHc1 and CcaHc2

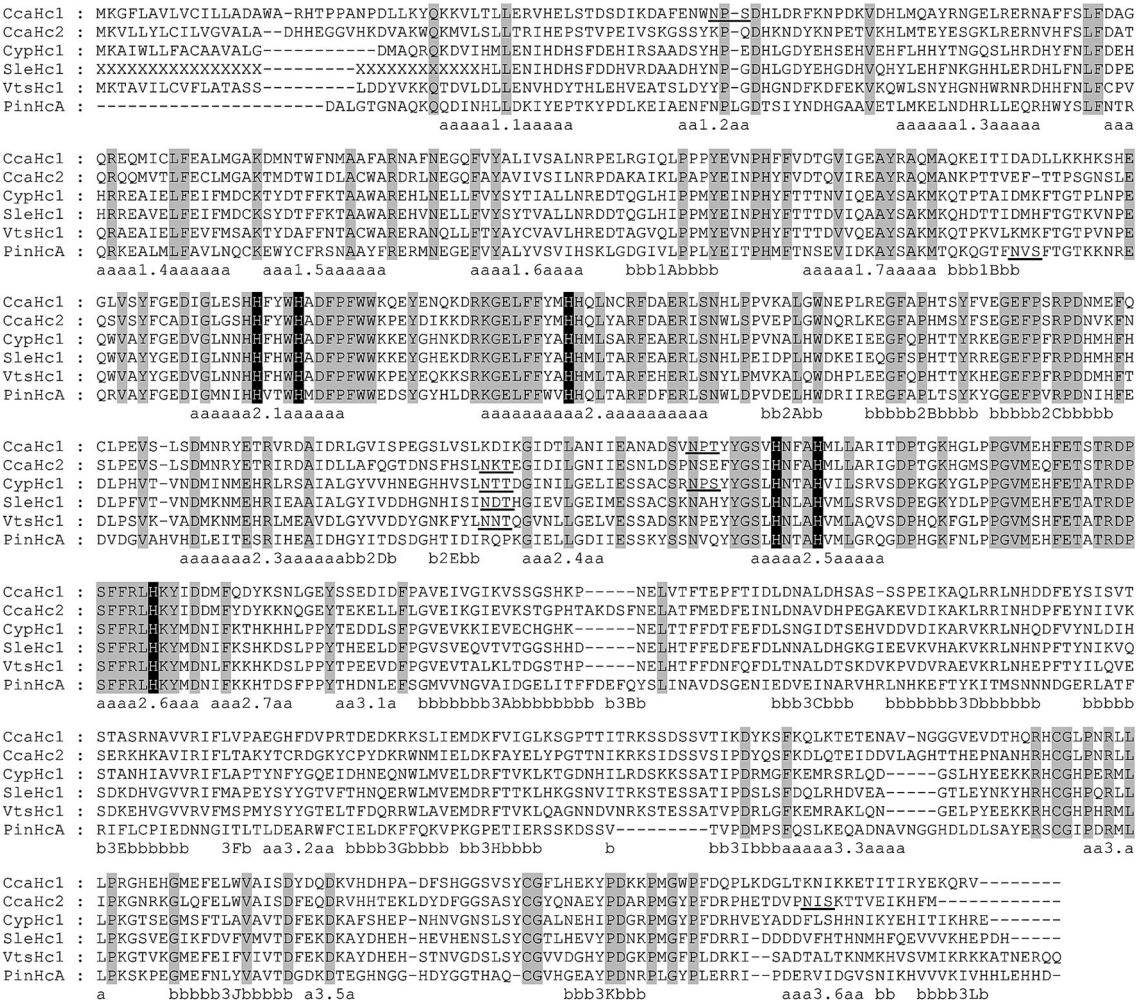


**Fig. 1** Multiple sequence alignment of Hc subunits from the ostracods *C. californica* (CcaHc1, CCaHc2), *Cypridininae* sp. (CypHc1), *S. lerneri* (SleHc1), and *V. tsujii* (VtsHc1), compared with the Hc α subunit of the decapod *P. interruptus* (PinHcA). Conserved amino acids are shaded gray, and the copper-binding histidines are shaded black. N-glycosylation sites are underlined, and the secondary structure elements of PinHcA (Volbeda and Hol 1989) are given below the sequences

and the myodocopid Hc subunits (CypHc1, SleHc1, and VtsHc1) were even lower and ranged from 43.6 to 48.6 % (65.9–73.6 % similarity). The Hc subunits of the Myodocopida share 62.9–69.2 % identical amino acids (79–83 % similarity).

## The Hemocyanin Protein in *S. lerneri*

The proteins of the whole-body homogenate of *S. lerneri* specimens were separated by SDS-PAGE. Hc subunits were visualized by Western blotting employing polyclonal antibodies against several pancrustacean hemocyanins. As expected, in the Coomassie stained gel, multiple bands were visible (Fig. 2). In the Western blot, a band at ~95 kDa reacted with the antisera against the Hcs from the cockroach *B. dubia* and the spiny lobster *P. elephas* but not with the antisera against the Hcs from the decapods *P. interruptus* and *A. astacus* (not shown).

The molecular masses of ostracod Hc subunits, as predicted from the cDNA sequences, were about 75 kDa (Table 2), as for most other pancrustacean Hc subunits (Markl 1986; Pick et al. 2009; Marxen et al. 2013). The difference in molecular mass of about 20 % compared to the putative Hc subunits in SDS-PAGE might be due to an unusually high glycosylation of the proteins. In fact, all ostracod Hc subunits possess at least one putative N-glycosylation site (Fig. 1 and Supplementary Fig. 2).

## Phylogeny of Ostracod Hemocyanin Subunits

Bayesian phylogenetic analyses were carried out on the basis of a multiple sequence alignment that covers 707 amino acid positions and 128 Hcs, including 13 ostracod Hc subunits

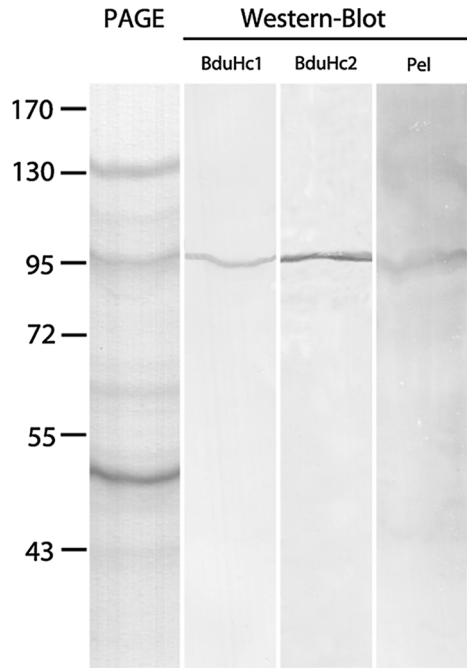


**Fig. 2** SDS-PAGE and Western blot of the *S. lerneri* total body homogenates. Several bands are visible after Coomassie staining. In Western Blot analysis, a band at 95 kDa was detected with antibodies against the two Hc subunits of the cockroach *B. dubia* (BduHc1, BduHc2) and the spiny lobster *P. elephas* (Pel)

(Fig. 3 in a simplified and Supplementary Fig. 3 in a detailed version). To evaluate the effect of the missing data, a second tree was constructed that only included the five (nearly) complete ostracod sequences (Supplementary Fig. 4). With the exception of the additional ostracod Hc subunits, the tree topologies were identical.

We found high support (1.0 Bayesian posterior probability [BPP]) for a clade of pancrustacean Hc subunits (including decapod pseudohemocyanins, which are copperless, non-respiratory storage proteins [Burmester 1999; Terwilliger et al. 1999]). Likewise, monophyly of a clade consisting of hexapod and remipede Hc subunits, separated in type I and type II subunit (Ertas et al. 2009) on the one hand, and malacostracan Hc subunits plus pseudohemocyanins on the other hand (Burmester 2002; Scherbaum et al. 2010; Marxen et al. 2013) received maximum support. However, ostracod Hc subunits were not monophyletic, with the myodocopid Hc subunits being more closely related to the malacostracan Hc subunits (1.0 BPP) than to those of the platycopid. Platycopid Hc subunits (CcaHc1 and CcaHc2) may either be a sister group of (i) the other ostracod plus malacostracan Hc subunits (0.88 BPP in the first tree, 0.93 BPP in the second tree) or (ii) all other pancrustacean Hc subunits. Within myodocopid Hc subunits, three well-supported clades were identified. The first clade included SleHc1, SleHc3, and SleHc4 (1.0 BPP), the second clade CypHc1, EmoHc2, EmoHc3, and SleHc2 (1.00 BPP), and the third EmoHc1, SleHc5, SleHc6, and VtsHc1 (0.98 BPP). The relationships among these three clades, however, were not resolved. ML analyses (Supplementary Fig. 5) retrieved a very similar tree which differs in the topology of the poorly resolved node at the base of the ostracod Hcs, but which also did not identify monophyletic ostracod Hcs.

## Discussion

Within the crustaceans, Hc has initially only been identified in Malacostraca (Mangum 1985; Markl and Decker 1992; Burmester 2002). In other crustacean classes, either hemoglobin serves as an oxygen carrier or a respiratory protein is lacking altogether (Mangum 1985). Hc has been reported in the parasitic cirripede *Sacculina carcini* (Herberts and de Frescheville 1981), but it is uncertain whether this is an endogenous protein or actually derives from the decapod host, *Carcinus maenas* (Burmester 2002). Hc subunits have been obtained from *Speleonectes tulumensis*, providing conclusive evidence that this respiratory protein also occurs in Remipedia (Ertas et al. 2009). This was the first report that Hc occurs in crustacean classes others than Malacostraca. Here, we extend this observation, reporting for the first time the presence of Hc also in ostracod crustaceans.

### Hemocyanin is the Respiratory Protein of Ostracoda

Based on a microspectroscopic technique, Fox (1957) noted the presence of hemoglobin in the ostracods *Cypria* and *Pseudocypris*, but no further details have been reported in the literature. In our analyses, BLAST searches actually identified globin sequences in some ostracod EST datasets: Putative orthologs of a membrane-bound globin of the crab *Carcinus maenas* (Ertas et al. 2011) were found in *Puriana* sp. and *Cypridininae* sp., and a globin with the highest similarities to the nerve globin of bivalve mollusc *Spisula solidissima* was seen in *C. californica* (Dewilde et al. 2006) (data not shown). None of these globin sequences resemble a typical hemoglobin found in other crustaceans (Weber and Vinogradov 2001). Although our inability to find hemoglobin in ESTs is not conclusive evidence of absence, the results by Fox (1957) cannot be validated. We consider it unlikely that ostracods use hemoglobin as respiratory protein that transports $O_2$ in the hemolymph.

In contrast to the lack of evidence for hemoglobin, we used a combination of database searches, RT-PCR, and protein biochemical methods to demonstrate the presence of Hc in ten ostracod species (Table 1). Five of them belong to the order Myodocopida within the subclass Myodocopa. Species of the other myodocope order (Halocyprida) were not available for this study. Among the subclass Podocopa, Hc was detected in members of the order Platycopida

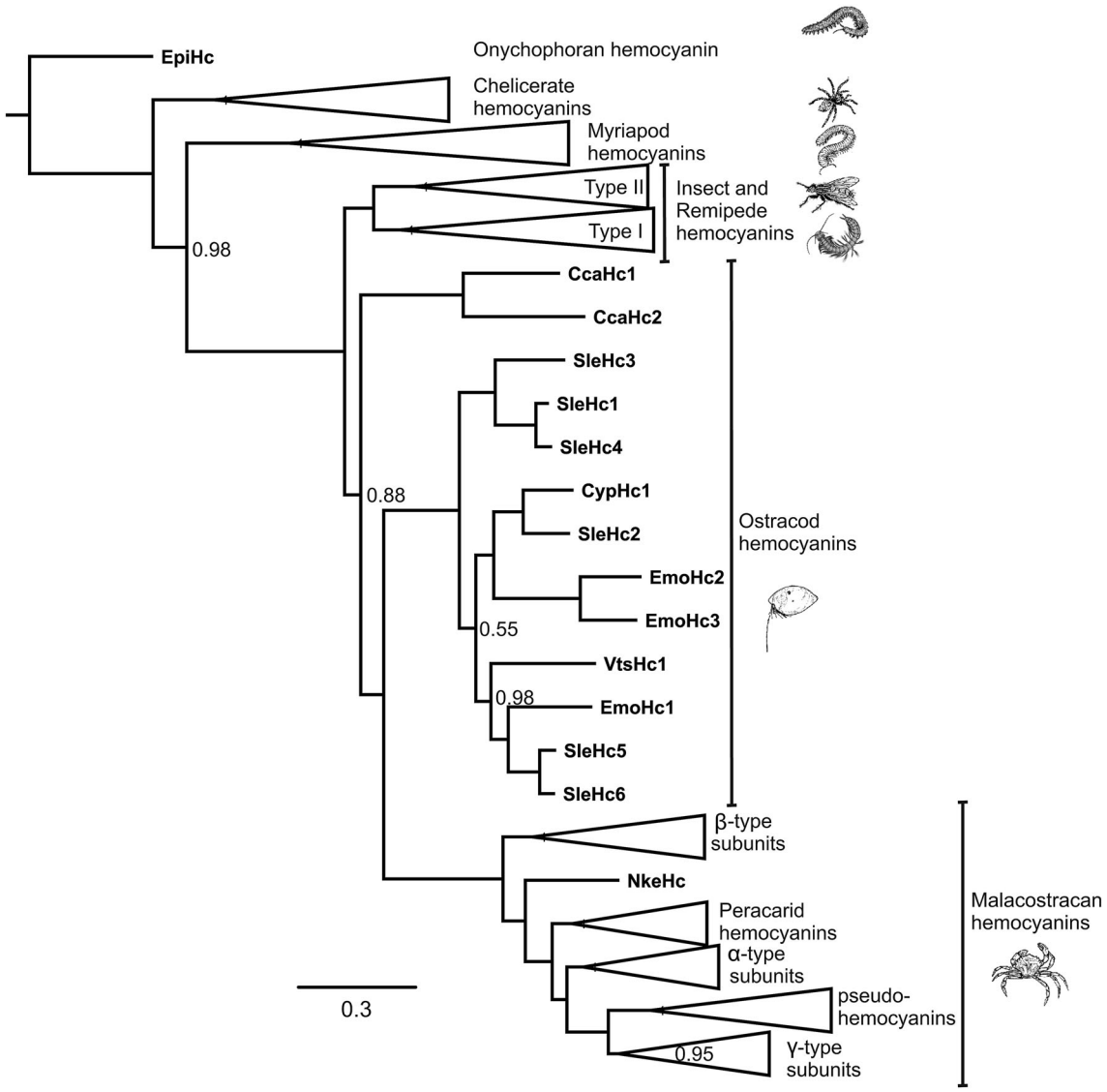


**Fig. 3** Simplified Bayesian tree of 128 arthropod Hc subunits and pseudohemocyanins, including 13 ostracod Hc subunits (full list see Supplementary Table 2). The tree was rooted with the Hc subunit of the onychophoran *Epiperatus* sp. The Bayesian posterior probabilities are 1.0 unless otherwise given. The *bar* indicates 0.3 PAM distance

(represented by *C. califonica*) but not in members of the order Podocopida, suggesting loss in the latter taxon. The absence of a respiratory protein agrees with the physiology of podocopids, which also lack a cardiovascular system and any regulatory mechanism to adapt their ventilation to water oxygenation (Corbari et al. 2004a).

## Early Divergence of Ostracod Hemocyanin Subunits

Arthropod Hcs are hexamers or oligohexamers with subunits that are either encoded by a single gene or derive from different paralogous genes. Thus, multiple distinct Hc subunits may be detected in a single species. Within the malacostracan crustaceans, three distinct subunit types (α-γ) occur that diverged more than 450 MYA (Marxen et al. 2013), which assemble to quaternary structures that may even differ within species (Markl 1986). Likewise, in remipedes and insects, two (orthologous) subunits occur (Ertas et al. 2009; Pick et al. 2009). Thus, the presence of distinct subunit types is a common feature of arthropod Hc but emerged independently in the different subphyla.

A similar diversity occurs in the ostracods, in which up to six distinct Hc subunits were observed in *S. lerneri*. In our phylogenetic analyses, the clades of ostracod Hc subunits are not nested within the Hc subunits of the other crustacean classes. Thus, distinct subunit types evolved independently within the ostracods after they diverged from the other pancrustacean classes. In addition, we found ostracod Hcs paraphyletic, indicating a complex and partially independent evolution of Hc within this taxon. Although the phylogenetic

position of the *C. californica* Hc subunits could not be conclusively resolved, they do not associate with the other ostracod Hc subunits (Fig. 3), indicating that the Hc subunits from Platycopida (*C. californica*) and Myodocopa (*Cypridininae* sp., *E. morini*, *S. lerneri* and *V. tsujii*) emerged from distinct subunit types that split before Ostracoda and Malacostraca diverged more than 500 MYA. Further, the subunit type represented today by *C. californica* Hc subunits appears to have been lost in the Malacostraca.

Finally, we note that the inclusion of ostracod Hc subunits in the phylogenetic analyses confirms earlier conclusions drawn from arthropod Hc phylogeny (Kusche and Burmester 2001; Kusche et al. 2003; Ertas et al. 2009). For example, the association of myriapod and pancrustacean Hc subunits receives high support, which is in line with the Mandibulata hypothesis (e.g., Regier et al. 2010). Likewise, the very close relationship of the Hc subunits of Hexapoda and Remipedia supports the notion of a close relationship of these taxa (Ertas et al. 2009; Regier et al. 2010; von Reumont et al. 2012).

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