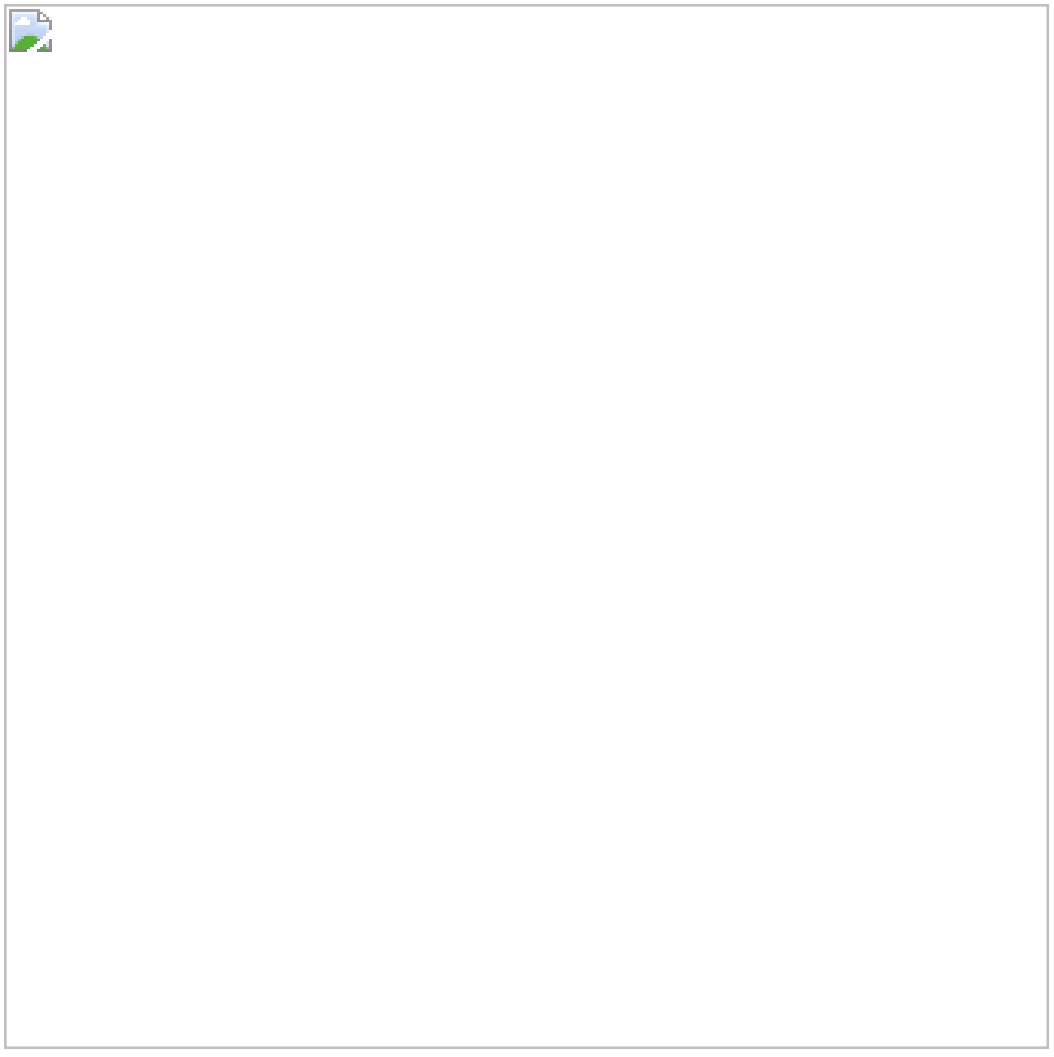

N-Terminal Amino Acid Sequence Comparison of Asian Horseshoe Crab Hemocyanins: Immunologically Identical Hemocyanin Subunits Are Orthologous in Asian Horseshoe Crabs

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N-Terminal Amino Acid Sequence Comparison of Asian Horseshoe Crab Hemocyanins: Immunologically Identical Hemocyanin Subunits Are Orthologous in Asian Horseshoe Crabs

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ABSTRACT—Each hemocyanin of the 3 Asian horseshoe crabs is composed of 6 immunologically different subunits, each subunit of which is immunologically identical with the comparable subunits of 2 other species. These immunologically identical subunits show 0–15% N-terminal sequence differences between *Carcinoscorpius rotundicauda* and *Tachypleus gigas*, while the immunologically different subunits show 25–74% sequence differences within species and 25–72% between *C. rotundicauda* and *T. gigas*. From the N-terminal sequence comparison and immunological comparison of hemocyanin subunits, it is evident that Asian horseshoe crabs share 6 orthologous hemocyanin subunits which are immunologically identical. We may produce 6 phylogenetic trees of Asian horseshoe crabs using the 6 sets of orthologous hemocyanin subunits and an evolutionary tree of horseshoe crab's hemocyanin molecules.

INTRODUCTION

Hemocyanins are the blue respiratory pigments freely dissolved in the hemolymph plasma of many arthropods and molluscs. Arthropodan and molluscan hemocyanins differ fundamentally in their quaternary structure (Van Holde and Miller, 1982, 1995; Ellerton *et al*., 1983) and there is very little sequence similarity between arthropodan subunits and molluscan functional units (Drexel *et al*., 1987). On the contrary, the functional units of molluscan hemocyanins show amino acid sequence similarity with tyrosinases from a bacterium, a mold and a mouse (Drexel *et al*., 1987), while the hemocyanin subunits of arthropods are similar in the amino acid sequence to insect storage proteins (hexamerins) (Willott *et al*., 1989; Beintema *et al*., 1994) and insect pro-phenol oxidases (Fujimoto *et al*., 1995; Kawabata *et al*., 1995). Therefore, arthropodan and molluscan hemocyanins are thought to have evolved by a quite different and independent evolution, that is, convergent evolution, although they both are oxygen transporting molecules containing copper atoms.

Arthropodan hemocyanins consist of hexamers or multihexamers of subunit chains of about 75 kDa and the heterogeneity of the subunits was demonstrated by means of polyacrylamide gel electrophoresis using hemocyanins from Chelicerata (Sugita and Sekiguchi, 1975; Markl *et al*., 1979a) and Crustacea (Murray and Jeffrey, 1974; Markl *et al*., 1979b).

The complete amino acid sequences of the subunits were determined for the chelicerate hemocyanins from 2 horseshoe crabs, *Tachypleus tridentatus* (Takagi and Nemoto, 1983) and *Limulus polyphemus* (Nakashima *et al*., 1986), a tarantula spider, *Eurypelma californicum* (Schartau *et al*., 1983; Schneider *et al*., 1983; Voit and Feldmaier-Fuchs, 1990) and a scorpion, *Androctonus australis* (Buzy *et al*., 1995). Pairwise distances between these sequences range from 35% to 47% (Linzen *et al*., 1985; Voit and Feldmaier-Fuchs, 1990; Beintema *et al*., 1994; Buzy *et al*., 1995), showing a considerable degree of similarity within a species (the tarantula spider), between genera (the horseshoe crabs), between orders (the spider and the scorpion) and between classes (the horseshoe crabs and the spider or scorpion). Phylogeny inference programs produced branching patterns for these 5 hemocyanin subunits except the scorpion hemocyanin (Beintema *et al*., 1994; Burmester and Scheller, 1996) but the patterns could not give any evolutionary relation between animals having these subunits, showing a terminal node which included the *L. polyphemus* hemocyanin subunit and one of the *E. californicum* hemocyanin subunits instead of the *T. tridentatus* hemocyanin subunit. They discussed the relation between hemocyanin and hexamerin but did not have any discussion on evolution of hemocyanin subunits and animal species. To make phylogenetic tree of animal evolution, therefore, we must choose orthologous hemocyanin subunits from many subunits between species.

On the other hand, Sugita and Shishikura (1995) reported that a subunit of 6 immunologically different hemocyanin subunits from Asian horseshoe crab, *Carcinoscorpius rotundicauda* showed about 97% sequence similarity with the *T. tridentatus* hemocyanin subunit, and that those 2 subunits were immunologically identical. Furthermore, Sugita (1988) reported that

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the 6 subunits of *T. tridentatus* hemocyanin differed entirely from one another in antigenic properties. Another Asian horseshoe crab, *T. gigas* had 5 hemocyanin subunits which exhibited no immunological cross reaction within species. However, each of these hemocyanin subunits from the 3 Asian species was immunologically identical to the comparable subunits from each of the other 2 species (Sugita, 1986, 1988).

To examine whether immunologically identical hemocyanin subunits are orthologous or not, N-terminal amino acid sequences of hemocyanin subunits from these 3 Asian horseshoe crabs were analyzed and orthologous relationships between these subunits were discussed under the existence of many homologous sequences in species.

MATERIALS AND METHODS

Preparation of hemocyanin sample

The Japanese horseshoe crabs, *Tachypleus tridentatus*, were obtained from the vicinity of Imari and Fukuoka, Japan and the Southeast Asian horseshoe crabs, *T. gigas* and *Carcinoscorpius rotundicauda*, were obtained from the vicinity of Bangsaen, Thailand. Hemolymph was collected by puncturing the heart with a sterilized syringe to prevent the hemocytes from exploding. After sedimentation of hemocytes at 4°C, the blood plasma was pushed out of the syringe and kept with a roughly equal volume of glycerin at –20°C.

Preparation of antiserum and agar plate

Antiserum to pure hemocyanin of *T. tridentatus* was prepared as described previously (Sugita and Sekiguchi, 1975). Hemocyanin subunits used as antigens were cut out from acrylamide disc gels containing the respective subunits and put in wells. A double immunodiffusion test was carried out in a moisture chamber at 5°C using 1% agar plate prepared with 52 mM Tris-glycine buffer, pH 8.9, containing 10 mM EDTA and 0.01% thimerosal. The process of immunoprecipitin line formation was recorded every day.

Polyacrylamide gel electrophoresis

7.5% disc and slab gels at pH 8.9 were prepared according to the method of Davis (1964) and electrophoresis of native hemocyanin subunits was carried out in a disc gel and a slab apparatus using Davis's tank buffer.

Electrophoretic blotting

The hemocyanin subunits in a disc gel were re-electrophoresed into a slab gel and the subunits in the slab gel were transferred to a polyvinylidene difluoride (PVDF) sheet which was activated for 1 min in 100% methanol and soaked in transfer buffer. The transfer buffer contained 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol and 0.02% sodium dodecyl sulfate. Electrophoretic transfer was carried out for 7 hr at 2.5 mA/cm^2 in a blotting apparatus (Towbin *et al.*, 1979). Subsequently, the PVDF sheet was washed for 10 min in 10 mM sodium borate solution containing 25 mM NaCl. After a rinse with distilled water, proteins on the PVDF sheet were stained for 5 min with a Coomassie blue solution (0.1% in 42% methanol/17% acetic acid) and destained for a few minutes with 90% methanol, washed with distilled water and then dried overnight.

Amino acid sequence determination

The portion of the PVDF sheet containing the hemocyanin subunit was cut out and mounted in the reaction chamber of a protein sequencer. N-terminal sequence analysis was performed with an Applied Biosystems model 477A gas phase sequencer equipped with an online model 120A PTH-analyzer.

RESULTS

Chelicerate hemocyanins electrophoresed using the buffer system of Davis (1964) were dissociated into monomer subunits (Sugita and Sekiguchi, 1975). When hemocyanins from 3 Asian horseshoe crabs were electrophoresed in that buffer system, they gave electrophoretic patterns shown Fig. 1. After electrophoresis of those hemocyanins, subunit bands containing the respective subunits were cut out and used for antigen analysis (Fig. 2). Reacting with antiserum, subunits HG3

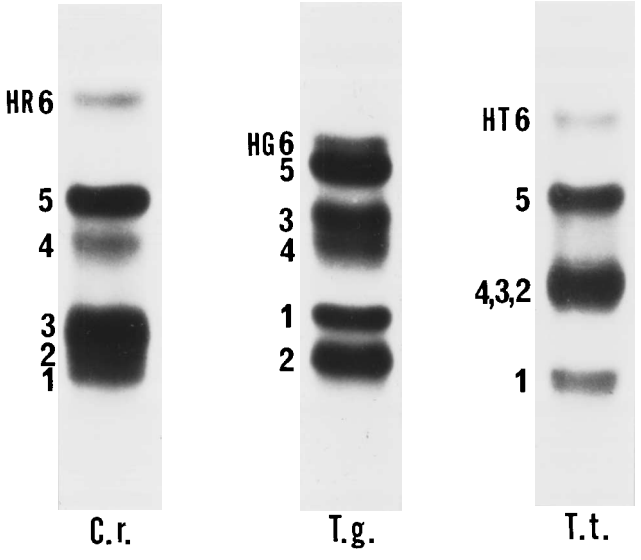

Fig. 1. Acrylamide gel electrophoretic patterns of Asian horseshoe crab's hemocyanin subunits. *C. rotundicauda* (C.r.), *T. gigas* (T.g.) and *T. tridentatus* (T.t.) hemocyanins consist of 6 immunologically different subunits HR1–6, HG1–6 and HT1–6, respectively. Similarly numbered subunits are immunologically identical between species (Sugita, 1988 and this work).

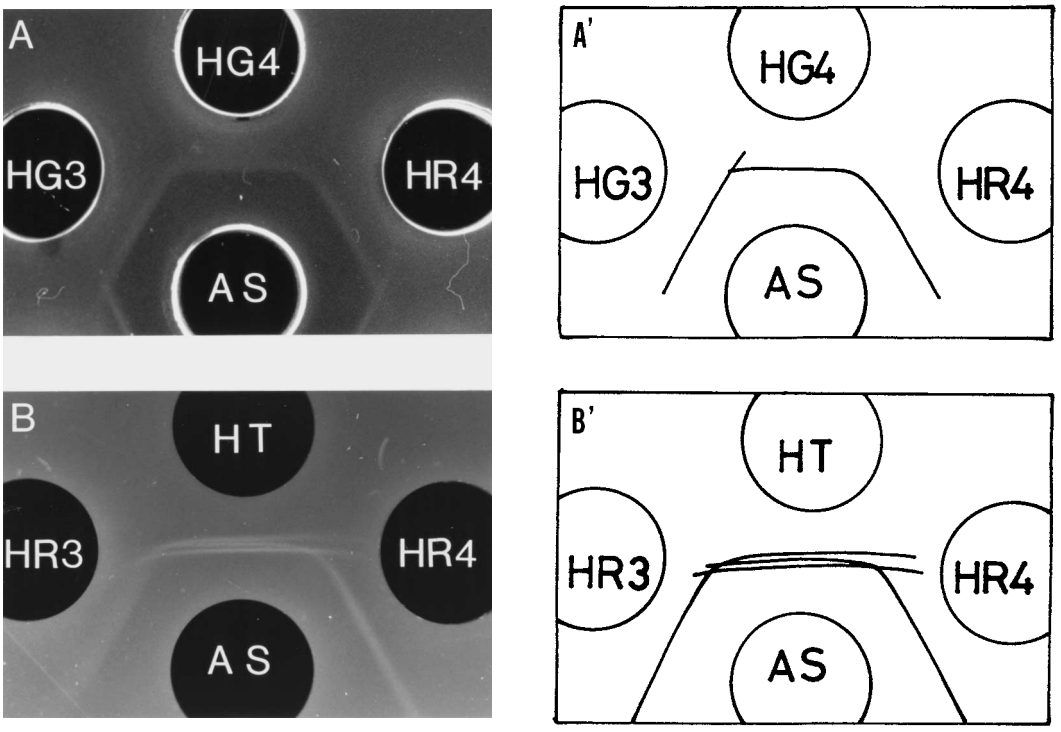

Fig. 2. Antigen analysis of hemocyanin subunits. AS, antiserum against *T. tridentatus* hemocyanin. HG3 and HG4, *T. gigas* hemocyanin subunits HG3 and HG4. HR3 and HR4, *C. rotundicauda* hemocyanin subunits HR3 and HR4. HT, *T. tridentatus* hemocyanin subunits from the second lowermost electrophoretic band. The results of immunodiffusion in agar plates (**A** and **B**) are schematized (**A'** and **B'**).

and HG4 formed immunologically different immunoprecipitin lines and the precipitin line due to HG4 fused with that due to HR4 without any spur (Fig. 2A, A'). The subunits contained in the second lowermost electrophoretic band of *T. tridentatus* hemocyanin formed 3 immunoprecipitin lines reacting with antiserum (Fig. 2B, B'). The central precipitin line of those was completely continuous with the precipitin line due to HR4 and the precipitin line formed on the side of antigen well was completely continuous with that due to HR3.

Hemocyanin subunits HG3 and HG4 have been regarded as immunologically identical with HR3 until now (Sugita ,1988). However, they differ immunologically from each other and HG4 is immunologically identical with HR4 (Fig. 2A, A'). Furthermore, the second lowermost electrophoretic band of *T. tridentatus* hemocyanin is composed of 3 immunologically different subunits, two of which are immunologically identical with HR3 or HR4 (Fig. 2B, B'). The rest of the 3 subunits was immunologically identical with HR2 (Sugita, 1988). Thus, from immunological comparison studies of these hemocyanin subunits and the other subunits (Sugita, 1988), it is evident that *T. gigas* and *T. tridentatus* hemocyanins consist of 6 immunologically different subunits, each one of which is immunologically identical with the comparable subunits of *C. rotundicauda* hemocyanin. Their immunological relations between species are shown in Fig. 1. In this nomenclature of hemocyanin subunits, the same numbered ones, e.g. HR6, HT6 and HG6, are immunologically identical with each other. Thus, it is clear that the 3 Asian horseshoe crabs share a set of 6 immunologically different hemocyanin subunits.

```
                1                  10                  20                  30                  40
HR1     TLHDKQVRVXHLFEQLSSATVT
HR2     TVKEKQSRLLPLFEHLTSAT
HR3     TIQEKQNKILSLLEHLNNLTKHQ-LPVDERD
HR4   VLSVLQKQLRVLPLFETATVPTXXXFALKAYRDH
HR5     TLKEKQDRILALFEHLTSLTKHQ-LPEEQRDARLHDLGHLL
HR6     TIKEKQASILALFEHLTSVPKQH-IPEKERDNRLHDVGHLS

HG1     TLHDKQVRVXHLFEQLSSATVTG
HG2     TVKEKQSRLLPLFQHLTXXXKDQ
HG3     TIQEKQNKILSLLEHLNNLTKHQ-LPVDERDA
HG4   VLDVLQKQLRVLPLFEYATI
HG5     TLKEKQDRILALFEHLTSLTKHQ-LPEEQRDARLHDSGH
HG6     TIKEKQASILALFEHLTSVPKQH

HT1     TLHDKQVRVXHLFEQLSSATVTGS
HT2     -VKEKQSRLLPLFKHLTRLSRDR-LPVS
HT3     TIQEKQNKILTLLEHLNSLSKHQ-LPVDERD
HT5     TLKEKQDRILALFEHLTSLTKHQ-LPEEQRDARLHDLGH
HT6     TIKEKQASILALFEHLTSVPKQH-IPEKERDNRLHDVGHLS
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Fig. 3. N-terminal amino acid sequences of hemocyanin subunits from *C. rotundicauda* (HR1–6), *T. gigas* (HG1–6) and *T. tridentatus* (HT1–3,5,6). Sequence data of HR6 and HT6 are from Sugita and Shishikura (1995) and Linzen *et al.* (1985). Amino acid residues indicated by X are ambiguous. Dashes represent gaps introduced under the necessity of comparing all N-terminal sequences.

Table 1. N-terminal % difference scores among hemocyanin subunits

	HR1	HR2	HR3	HR4	HR5	HR6	HG1	HG2	HG3	HG4	HG5	HG6	HT1	HT2	HT3	HT4	HT5	HT6
HR1	0																	
HR2	42	0																
HR3	67	50	0															
HR4	58	45	71	0														
HR5	48	25	37	62	0													
HR6	62	40	43	69	30	0												
HG1	0	42	68	58	50	64	0											
HG2	61	6	50	47	30	40	63	0										
HG3	67	50	0	72	35	45	68	50	0									
HG4	59	44	72	15	56	61	59	47	72	0								
HG5	48	25	37	62	3	29	50	30	35	56	0							
HG6	62	35	43	65	26	0	64	40	43	61	26	0						
HT1	0	42	70	60	50	63	0	63	70	59	52	64	0					
HT2	71	25	59	64	48	59	73	20	59	50	48	57	74	0				
HT3	67	50	10	71	37	40	68	60	10	72	37	39	70	56	0			
HT4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		
HT5	48	25	37	62	0	29	50	35	35	56	3	26	52	48	37	–	0	
HT6	62	35	43	69	30	0	64	40	45	61	29	0	65	59	40	–	29	0

In pairwise comparison, all comparable residues between 2 sequences are counted. When there are HQ and QH in positions 22–23 of 2 sequences, the difference score is counted as 1. HR, HG and HT denote hemocyanin subunits of *C. rotundicauda, T. gigas* and *T. tridentatus*, respectively.

Direct protein sequencing of the hemocyanin subunits gave the sequences for the first 17–41 amino acid residues as shown in Fig. 3. All the subunits possessed lysine-glutamine residues in positions 5-6 and a leucine residue in position 12. Position 13 contained a phenylalanine except for subunits HR3, HG3 and HT3, which was leucine. Subunits HR4 and HG4 were 2 residues longer on the N-terminal and HT2 was a residue shorter than other subunits. Although the subunits contained in the second lowermost electrophoretic band of *T. tridentatus* hemocyanin could not be separated apparently, protein sequencing of subunits from both ends of the band in long disc gel gave the 2 sequences similar to HR2 or HR3.

When the N-terminal sequences of *C. rotundicauda* hemocyanin subunits are compared within species, the difference ranges from 25% to 71% and hemocyanin subunits from *T. gigas* and *T. tridentatus* show sequence differences of 26–72% and 29–74% within species, respectively (Table 1). Furthermore, when the N-terminal sequences of *C. rotundicauda* and *T. gigas* hemocyanin subunits are compared, the sequence difference is 25–72% between different numbered subunits but the difference is 0–15% between the same numbered ones (Table 1). The N-terminal sequence comparison between hemocyanin subunits of *T. tridentatus* and these 2 species give similar results.

DISCUSSION

Although the N-terminal sequence of HT4 could not be revealed in this work, we can infer from the relation between N-terminal sequences and antigenicities in other subunits that HT4 probably has an N-terminal amino acid sequence similar to HR4 and HG4. Therefore, it seems that the 6 hemocyanin subunits found in each species are products of 6 different genes at distinct loci, because the 6 subunits are different in their N-terminal sequences and antigenicities from each other. This is supported by results of acrylamide-gel electrophoresis of hemocyanin subunits from interspecific hybrids. The results showed that any interspecific hybrid between Asian horseshoe crabs has 2 complete sets of hemocyanin subunits derived from the parents (Sekiguchi and Sugita, 1980). Thus, it is evident that Asian horseshoe crabs have 6 orthologous hemocyanin subunits derived from their common ancestor. These orthologous subunits are immunologically identical with each other.

In some work, hemocyanin subunits from chelicerates showed 35–47% difference in complete amino acid sequences (Linzen *et al*., 1985; Voit and Feldmaier-Fuchs, 1990; Beintema *et al*., 1994; Buzy *et al*., 1995) and gave evolutionary trees of hemocyanin molecules but could not gave phylogenetic tree of animals having these subunits (Beintema *et al*., 1994; Burmester and Scheller, 1996). On the other hand, Sugita and Shishikura (1995) determined about 83% of the amino acid sequence of HR6 and reported that there was a difference of about 43% between HR6 and hemocyanin subunits from the American horseshoe crab, *L. polyphemus* and a tarantula spider, *E. californicum* and that immunologically identical subunits HR6 and HT6 showed 2.7% sequence difference. Furthermore, they showed that these subunits HR6 and HT6 could be used to estimate divergence time of *C. rotundicauda* and *T. tridentatus*. Therefore, to study the divergence pattern of animal species with many homologous molecules such as hemocyanins, orthologous molecules or genes should be selected. In the case of the horseshoe crab hemocyanins, immunological analysis is a good method to

find orthologous subunits in many homologues. If complete sequence data of the 6 sets of orthologous hemocyanin subunits of the horseshoe crabs are generated, we can infer how the 6 subunits arose due to gene duplication and obtain better estimates of the divergence times of the subunits and the horseshoe crab species. Furthermore, we may test the molecular clock by comparing 6 phylogenetic trees which are produced using complete amino acid sequences of these 6 sets of orthologous hemocyanin subunits.

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