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VARIATION OF CARBONIC ANHYDRASE (CA) ISOZYME IN MUSCLES OF FIVE SPECIES OF PORTUNID CRABS FOUND IN COASTAL WATERS OF PAKISTAN

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ABSTRACT

Portunid crab species were collected from different localities throughout their distribution range along the Sindh and Balochistan coast of Pakistan. Genetic and biochemical variations of Carbonic anhydrase were determined in five economically important crab species (*Portunus pelagicus, P. sanguinolentus, Charybdis feriatus, Scylla serrata* and *S. olivacea*) of family Portunidae: by the use of SDS and native PAGE, vertical slab gel electrophoresis. Crabs muscle samples were homogenized, centrifuged and run on a 10% vertical polyacrylamide gel in the discontinuous buffer system and stained for CA isozyme variability. The obtained band patterns revealed the biochemical and genetic variability of Carbonic anhydrase in five species of Portunids. They displayed polymorphic loci (50%), (25%), (16.67%) and (20%), in *Portunus pelagicus, P. sanguinolentus, S. serrata* and *S. olivacea* and the expected heterozygosity was 0.015, 0.016, 0.025, 0.022 in *Portunus pelagicus, P. sanguinolentus, S.serrata* and *S.olivacea*, respectively.

Key-words: Portunid, native PAGE, electrophoresis, Carbonic anhydrase (CA), Pakistan.

INTRODUCTION

The crabs belong to family Portunidaeand constitute one of the dominant aqua terrestrial group that play an important role in the near shore and off shore coastal areas food web and in swamps as scavengers (Takween and Qureshi, 2001). The swimming crabs of the family Portunidae Rafinesque, 1815 are common in the coastal habitat of Pakistani waters and are a key resource in local fisheries (Rasheed and Mustaquim, 2010). The species mainly belongs to genus *Scylla* and *Portunus* commercially important species that found in Pakistani waters and constitute about 63% of the total catch of the fishery (Khan, 1975; Mustaquim and Rabbani, 1976; Tirmizi and Kazmi, 1996; Takween and Quresh, 2001). Despite the highest economic and commercial importance of Portunids, there is no previous study available on the genetic diversity, protein and biochemical variations of these species.

The biochemical characters like isozyme variations (molecular markers) are species specific,but express interspecific or intraspecific variations at different ecological levels (Mustaquim,1988; Carlile and Watkinson, 1994). High resolution of polyacrylamide gel electrophoresis constitutes a valuable technique for separation and characterization of these molecular markers and also can be used to determine some of their physico-chemical properties (Mateus *et al*., 2009; Sujatha *et al*., 2011).These molecular markers are useful for estimating genetic variability and have become a significant tool for systematic studies. Various authors have discussed their importance in phylogenetic studies (Avise, 1975; Mustaquim, 1988; Sujhata *et al*., 2011).

The carbonic anhydrase (CA; E.C. 4.2.1.1) is one of the ubiquitous enzyme found in living organisms and form a family of enzymes (metalloprotein) that catalyze the rapid interconversion of carbon dioxide and water to bicarbonate and protons (Vitale *et al*., 1999). This enzyme also performs the physiological function to maintain acid-base balance in blood and transport carbon dioxide out from the tissues (Bottcher *et al*., 1991). As compared to morphological characters isozyme patterns showed higher varieties between species and individual (Bhuvanendra *et al*., 2010). The present study is an initial step to determine and evaluate the genetic diversity and protein variations in order to reveal the genetic structure through biochemical variations among the five species of Portunid crabs and will provide basic data for further advanced studies.

MATERIALS AND METHODS

Specimen collection

Five Portunidspecies i.e. *Portunuspelagicus, P. sanguinolentus, Charybdis feriata, Scylla serrata* and *S. olivacea* were selected for the general protein and carbonic anhydraseisozyme variability. Crabs were collected either directly from the field, fresh catch and purchased alive crabs (freshly hunted) from the local fishermen from

Sandspit, Korangi creek and Sonmiani areas along the coast of Pakistan.Alive crabs were killed by freezing,then transferred to the laboratory and kept in the freezer (-20ºC) until the process of tissue extraction. The muscle tissues were drawn out from the first walking leg of each crab. Approximately 250-300 mgs of tissues were removed from each specimen and homogenize in extraction (Tris-Citrate) buffer through hand Homogenizer. The homogenate was centrifuged at 13500 RPM for 15 min to remove solid tissue debris. The supernatant (enzyme extract) was filtered. This extract was either immediately used for electrophoresis or stored at -20°C. After muscles sample extraction the morphological identification of each crab was carried out according to available taxonomic keys (Tirmizi and Kazmi, 1996; Keenan *et al*.,1998; Lai *et al*., 2010).

Laboratory methods

SDS PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the general protein as described by (Laemmli, 1970) under reducing conditions in the discontinuous electrode buffer system at room temperature and the gelswere stained with Coomassie Brilliant Blue R-250.

Native polyacrylamide gel electrophoresis for Carbonic Anhydrase (CAE.C.4.2.1.1)

For the isozyme variations of Carbonic anhydrase, electrophoresis was performed in vertical native polyacrylamide gels by following (Laemmli, 1970) in the discontinuous buffer system. Individual samples of Portunid species were stacked in 5% slab gels, built up in 3.0 M Tris-HCl buffer (pH 8.8), followed by separation in resolving gels at 10% concentration, and prepared in Tris-HCl. Forisozyme activityof Carbonic anhydrase the gels were stained using Bromothymol blue as substrate according to (Hebert and Beaton,1993; Tiwari *et al*.,2006). Migrations for Carbonic anhydrase were measured in each gel and relative mobility (Rm) was obtained dividing protein migration by dye front (Bromophenol blue) migration.

Numerical analysis

The bands produced in each sample for each species were counted individually and the relative mobility (Rm) was estimated for all five studied species by following (Petrokas, 2008). Alleles at each locus were designated according to alphabetical order, starting with the allele encoding the most anodally migration isozyme.

Allele frequencies or genotype of each Individual's species was determined and genetic variability was calculated as proportion of polymorphic loci or polymorphism, average heterozygosity and hardy Weinbergequilibrium. Observed and expected proportions of heterozygous genotypes at each locus were averaged over loci to obtain means (Nei, 1978).

RESULTS

SDS-PAGE electrophoresis

SDS-PAGE resolved a total of 46 bands in five species of Portunid crab species and the relative mobility's of these bands varied from 0.17 to 0.95. *P. sanguinolentus* exhibited a maximum number ofbands as twelve polypeptide bands and relative mobility (Rm) of these bands ranged in between 0.17 to 0.91 followed by *S. serrata, S. olivacea, P. pelagicus,* and *C. Feriata* (Fig.1, Table 1).

The comparable patterns of isozyme (*CA*) were observed in five species of Portunids crabs. Carbonic anhydrase (*CA*), exhibited a monomorphic and polymorphic activity and was expressed collectively in 31 different scorable alleles at six loci (*CA-*1, CA-*2, CA-*3, CA-*4, CA-*5, CA-*6*), in all the five studied species. Only two loci (*CA-3* and CA-4**) were designated as a monomorphic. A total of 6 electrophoretic phenotypes was detected among five examined isolates. The mean number of alleles per locus was found to be highest in *S. serrata*.(Fig. 1, Table 1).

Carbonic anhydrase (*CA*) was shown variations in relative mobility among five species of Portunids crabs and ranged in between 0.18 - 0.76. The interspecific variations were observed for the relative mobility as for *P. pelagicus,* (0.19 - 0.44), *P. sanguinolentus* (0.18 - 0.75), *S. serrata* (0.23 - 0.69), *S. olivacea* (0.25 - 0.76) and *C. feriata* (0.21 - 0.58) were observed (Fig. 2, Table 1).

The maximum number of loci (six) was detected in three crab species (*P. pelagicus*, *S. serrata* and *S. olivacea*) studied in the present work (Table 2). The only one locus (*CA*-5*) was found to be polymorphic in all the three species, whereas the *CA*-1*, *CA*-3*, *CA*-4*, and *CA*-6* loci exhibited one allele per locus and were appeared as monomorphic. The locus *CA*-2* was found to be polymorphic in *S. serrata* whereas in *S. olivacea* and *P. pelagicus CA-2** appeared as monomorphic.The five different activity zones or loci were detected in *P. sanguinolentus*, only one locus *CA-4** appeared as polymorphic while the *CA*-2*, *CA*-3*, *CA*-5* and *CA*-6* exhibited single allele

andappeared as monomorphic (Table 2). In *C. feriata CA* appeared as a monomeric and coded by three loci (*CA-2**, *CA-4** and *CA*-6*).

Table 1. Relative mobility in SDS PAGE muscle protein and Carbonic anhydrase (CA) profile of five species of Portunid crabs found in coastal waters of Pakistan.

Population	General Protein (Rm)	Total bands	Carbonic anhydrase (CA) (Rm)	Total bands
Portunus pelagicus	0.17, 0.41, 0.47, 0.50, 0.67, 0.73 and 0.76	7	0.19, 0.20, 0.28, 0.33, 0.43 and 0.44	6
P. sanguinolentus	0.17, 0.27, 0.41, 0.46, 0.49, 0.51, 0.67, 0.77, 0.81, 0.84, 0.89 and 0.91	12	0.18,.42, 0.60, 0.71 and 0.75	5
Scylla serrata	0.20, 0.36, 0.58, 0.60, 0.67, 0.80, 0.84, 0.90, 0.91, and 0.95	10	0.23, 0.25, 0.44, 0.55, 0.65, 0.69	6
S. olivacea	0.20, 0.36, 0.58, 0.60, 0.67, 0.80, 0.84, 0.90, 0.91, and 0.95	10	0.25, 0.28, 0.50, 0.61, 0.68, 0.76	6
Charybdis feriatus	0.17, 0.41, 0.47, 0.50, 0.67, 0.73 and 0.76	7	0.21, 0.42 and 0.58	3

Table 2. Carbonic anhydrase (CA) allele frequencies, expressed as percentages, at six genetic loci in five species of Portunid crabs.

Locus	Allele	Species examined and allele frequency				
		P. pelagicus	*P. sanguinolentus*	*S.serrata*	*S.olivacea*	*C.feriatus*
CA-*1	a					
	b	100		1.000	1.000	
CA-*2	a	1.00	1.000	0.643	1.000	1.000
	b			0.356		
CA-*3	a		1.00		1.00	
	b	1.000		1.000		
CA-*4	a	1.000	0.973			
	b		0.026	1.00	1.00	1.000
CA-*5	a	0.347		0.1785	0.284	
	b	0.652	1.00	0.821	0.821	
CA-*6	a	1.00	1.000			
	b			1.00	1.00	1.000

Table 3. Genetic variability measures at each allozyme locus of the five species of family Portunidae collected from coastal waters of Pakistan.

	P. pelagicus	*P. sanguinolentus*	*S. serrata*	*S. olivacea*	*C. feriatus*
Total loci	6	5	6	6	3
Polymorphic loci	1	1	2	1	0
Allele expressed by loci	7	6	8	7	3
Percentage of loci polymorphic	16	20	33	16	0
Average number of Alleles per locus	1.16	1.2	1.33	0.166	0
Average Heterozygosity	0.01586	0.0160	0.02252	0.0221	0
X	0.000151	0.000263	0.00112	0.000785	

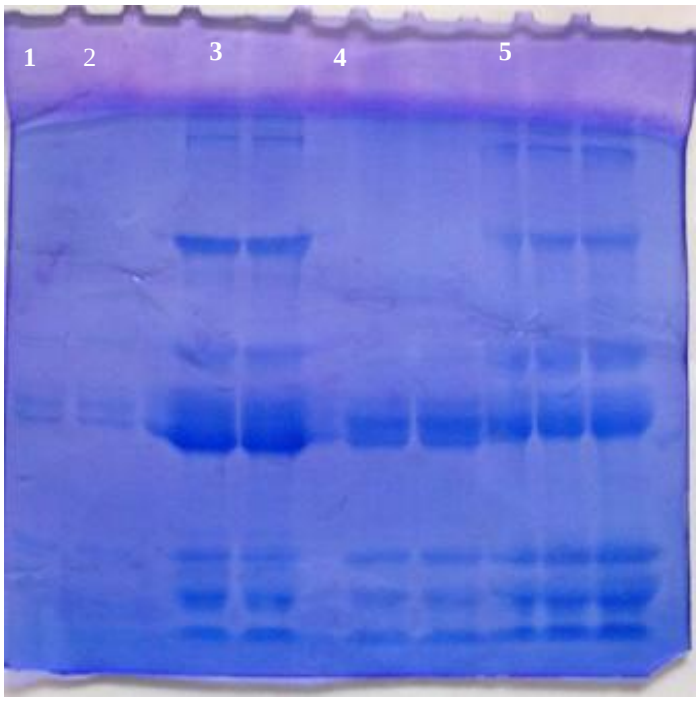
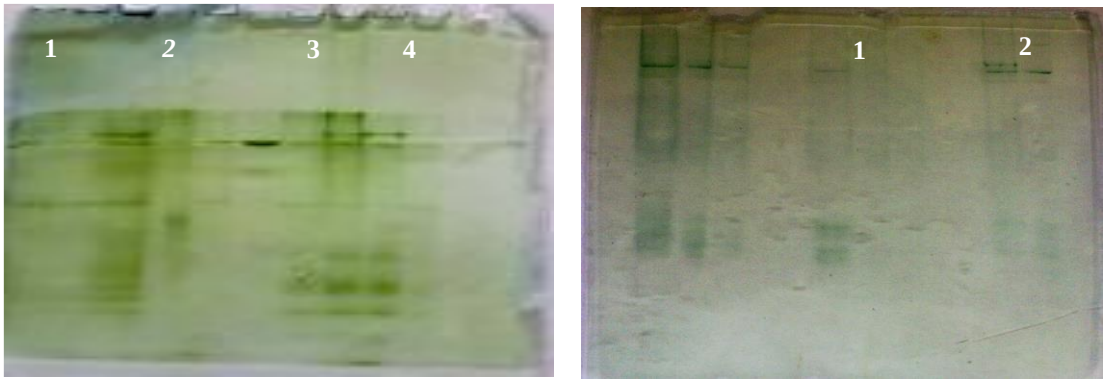


Fig. 1.General protein gel activities in*Scylla serrate* (1)and *S. olivacea* (2),*C. feriatus* (3), *P. pelagicus* (4) and *P. sanguinolentus* (5).



2a 2b

Fig. 2a.Carbonic anhydrase activities in *P. pelagicus* (1), *P. sanguinolentus* (2), *S. serrata* (3)and *S. olivacea* (4), (2b) *P. pelagicus* (1) and *C. feriatus* (2).

Genetic variation was estimated through the proportion of polymorphic loci, observed heterozygosity (H_o) and expected heterozygosity (H_e). The allele frequencies and observed heterozygosity (H_o) for each locus were determined by direct census of the data. Expected heterozygosity (H_e) of each population was calculated from alleles which was equal to or less than 0.99. *CA* displayed 5 polymorphic loci, one polymorphic locus was found in *P. pelagicus* (16%), one polymorphic loci was (20%) in *P. sanguinolentus* where as it was (33%) and (16%) in *S. serrata*and *S. olivacea*, respectively. Alleles expressed per polymorphic loci according to the interpretation of the produced banding patterns were 0.16, 0.20, 0.33 and 0.16. Mean number of alleles at per locus were found in *P. pelagicus* (1.16), *P. sanguinolentus*, (1.12*), S. serrata*, (1.133*), S. olivacea* (1.16) and in *C. feriata*(1.00). The expected heterozygosity varied from 0.16-0.50 for the polymorphic loci. These characteristics expected values were 0.50, 0.24, 0.167 and 0.20 in *P. pelagicus*, *P. sanguinolentus*, *S. olivacea* and *S. serrata*, respectively. The parameters calculated for genetic variations are given in (Table 3).

The average heterozygosity varied from 0.0158-0.0225 for the polymorphic loci. These characteristic values were 0.01586, 0.0160, 0.0255 and 0.02218 in *P. pelagicus*, *P. sanguinolentus*, *S. serrata*and *S. olivacea.* In each

sample with the exception of *C. feriata* there were no significant differences between observed heterozygosity (H_o) and expected heterozygosity (H_e) ($x^2 = 0.0$, df = 1) (Table 3).

DISCUSSION

This study provides the preliminary information on the biochemical structure and variations in iso enzymatic banding pattern of Carbonate anhydrase among five commonly found species of Portunid crabs. The allele frequencies and the observed heterozygosity for each locus were determined by direct census of the population data. A total of six loci was detected, of these three loci were monomorphic and the three loci (CA-2*, CA-4* and CA-5*) appeared to be polymorphic. A locus was considered polymorphic if the most common allele was equal to or less than 0.99. For the determination of genetic variability among different species the allele frequencies or genetic identity were calculated. The mean number of alleles per locus was found to be highest in *P. pelagicus* and *S. serrata*. Thorpe and Solecave (1994) suggested that in a general conspecific population tends to have mainly relatively small allele frequency differences at a few loci only. Between species in different genera most loci are usually entirely different (fixed for different alleles) but some may be identical.

The expected heterozygosity varied from 0.0799-0.1528 for the polymorphic loci in each species the χ^2 test for observed heterozygosity (H_o) and expected heterozygosity (H_e) showed no significant differences, defined that the allelic frequencies were in Hardy and Weinberg equilibrium at the Carbonic anhydrase locus. No polymorphic loci were found in *C. feriata* and therefore average expected heterozygosity could not be calculated. Crustacean species have shown the low genetic variability (Gooch, 1977; Turner and Lyerla, 1980; Nemeth and Tracey, 1979) and low mobility (Hedgecock *et al*., 1982) and the relatively low genetic variation is a phylogenetic character of decapods crustaceans (Gooch, 1997).

In present study, *S. olivacea* and *S. serrata* was showed mean heterozygosity 0.0225 and 0.0221, respectively. Fushimi and Watanabe (2000) calculated the mean high (0.108) heterozygosity for the genus *Scylla* (the whole population included three species) as compared to other crustacean. The mean values of heterozygosity in other crustacean species were lower than the *Scylla* population as were 0.007-0.014 in the swimming crab *P. trituberculatus*, 0.0004-0.02 in the snow crab *Chinonoecetesopilio*, 0.072-0.077 in the spider crab *C. japonicus*, and 0.023-0.032 in the hair crab *Erimarcus isenbeckii* Fushimi and Watanabe, 2000).

For genetic structure and population studies, the biochemical and molecular markers are being used as highly informative tools for identifications, estimate levels of genetic diversity and phenotypic relationships between interspecific and intraspecific populations (Majer *et al*., 1996; Brown, 1996; Mc Donald *et al*., 1999; Kumar *et al*., 2010). The allele frequencies and the observed heterozygosity for each locus were determined by direct census of the population data. Expected heterozygosity of each population was calculated from allele frequencies. Present study includes only five species of family Portunidae, representing three genera. An auxiliary study is going on to clarify the phylogenetic relationships of the Portunids species, including a broad representation of all species of the family Portunidae found along the coast of Pakistan.

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