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A Case of Orthologous Sequences of Hemocyanin Subunits for an Evolutionary Study of Horseshoe Crabs: Amino Acid Sequence Comparison of Immunologically Identical Subunits of *Carcinoscorpius rotundicauda* and *Tachypleus tridentatus*

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ABSTRACT—About 83% of the amino acid sequence of hemocyanin subunit HR6 from the Southeast Asian horseshoe crab, *Carcinoscorpius rotundicauda*, has been determined. There is a difference of about 43% between HR6 and complete sequences of chelicerate hemocyanin subunits from the American horseshoe crab, *Limulus polyphemus*, and a tarantula, *Eurypelma californicum*. However, the immunologically identical subunits HR6 and HT6 from *Tachypleus tridentatus* (Japanese horseshoe crab) show 2.7% sequence difference. Based on the amino acid sequences of HR6 and HT6, the divergence between *C. rotundicauda* and *T. tridentatus* occurred about 9.6 million years ago. In the case of horseshoe crab hemocyanin subunits, it seems that the orthologous homologues in many homologous subunits between species are immunologically detectable.

INTRODUCTION

Hemocyanins are the blue respiratory pigments found in the blood of many arthropods and molluscs. Arthropod and mollusc hemocyanins are different in molecular architecture [4, 30] and phylogenetic origin [3, 32]. Therefore, although they both are oxygen transporting molecules containing copper atoms, they are distinct molecules which have evolved by convergent evolution.

Arthropod native hemocyanins are made up of 6, 12, 24 or 48 subunits and molecular weight of the subunit is about 75,000 daltons [4]. Heterogeneity of the subunits was demonstrated by means of polyacrylamide gel electrophoresis using hemocyanins from Chelicerata [15, 25] and Crustacea [16, 18].

The complete amino acid sequences of 2 subunits were first determined for the hemocyanin from a tarantula, *Eurypelma californicum* [20, 21]. Three other complete sequences of chelicerate hemocyanin subunits, from horseshoe crabs, *Tachypleus tridentatus* and *Limulus polyphemus*, and the tarantula, *Eurypelma californicum* were reported, respectively, by Takagi and Nemoto [29], Nakashima *et al.* [19] and Voit and Feldmaier-Fuchs [31]. When the amino acid sequences were compared, a sequence similarity of about 55% was revealed in any pair of these 5 chelicerate hemocyanin subunits [14, 31]. All chelicerate hemocyanin subunits sequenced to date show a considerable degree of similarity within a species (a tarantula spider), between genera (*Limulus* and *Tachypleus*) and between classes (the spider and the horseshoe crab).

On the other hand, Sugita and Sekiguchi [25] showed that the 5 subunits of *Tachypleus tridentatus* hemocyanin differ entirely from one another in antigenic properties. Subsequently, immunological correspondence between chelicerate hemocyanin subunits was studied by many workers [6, 8–11, 17, 26–28]. Each of the 3 species of Asian horseshoe crabs had 5 or 6 hemocyanin subunits which exhibited no cross reaction in immunodiffusion tests, based on immunological comparisons using 3 antisera corresponding to whole serum hemocyanins [28].

In this paper, the partial amino acid sequence of *Carcinoscorpius rotundicauda* hemocyanin subunit is reported. It is compared with known sequences of immunologically identical and different subunits. The evolutionary implications of the existence of many homologous sequences in species is discussed.

MATERIALS AND METHODS

Preparation of hemocyanin sample

The Southeast Asian horseshoe crabs, *Carcinoscorpius rotundicauda*, were obtained from the vicinity of Bangsaen, Thailand. The Japanese horseshoe crabs, *Tachypleus tridentatus*, were obtained from Imari Bay, Japan. 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.

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Isolation of hemocyanin subunits

Hemocyanin samples were dialyzed against 50 mM Tris-HCl, 10 mM EDTA, pH 8.9 to dissociate hemocyanin molecules, then passed through a column of Sephadex G-50 (superfine), 2.6×34 cm, to eliminate small molecules. A fractionation of hemocyanin subunit mixture was performed by ion-exchange chromatography on a 1.5× 25 cm column of Q-Sepharose Fast Flow, equilibrated with 50 mM Tris-HCl, 10 mM EDTA, pH 8.9. Elution with a linear gradient of 0–0.5 M NaCl in 50 mM Tris-HCl, 10 mM EDTA, pH 8.9 was carried out at a flow rate of 33 ml per hour at room temperature.

Gel electrophoresis

7.5% acrylamide gels at pH 8.9 were prepared according to the method of Davis [2] and electrophoresis of native hemocyanin subunits was carried out in a slab gel electrophoretic apparatus using Davis's tank buffer without dilution. Gels were stained for protein with Coomassie brilliant blue according to the method of Weber and Osborn [33].

Preparation of antiserum and agar plate

Antiserum to pure hemocyanin of *T. tridentatus* was prepared as described previously [25]. Double immunodiffusion tests were carried out in 1% agar plates prepared with 52 mM Tris-glycine buffer pH 8.9 containing 10 mM EDTA and 0.01% thimerosal.

Digestion and peptide separation

Subunit HR6 dissolved in 6 M guanidine hydrochloride, 0.5 M Tris, 2 mM EDTA (pH 8.1) was denatured and completely reduced with dithiothreitol (DTT, 50 moles/mole of disulfide in the subunit) under nitrogen gas [7]. The number of disulfide bonds in subunit HR6 was estimated from known sequence data of hemocyanin subunits. After cooling to room temperature, iodoacetic acid (2 moles/mole of DTT) was added in a solution of reduced subunit HR6. The reaction was allowed to continue for 20 min in the dark. The mixture was dialyzed against 0.1 M NH_4HCO_3 in the dark at 4°C. After repeated dialysis, the reduced and carboxymethylated peptides were freeze-dried.

Freeze-dried HR6 peptides were dissolved to a concentration of 1% (w/v) in 70% formic acid. A 4-fold (w/w) amount of CNBr was added and the reaction proceeded for 20–24 hours at room temperature [34]. The reaction was terminated by the addition of 9 volumes of distilled water and freeze-dried after dialyzing against 0.1 M NH_4HCO_3. The dried samples were dissolved in 70% formic acid and then subjected to gel filtration on either Sephadex G-50 or Superose 12 column equilibrated and developed with the same solvent of 10 or 70%, respectively.

CNBr fragments were digested with the V8 protease from *Staphylococcus aureus* in 50 mM ammonium acetate, pH 4.0 at a substrate/enzyme ratio of 50/1 (w/w) for 18 hours at 37°C [34]. Digestion with trypsin (Worthington, TPCK grade) was performed as described [13], except that the freeze-dried samples were dissolved in 0.1 M NH_4HCO_3.

Separation of digested peptides was carried out by reverse phase FPLC using a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. Re-chromatography of selected peptide fractions was performed under the gradient conditions as described by Shishikura *et al.* [23].

Amino acid sequence determination

Sequence determination was performed using an Applied Biosystems model 477A gas phase sequencer equipped with an online model 120A PTH-analyzer.

Computer analysis of aligned sequences

Sequences aligned by hand were analyzed using computer programs Protdist under the Dayhoff PAM matrix option and Neighbor

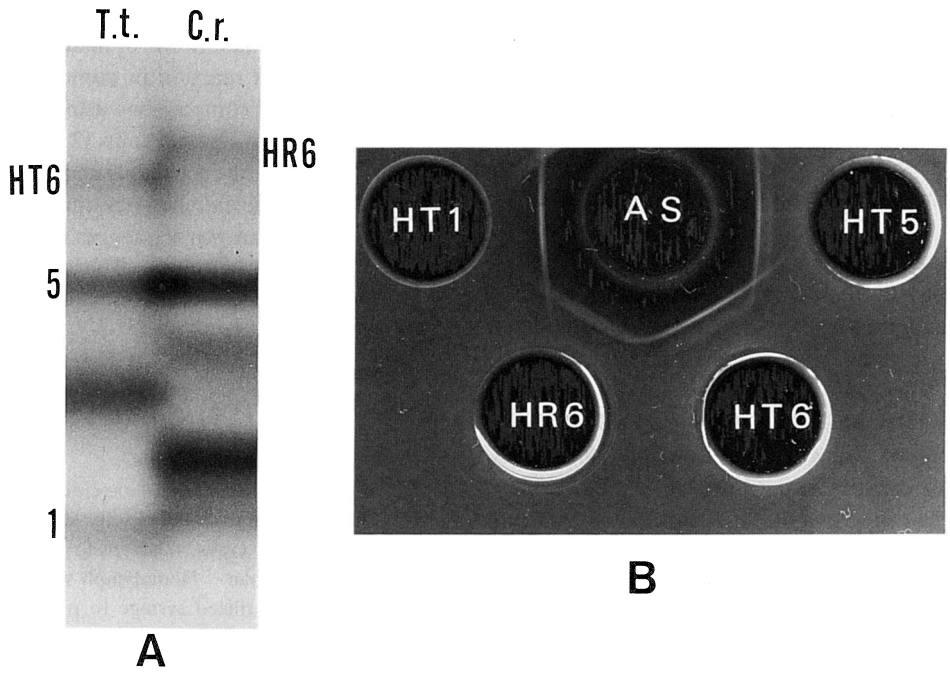

FIG. 1. Electrophoretic patterns of native hemocyanin subunits (A) and antigenicity analysis in double-diffusion plate (B). The slowest subunits of *Carcinoscorpius rotundicauda* (C.r.) and *Tachypleus tridentatus* (T.t.) hemocyanins were designated as HR6 and HT6, respectively. AS, antiserum against *T. tridentatus* hemocyanin.

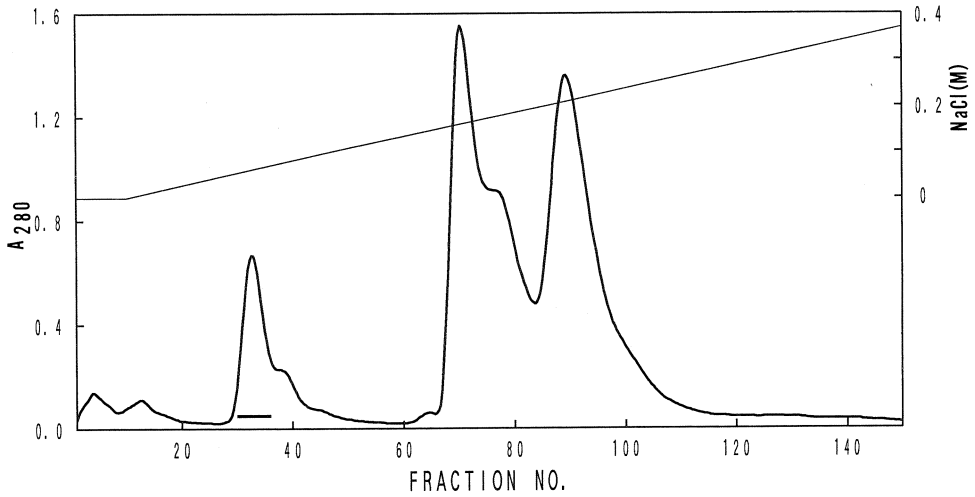

FIG. 2. Fractionation of *C. rotundicauda* hemocyanin subunits on a 1.5×25 cm column of Q-Sepharose Fast Flow with a linear gradient of 0–0.5 M NaCl in 50 mM Tris-HCl, 10 mM EDTA, pH 8.9. Elution rate was 33 ml/hr. Fractions 30–36 (indicated by bar) were freeze-dried for sequence analysis.

under the UPGMA and Neighbor-Joining options of the Phylip package [5].

RESULTS

Carcinoscorpius rotundicauda and *Tachypleus tridentatus* have 6 different hemocyanin subunits [28]. The slowest subunits in polyacrylamide electrophoresis are designated HR6 (*C. rotundicauda*) and HT6 (*T. tridentatus*), as shown in Figure 1A. Reacting with anti-*T. tridentatus* hemocyanin antiserum, subunit HR6 forms a precipitin line which is immunologically identical with the precipitin line of subunit HT6 but not with that of subunit HT1 (Fig. 1B). Figure 1B also shows that HT5 and HT6 are not immunologically cross-reactive. The minor precipitin lines between wells of HT5 or HT6 and antiserum are thought to be produced by aggregated molecules of subunits.

In order to compare the amino acid sequence of HR6 with the known sequence of HT6 (which was referred as Tachy α by Linzen *et al.* [14]), HR6 was separated from subunit mixture of *C. rotundicauda* hemocyanin on Q-Sepharose Fast Flow column. The fractionation pattern is shown in Figure 2 and the electrophoresis patterns of proteins in 5 fractions are presented in Figure 3. Because fraction 33 contained pure subunit HR6, fractions 30–36 were freeze-dried and the dried sample and its digested peptides were used for sequence analysis.

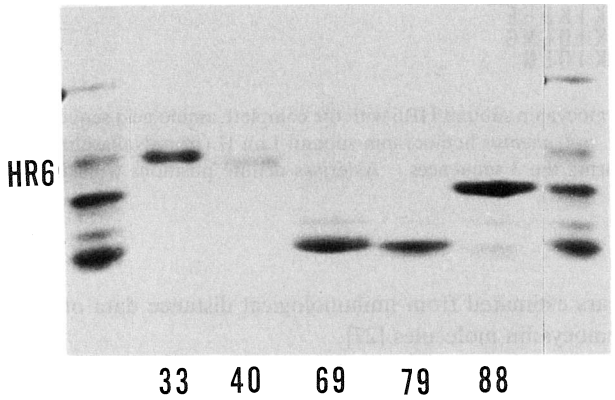

FIG. 3. Electrophoretic patterns of the 5 fractions (numbered lanes) on Q-Sepharose FF (see Fig. 2) and whole native subunits (outside lanes).

Sequencing of subunit HR6 resulted in the determination of 515 amino acid residues (Fig. 4), which is about 83% of the 622 residues in the immunologically identical subunit HT6 which has been completely sequenced. For determining the position of digested peptides, sequence similarity to HT6 was utilized. The alignment of the peptides of HR6 is shown with the HT6 sequence in Figure 4.

DISCUSSION

Table 1 lists the percent differences and protein distances between complete amino acid sequences of chelicerate and spiny lobster hemocyanin subunits and between partial sequence of HR6 and complete ones. The difference scores (40–45%) between HR6 and chelicerate subunits except for HT6 are equal to those (40–47%) between complete subunits of chelicerates. Therefore, the partial sequence of HR6 is a reliable representation of the whole subunit. Thus, the difference (2.7%) between HR6 and HT6 is used for calculating the divergence time of these 2 subunits.

UPGMA (unweighted pairgroup method with arithmetic means) analysis [5] of distance matrices shown in the lower left half of Table 1 produced a rooted tree with the assumption of an evolutionary clock (Fig. 5). Furthermore, neighbor-joining analysis [5] produced the same tree topology as the UPGMA analysis. Evolutionary rates of hemocyanin subunits were calculated by assuming that the divergence between the Crustacea and the Chelicerata occurred about

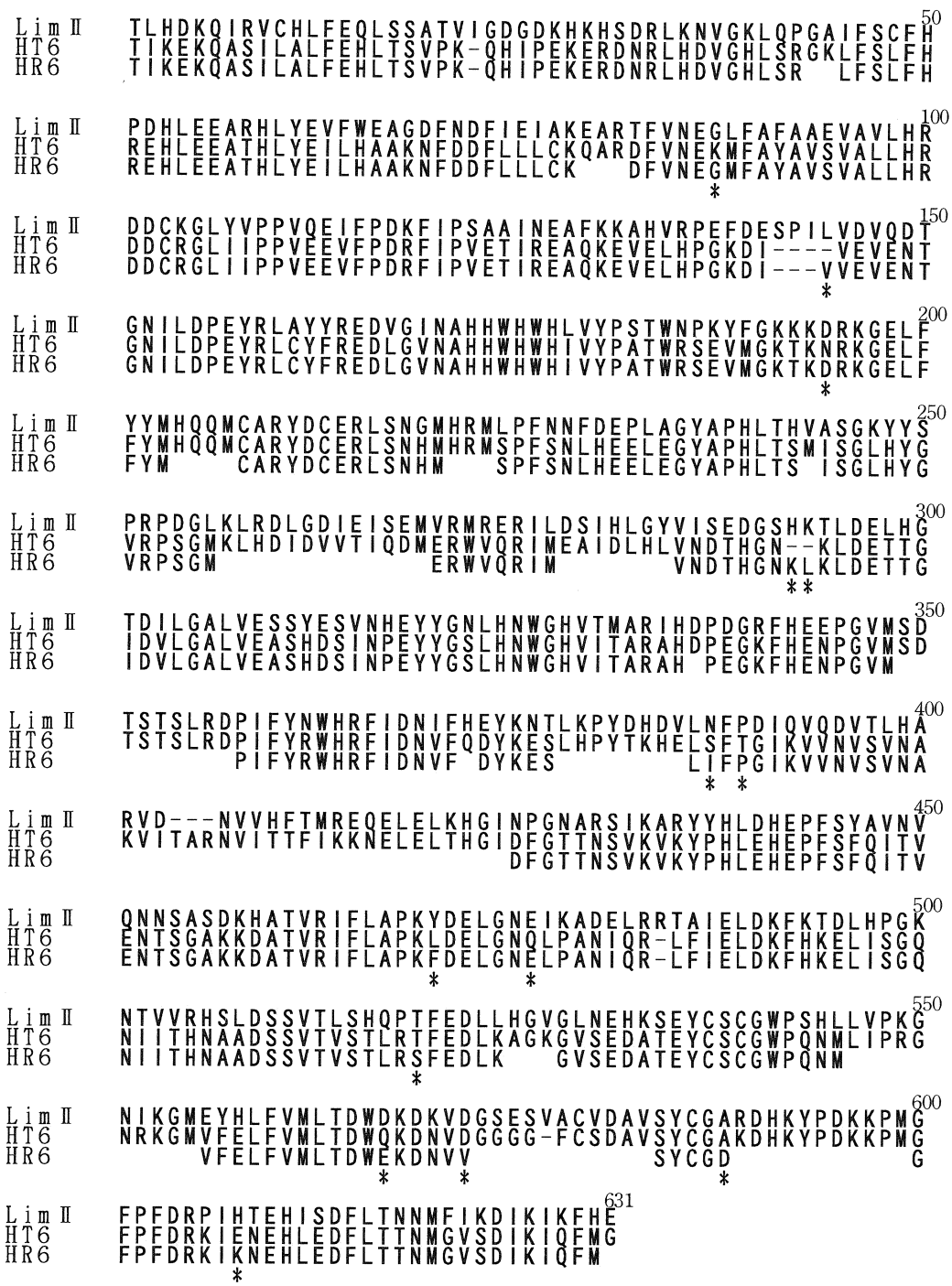

FIG. 4. Comparison of aligned sequence of peptides from *C. rotundicauda* hemocyanin subunit HR6 with the complete amino acid sequences of *T. tridentatus* hemocyanin subunits HT6 (from Linzen *et al.* [14]) and *L. polyphemus* hemocyanin subunit Lim II (from Nakashima *et al.* [19]). Dashes represent gaps introduced under the necessity of comparing the 3 sequences. Asterisks denote positions with different residues or inserted gaps between the 2 sequences of HR6 and HT6.

600 million years ago [14]. This gave an estimated divergence time of HR6 and HT6 of 9.6 million years ago; all other nodes were between 200 and 300 million years ago. This divergence time of HR6 and HT6 is shorter than the 36.3 million years calculated from the amino acid sequences of coagulogens [24] and somewhat longer than the 8.2 million years estimated from immunological distance data of whole hemocyanin molecules [27].

From the electrophoretic patterns of hemocyanin subunits from hybrid horseshoe crabs, it is evident that the subunits are the products of genes at distinct loci [22], and the gene products of the subunits did not show any polymorphism

TABLE 1. Pairwise distances between amino acid sequences of chelicerate and spiny lobster hemocyanin subunits

	HR6	HT6	Lim II	Eury a	Eury d	Eury e	Pint a
HR6	—	2.7	43	45	44	40	67
HT6	0.023	—	43	46	44*	40*	67*
Lim II	0.647	0.641	—	40**	47	45**	67*
Eury a	0.687	0.682	0.542	—	47	46**	68*
Eury d	0.692	0.688	0.780	0.721	—	44*	68*
Eury e	0.597	0.589	0.686	0.710	0.703	—	67*
Pint a	1.416	1.408	1.469	1.510	1.474	1.509	—

Values in the upper right half of the table are the percent differences between amino acid sequences. Percent scores are calculated from complete sequence data except HR6. Values in the lower left half of the table are distances computed from amino acid sequences corresponding to 515 sites of HR6 by the program Protdist under the Dayhoff PAM matrix option [5].
*Scores from Beintema *et al.* [1]. **Scores from Voit and Feldmaier-Fuchs [31]. HR6, HT6 and Lim II are subunits of horseshoe crab hemocyanins from *C. rotundicauda, T. tridentatus* and *L. polyphemus*, respectively. Eury a, d and e are subunits a, d and e of a tarantula, *Eurypelma californicum*, hemocyanin, respectively. Pint a is a subunit of a spiny lobster, *Panulirus interruptus*, hemocyanin.

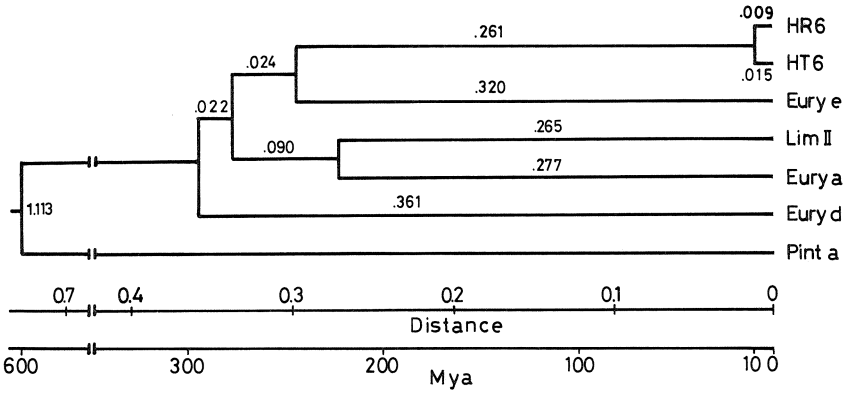

FIG. 5. A rooted tree produced from distance matrices shown in the lower left half of Table 1 by the program Neighbor under the UPGMA option of the Phylip package [5]. Branch lengths are proportional to protein distances at the bottom of the tree. Also shown on the tree are individual branch lengths from the neighbor-joining analysis [5] that produced the same branching structure as the UPGMA analysis. Mya, million years ago. Hemocyanin subunits abbreviated as HR6: *C. rotundicauda*; HT6: *T. tridentatus*; Lim II: *L. polyphemus*; Eury a, d, e: *E. californicum*; Pint a: *P. interruptus*.

in acrylamide gel electrophoresis. Therefore, the similarity between the 2 subunits from *C. rotundicauda* and *T. tridentatus* indicates a recent divergence from an ancestor. Minute antigen analysis of *T. tridentatus* hemocyanin revealed that it consisted of 6 immunologically different subunits, each one of which was immunologically identical with the comparable subunits of *C. rotundicauda* hemocyanin [28]. Consequently, it is concluded that these 2 horseshoe crabs have a set of 6 immunologically different subunits which is descended from their recent ancestor. Therefore, it is suggested that these 2 species share 6 pairs of orthologous hemocyanin subunits including a pair of HR6 and HT6, and each species has 6 subunits produced from multiplicated genes.

Thus, it is expected that a study of the molecular evolution of chelicerate hemocyanins will give evidence when gene duplications leading to separate genes (or subunits) occurred in each lineage. Eight immunologically different subunits of the scorpion, *Androctonus australis* [8] and 7 immunologically different subunits of the tarantula, *Eurypelma californicum* [9] are good materials for that purpose.

However, in order to investigate the divergence pattern of species with homologous genes, appropriate genes or molecules to analyze should be selected by a method by which the orthologous relationship between homologous genes can be detected. In the case of the horseshoe crab hemocyanin subunits, it has been suggested that the orthologous subunits between *C. rotundicauda* and *T. tridentatus* are immunologically identical. Thus, although immunological analysis is regarded as a good method to find orthologous subunits in many homologues, the reason why the orthologous hemocyanin subunits carry an identical antigenicity is unknown.

When the sequence of HR6 is compared with the N-terminal sequences of 8 different subunits from *L. polyphemus* hemocyanin [12], the first 24 amino acid residues of subunit Lp I are the same as those of HR6 except for the 4th and 19th positions from the N-terminal. The N-terminal sequence difference between HR6 and Lp I is 8.3%, while

those between HR6 and 7 other *L. polyphemus* subunits are 30% (Lp IIA) to 67% (Lp II). There is a possibility that Lp I subunit is equal to the *Limulus* hemocyanin subunit which is immunologically similar to HT6 [26]. Moreover, the Southeast Asian horseshoe crab, *T. gigas*, has a hemocyanin subunit (HG6) that is immunologically identical with HR6 and HT6 [28]. Therefore, the evolutionary divergence pattern of the extant horseshoe crabs will be studied using the sequence data from the subunits HR6, HT6, HG6 and Lp I. Moreover, we may produce 6 phylogenetic trees using 6 sets of immunologically different subunits.

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