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Hemocyanin subunits composition as a molecular marker for identification of Intra-specific variations of *Gammarus*

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Abstract

Crustacean hemocyanin represents a unique case of molecular heterogeneity among oxygen-carrying proteins. Two populations of *Gammarus lacustris* and six populations of *Gammarus komareki* were identified and isolated from aquatic habitats of Ardabil (northwest of Iran). The populations were nominated based on the name of the isolated region. The Principal component analysis of morphological absolute attributes showed that the populations were relatively dissociated. The Meshkin population of *G. komareki* was completely different from the other populations. The analysis of relative attributes did not show any dissociation. The Hemolymph profile analysis using native PAGE revealed 5 to 7 hemocyanin subunits for four populations of *G. komareki* and 8 subunits for two populations of *G. lacustris.* The Detection of copper ions using bathocuproin sulfonate on the native PAGE surface confirmed the hemocyanin bands. Using SDS PAGE, 1-3 and 1 protein bands were separated for hemolymph samples from *G. lacustris* and *G. komareki*, respectively. Molecular weights of the bands were 75 - 80 kDa. Comparison of our results with the previous published data confirms the species specificity of hemocyanin subunit composition. These results imply that the ecological variation may affect the morphological traits, but the hemocyanin profiles are useful as a molecular marker for the taxonomic studies and identifying very closely-related species of *Gammarus*.

Keywords**:** *Gammarus lacustris*, *Gammarus komareki*, hemocyanins, Ardabil

Introduction

Arthropods hemocyanins are copper-containing and multimeric proteins which are dispersed in the hemolymph and transport oxygen in the hemolymph of many chelicerate, crustacean and also some insect species. The arthropod hemocyanins are the member of a large protein superfamily that also includes the arthropod phenoloxidases and certain crustacean and insect storage proteins (Burmester, 2002).

Heterogeneous subunits compositions of hemocyanin in some arthropods species, specially, between-species or between-individuals comparisons within a single population were previously studied (Markl and Deker, 1992; Markl, 1996; Mangum and Greaves, 1996; Mangum and McKenney, 1996). The study of between population variation based on hemocyanin subunit composition information is restricted to a few amphipod and decapods species (Spicer and Hodgson, 2003). Markl et al. (1979) have shown that quaternary structures and subunit compositions are highly variable among the hemocyanin of malacostracan Crustacea (Markl et al. 1979).

Because of the species-specific property, the *Gammaridea* hemocyanin subunits composition could be a potentially useful discriminatory tool for taxonomists. In this research morphological traits and hemocyanin subunits composition of *Gammarus* populations, as a marker were used to identify different species of this genus.

Materials and Methods

Animal Materials

Twelve aquatic regions in Ardabil, northwest of Iran, were selected for sampling. Six populations of *Gammarus* were found, sampled and transferred to the Lab. The samples were nominated in terms of their source location: Ne (neor lake), Sa (sarein lagoon), Kh (Khalkhal spring), Me (Meshkin spring), Ba (Baleklo river) and Hi (Hir spring).

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Morphological studies

Identification of the samples was carried out using morphological traits based on definition resources (Stock, 1974; Karaman and Pinkster, 1977). Six adult individuals from each sample were examined for species identification. 44 absolute attributes and 16 relative attributes were measured for evaluation of intraspecific variations. The data were analyzed using the Principal Component Analysis (PCA).

Isolation of Hemocyanin

Individuals of the populations were brought to the laboratory, about 5h of capture, and their hemolymph were sampled. The samples bled by piercing the ventral abdominal sinus with a Hamilton syringes. Haemolymph extraction and preparation was carried out exactly as described by Hodgson and Spicer (2001). The prepared samples were frozen immediately at -20ºC. Protein concentrations of the properly diluted samples were appraised by the Bradford method (1979).

Hemolymph preparation

The samples of hemolymph were prepared for electrophoretic separation as described by Mangum (1993). Briefly, frozen samples were thawed while incubated on ice and then, about 15 µg protein were diluted (1:100) with dissociating buffer (10 mM EDTA and 50 mM Tris-(hydroxymethyl)-aminiomethane adjusted to pH 9.1). The diluted samples were kept at 4ºC for overnight. Glycerol was added to each sample (up to 20 percent) to prevent diluted samples from diffusing out of the wells of the gel.

Denaturing and nondenaturing gel electrophoresis

SDS-PAGE electrophoresis of the prepared hemolymph was carried out at constant current according to Laemmli (1970) with some modifications. The gels (150 mm × 0.75 mm) were composed of a stacking gel (4%, pH 8.3), over a resolving gel (8.5%, pH 9.5). After loading the samples and running the electrophoresis, the gels were stained immediately for 2 h with Coomassie Blue R250 solution of 0.025 % and subsequently de-stained using standard methods. An SDS-PAGE molecular weight marker (26-116 kDa, Fermentas, SM0431) was used for the detection of molecular weights.

The electrophoresis of the dissociated samples was carried out in its native conformation under the high pH discontinuous system described by Hames and Rickwood (1995). The prepared Native Page (dimensions: 150 mm × .075 mm) was composed of a stacking gel (4%, pH 8.3) over a resolving gel (8.5%, pH 9.5). Eighteen microliter of the diluted haemolymph was loaded into each well. Electrophoresis was carried out at a constant temperature (4ºC) and current (60 mA per tow gels) for 120 min. Gels were stained and de-stained exactly as described above for SDS-PAGE.

Detection of copper ions on polyacrylamide gels

The banding patterns of haemolymph of the species were characterized using the Native Page but for confirmation of the hemocyanin bands, presence of Cu in each band was examined by the bathocuproine sulfonate method (1978). Briefly, a replicate of nondenaturing gel electrophoresis were also run, and because of low sensitivity of the bathocuproine sulfonate, amount of the diluted hemolymph loaded to the gel was increased up to five times.

The gels were first soaked for 1 min at 16 mM ascorbate in glacial acetic acid to denature proteins and reduce copper ions and then were immersed in a 0.28 mM aqueous bathocuproin sulfonate solution for 8 hours. The staining pattern on the gels was studied under a UV light cabinet and the results were compared with the banding pattern of non denaturing gel electrophoresis for verification of hemocyanin banding pattern. This experiment was carried out triplicate to gain confidence of the results.

Data analysis

For evaluation of the results, they were compared with the previous reported results. Hodgson and Spicer (2001) have previously reported the hemocyanin bands number, positions and the percent contribution for 6 species of *Gammarus*. A binary matrix was produced for the 6 reported species and these 6 populations. Presence and absence of a band marked with 1 and zero, respectively. The data was analyzed by hierarchical cluster using between linkage methods and Pearson correlation intervals.

Results

The six selected samples of *Gammarus* populations were isolated from aquatic habitats of Ardabil and nominated in terms of their sampling locations. The four samples of Hi, Ba, Me and Kh were recognized as *G. komareki* and the two samples of Ne and Se were recognized as *G. lacustris.* The principal component analysis of morphological absolute and relative attributes

imply that the Me population was completely different from the other populations of *G. komareki* but the relative attributes did not depict any significance within species variations (figure 1). However, two populations of *G. lacustris* were relatively dissociated by the same analysis.

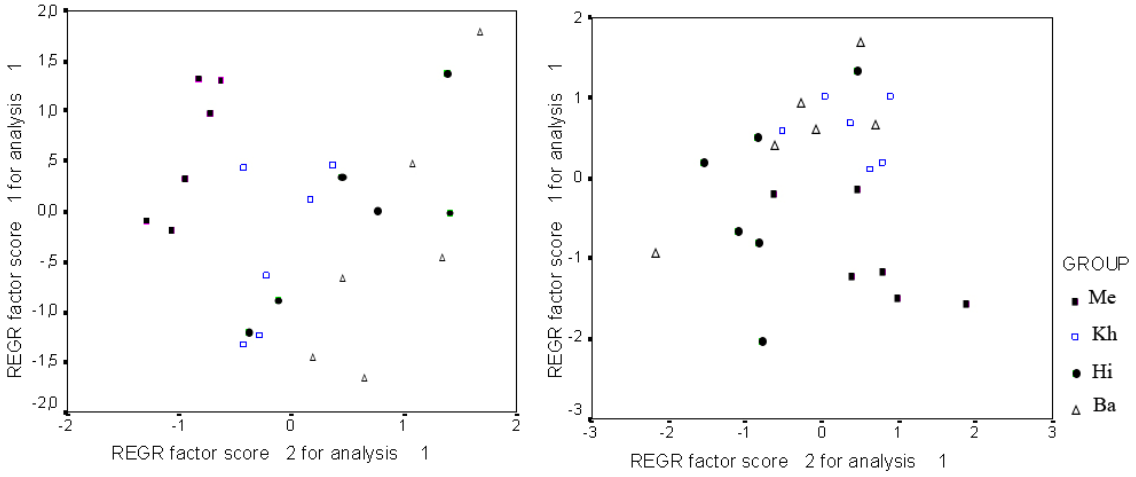

Figure 1. Principal component analysis of morphological absolute (left) and relative attributes (right) of *G. komareki*

The study of SDS-PAGE electrophoretic pattern of their hemolymphs showed proteins bands with molecular weight range of 75 – 80 kDa. There were 2 - 3 protein bands in four populations of *G. komareki* and one protein band in two populations of *G. lacostris*. Repetitions of this experiment authenticated these results (figure 2).

Analysis of the results of non-denaturing polyacrylamide gel electrophoresis showed significant subunit composition variations between the two species. Irrespective of the percentage contribution of each band, there were different relative mobility and bands number for each species and these results showed between species difference (figure 2). The terminal two bands revealed as one intense band, but numerous repeated experiments with different concentrations of polyacrylamide gels revealed these two bands.

Copper ions detection on Non-denaturing polyacrylamide gels confirmed the above results. These results depicted that there were 8 and 5-7 Cu-positive bands in *G. komareki* and *G. lacostris*, respectively. Comparison of Comasee blue and bathocuproine sulfonate stained gels revealed that all above mentioned bands are Cu-positive.

The presence of copper was not detected on our SDS-PAGE gels. However, there was comparatively same banding pattern for each species. Comparison of Non-denaturing Hemocyanin subunits pattern of *G. komareki* populations depicted 6, 7, 6 and 5 protein bands for Hi, Ba, Me and Kh population, respectively. However, the band number 3 was not observed in the Kh population.

These finding imply that eight hemocyanin subunits of *G. lacostris* have the same molecular weight.

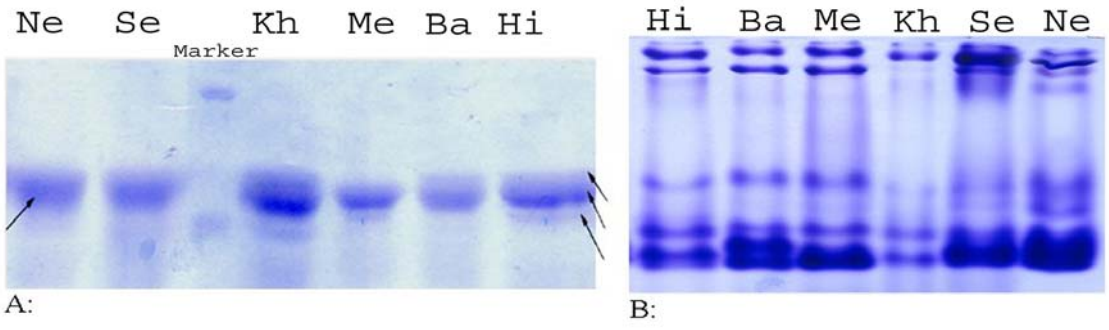

Figure 2. (A) An example of SDS-PAGE gel electrophoretic patterns of hemolymph which was stained using Coomassie blue. (Two bands of molecular weight marker were shown: 116 kDa and 66.2 kDa). (B) An example of non-denaturing polyacrylamide gel electrophoretic patterns obtained after alkaline dissociation of hemolymph proteins. The

samples were nominated in terms of their source location: Ne (neor lake), Sa (sarein lagoon), Kh (Khalkhal spring), Me (Meshkin spring), Ba (Baleklo river) and Hi (Hir spring).

Comparison of the results with previous published data were performed using hierarchical cluster analysis and between linkage methods. The obtained dendrogram divided the 12 population in 8 distinct groups (figure 3). This data showed that Sa and Ne populations were located at the same group and other four population made a second group, but the comparison with the previous reported data showed that *G. pulex* and *G. lacostris* have the same subunit composition and Hi and Me populations (*G. komareki*) were different from the two other populations.

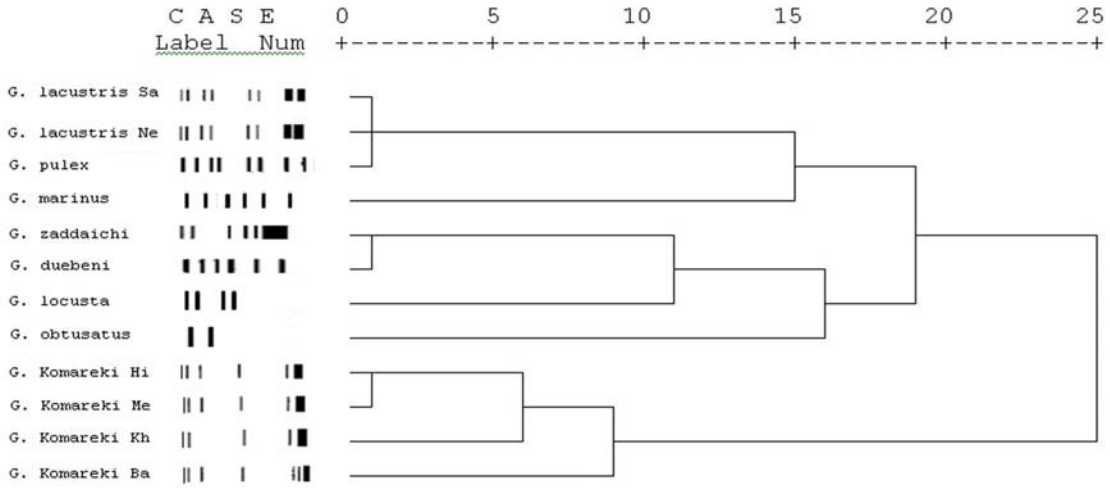

Figure 3. The dendrogram of hemocyanin subunit composition of this study and previous reported data obtained from hierarchical cluster analysis.

Discussion

One of the main objectives of a systematic science is identification of species and its taxons. Numerous reports showed that morphological traits can be affected by ecological factors and sometimes, are not reliable (Miller and Buikema, 1977). Thus, molecular and biochemical factors can be used as important factors in species identification. Spicer and Hodgson (2003) described that the Hemocyanin molecule may be a useful tool for investigating speciation and speciation events in crustacean arthropods and it is better than morphological traits. However, some previous studies depicted that hemocyanin subunits, composition heterogeneity is an essential condition for ecological adaptation and hemocyanin-oxygen binding properties (Mason et al., 1983; Mangum and Rainer, 1988; DeFur et al., 1990). Our results indicate that the SDS PAGE electrophoresis is unable to isolate the hemocyanin subunits, but they were isolated efficiently using nondenaturing gel electrophoresis under the above mentioned condition. Then, it was clear that the hemocyanin subunits had nearly the same molecular weight but with different net charge. Burmester and Scheller (1996) showed that the first band on the native PAGE was not Cu-positive and they supposed it as a Cryptocyanin, but the reiteration of these experiments did not reveal any obvious Cu-negative band.

Terwilliger et al. (1999) suggested that the variation in the previous may had represented intermediate association phenomena rather than actual subunit distinction. However, dissociation was likely completed under conditions of our investigation. Repetitions of the dissociation experiments at a time course led to repeatable results after 24 hours. Comparison of these data and the reports of Spicer and Hodgson (Spicer and Hodgson, 2003) showed that the hemocyanin subunit composition is useful but not sufficient for discrimination of the species. The results clearly showed that the morphological attributes were affected by ecological conditions but the hemocyanin subunit composition was not significantly variable between populations.

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