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Isozyme analysis and genetic variability among semiterrestrial crabs of the family Camptandriidae (Decapoda: Brachyura: Ocypodoidea) from the coastal areas of Pakistan

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Genetic diversity within different Camptandriid species and their populations have been investigated using isozyme electrophoresis from the North Arabian coast of Pakistan. Patterns of a total of eight isozymes including Amylase, Carbonate anhydrates, Catalase, Creatine kinase, Glucose 6 phosphate dehydrogenase, Octanol dehydrogenase, Peroxidase and a general protein were analyzed within three Camptandriid crab species (*Manningis arabicum* (DA Jones & Clayton, 1983), *Opusia indica* (Alcock, 1900) and *Nasima dotilliformis* (Alcock, 1900)) and their populations. The calculated mean number of alleles per locus was 1.26±0.1; the percentage of polymorphic loci per population ranged between 7.45 – 33.3 %; the observed heterozygosity ranged between 0.033 – 0.124; and the expected heterozygosity ranged from 0.032 – 0.14. Intraspecific studies showed that the coefficient of gene differentiation among populations was low ($F_{ST} = 0.032$), indicating that only 3.2 % of the total genetic diversity comes from inter-population differences; while the remaining 96.8 % comes from intra-population differences. The intraspecific genetic distance was calculated as 0.000 – 0.008 (average = 0.002), and the gene flow among the populations of *O. indica* and *N. dotilliformis* was calculated as 1.57 to 0.824, respectively. Interspecific Nei's genetic distance calculated using allele frequencies were 0.474 between *N. dotilliformis* and *M. arabicum*, 0.505 between *N. dotilliformis* and *O. indica*, and 0.658 between *O. indica* and *M. arabicum* which showed that *O. indica* and *M. arabicum* is genetically closer species.

[**Keywords**: Camptandriid, Gene flow, Interspecific, Intraspecific, Isozyme, North Arabian]

Introduction

Biochemical marker, *i.e.* isozyme, has its potential in systematic studies as it examines the genetic diversity within and among populations of different species by detecting allelic variation at the enzyme level, which is the reflection of the genome[1,2]. The electrophoretic data analysis is based on the assumption that greater the level of divergence in isozymes bands, lower will be the taxonomic affinity between species or populations[3,4]. Nowadays, isozyme markers have been widely applied for species identification, gene linkage analysis, environmental physiology, interbreeding research, and population inheritance structural analysis of marine habitats[2].

An intertidal estuarine area around the world represents spatially discrete habitats isolated from the adjacent areas by barriers to dispersal, which results in population subdivision in marine species[5,6]. Species with restricted dispersal capabilities will show significantly greater population genetic structure than those without such limitations. The absence of selective pressures and reduced geneflow results in marked patterns of intraspecific genetic divergence[7].

Mangrove intertidal ecosystems of Pakistan comprise a variety of brachyuran fauna, including small-sized sluggish crabs of the family Camptandriidae[8]. These small-sized, numerically dominated macrobenthos are an important ecological recycler and bioturbators in softer muddy shores that effect energy flows of intertidal detrital food chain[9,10]. The burrowing activities, away of refuge from predators and inactivity during ebb tide time, cause mixing of the topmost layer of the sediments[11-13].

From Pakistan's intertidal coast, three species of Camptandriid crabs were reported, including *Opusia indica*, *Nasima dotilliformis* and *Manningis arabicum*[8,14]. The first two are common and continuously distributed along the intertidal habitats throughout the coast; while the latter species is only reported from the Sonmiani Bay lagoon.

A lot of work has been done previously in the biology and ecology of small intertidal crab species. Snowden *et al.*[13] described the population biology and ecology of *Ilyoplax stevensi* and *Tylodiplax indica*. Further, Jones & Clayton[10,15] described the ecology and taxonomic status of *N. dotilliformis* and *M. arabicum* from Kuwait mud flats. Although these have been well-studied species on both ecological and physiological grounds, review of literature showed no previous work on the taxonomic relationship among these species based on modern molecular markers (isozyme and allozyme). In the last two decades, taxonomical studies have revised the species status and transferred some to new monotypic genera[15-18]. Still, the intergeneric relationship within these crabs so far is not clear. Analyses based on electrophoretically detected enzyme polymorphism help to estimate the degree of genetic divergence among different species, the gene flow and recruitment in a species. Hence, the current study aims to examine the genetic properties (interspecific relationship) in selected semiterrestrial crab species by using isozyme electrophoresis, a proven tool to determine population structure and estimate interspecific variation in various organisms.

Materials and Methods

Study area and sampling

Pakistan is a subtropical country with semi-arid temperature, influenced by strong monsoon regime. The coastline is bordered by the Arabian Sea along the northern sites, which stretches along the two provinces (Sindh and Baluchistan) that includes mud flats, backwaters, creeks and sandy shores where the vast number of mangrove trees, especially *Avicenna marina* provides diverse habitat for abundant intertidal Camptandriid brachyuran fauna. During March 2015 to July 2017, crab species were collected randomly along different sites (Table 1) by digging the intertidal sediment during ebb tide. Specimens were ice-killed and transported to the laboratory for further processing.

Enzyme electrophoresis

Tissue sample for electrophoresis were obtained by grounding individual specimen using a ceramic mortar and pestle in tris-citrate grinding buffer (pH: 8). Homogenates were then centrifuged at 13,000 rpm for 15 min. The supernatant was kept frozen at -20 °C until electrophoresis. Vertical polyacrylamide gel electrophoresis (10 %) was performed using a discontinuous tris-citrate buffer system at 130 – 170 V for 2 – 3 h(ref. 19). Enzyme systems investigated for screening isozyme variations were: a-Amylase (AMY), Carbonate Anhydrates (CD), Catalase (CAT), Creatinine Kinase (CK) Peroxidase (PER), Octanol Dehydratase (OD), Glucose 6 phosphate dehydrogenase (G6PDH), and a General Protein (GP). Staining protocols followed as described previously with slight modifications[20-22]. The names, numbers and abbreviations of the enzymes, the method of interpretation of gel-banding patterns and locus nomenclature was done following Shaklee *et al.*[23]. Each locus was assigned based on its relative mobilities; the band closest to the anode was considered as one and so on. Locus was considered polymorphic if more than one allele was detected. At each locus (bands), the alleles were indicated by alphabet, with the most anodal migrating allele designated as A and so on.

Data analysis

The diversity indices, including allele frequencies, mean number of alleles per loci, observed heterozygosity, expected heterozygosity, and percentage of polymorphic loci (0.99 criteria), were calculated to analyze the data. Polymorphic loci were tested for conformance to Hardy-Weinberg expectations of random mating. F-statistics[24] were used to assess the variation within and among the populations of species, including F_{IS} (inbreeding coefficient), F_{IT} (total inbreeding), and F_{ST} (interpopulation divergence). The degree of genetic differentiation among different species of Camptandriid crabs was estimated from the values of genetic identity I and Nei's genetic distance $D^{(25)}$

Table 1 — Summary of sample locations, substrate type, grid references and species of Camptandriid crabs collected along the coast of Pakistan

Sr. No.	Site	Substrate	Coordinates	Crab species
1	Korangi creek (KC)	Soft sediment	24°79′ N, 67°20′ E	*N. dotilliformis*, *O. indica*
2	Bhambore (BHM)	Muddy Sandy	24°43′ N, 7°35′ E	*O. indica*
3	Dhabiji (DBJ)	Muddy	24°48′ N, 7°29′ E	*N. dotilliformis*
4	Sonmiani Bay (SB)	Sandy muddy	25°26′ N, 6°35′ E	*M. arabicum*

using allelic frequencies. A dendrogram of genetic relationships among the species was constructed by using the UPGMA method (Software PopGen32).

Results

Isozyme polymorphism

Analysis based on 24 loci coding of 7 enzymes and a general protein system showed 19 loci were polymorphic in at least one population or species and could be consistently scored across the gel. The remaining five loci (*Cd-1*, *Ck-1*, *Per-1*, *Odh-1*, *G6-Pdh*) were monomorphic for the same allele in reported species of Camptandriid crabs. Within *O. indica*, nine loci (in one or both populations), 7 loci in *N. dotilliformis* and two loci in *M. arabicum* were variable. *Nasima dotilliformis* and *M. arabicum* share common alleles at ten loci (*Cd-1*, *Cat-3*, *Ck-1*,*3*, *Per-1*, *Odh-1*, *G6pdh*, *GP-4*,*5*,*7*); nine loci (*Amy-2*, *Cd-1*, *Ck-1*,*2*, *Per-1*, *Odh-1*, *G6pdh*, *GP-3*,*7*) shared with common alleles by *O. indica* and *M. arabicum*; eight loci (*Cd-1*, *Cat-1*, *Ck-1*, *Per-1*, *Odh-1*, *G6pdh*, *GP-3*,*6*) were common between *N. dotilliformis* and *O. indica*, while the remaining loci were distinct among the three species. Allele frequencies of all the polymorphic loci, observed and expected heterozygosity, mean number of alleles per loci and percent polymorphic loci for three species were presented in Table 2 which is useful to estimate diversity indices. According to Hardy and Weinberg's law there are no significant differences between the observed and expected heterozygosity values within each species (chi-square test, $P > 0.05$).

Within different Camptandriid populations, genetic variability was highest in *O. indica* with an average observed heterozygosity and percent polymorphism as $Ho = 0.137$ and $\% P = 29$, followed by *N. dotilliformis* ($Ho = 0.097$, $\%P = 25$), and *M. arabicum* ($Ho = 0.038$, $\%P = 25$). The F statistics calculated to measure intraspecific genetic variation among and within populations were insignificant (Table 3). Mean F_{ST} values were ranged between 0.009 – 0.17 among different species of Camptandriid crabs, with gene flow values (Nm) calculated as 0.529 for *N. dotilliformis* and 0.66 for *O. indica* populations.

Mean genetic similarity (I) among different population and species ranged from 0.535 – 0.980; while genetic distance varied from 0.019 – 0.624 (Table 4). The dendrogram of genetic relationships among all studied species of the family Camptandriidae were shown in Figure 1.

Discussion

The present biochemical study determines genetic variation by calculating allelic variation, which is used to compute genetic divergence among populations and species of Camptandriid crabs from the coastal areas of Pakistan. Among all isozyme loci assayed, observed heterozygosity values ranged between 0.030 – 0.160 in different Camptandriid crab species. The average number of alleles per locus was also low (1.16 to 1.33), indicating that most loci within a species were monomorphic. Low estimates of allozyme diversity and average heterozygosity are similar to the previous studies in different brachyuran crabs[26,27]. Irawan *et al.*[28] calculated low heterozygosity values (0.072 – 0.077) in the spider crab species. Further, Irawan & Kijima[29] also estimated a low level of mean heterozygosity ($He = 0.004 – 0.006$) among the three species of Grapsid crabs. Moreover, Fuseya & Watanabe[30] also calculated similar values of heterozygosity (0.004 – 0.049) among different swimming crab species of genus *Scylla.* Additionally, Gao & Watanabe[31] also reported low levels of heterozygosity (0.021 – 0.034) in mitten crabs from China and Japan. The low levels of genetic diversity in crustaceans recorded in the above studies are a result of genetic drift, trophic stability and elimination of mutational variation in the majority of loci[27,32].

A population genetic study was conducted among the populations of *N. dotilliformis* and *O. indica*, as they are among the most common and widespread species along the coast. Each of the two species was divided into two groups: the central (Korangi Creek, Karachi) and eastern group (Dhabiji, Bhambore). Low levels of average heterozygosity among populations of *N. dotilliformis* (0.09) and *O. indica* (0.10) were obtained during the present study, and the mean F_{ST} values also showed that there was no significant genetic variation among populations of these crabs which was reported over the same geographic range[33]. The rate of gene flow was found to be high among populations of both species (*N. dotilliformis*: $Nm = 0.529$; and *O. indica*: $Nm = 0.66$), similar to the results obtained by Somasundaram *et al.*[34], who calculated a high rate of gene flow ($Nm = 0.6297$) among the population of silkworms, which might be the reason for the low level of genetic differentiation among the populations. The intraspecific genetic identity (I) was high for *N. dotilliformis* (0.979 – 0.980) and *O. indica* (0.964) and showed little or no

Table 2 — Summary of genetic structure *i.e.* allele frequency, proportion of polymorphic loci and average heterozygosity among different samples of Camptandriid crabs. *N* = Mean sample size, *%P* = Percentage of polymorphic loci; *Ho* = Mean observed heterozygosity; *He* = Mean expected heterozygosity; *KC*: Korangi creek; *BHM*: Bhambore; *DBJ*: Dhabji; and *SB*: Sonmiani Bay

Locus	Allele	*O. indica*		*N. dotilliformis*			*M. arabicum*
		KC	*BHM*	*DBJ*	*BHM*	*BHM*	*SB*
Amy-1	**a*	0.833	0.650				1.000
	**b*	0.166	0.350	1.000	1.000	1.000	
Amy-2	**a*	0.833	1.000				0.500
	**b*	0.083		0.916			0.250
	**c*	0.083			1.000	1.000	0.250
	**d*			0.083			
Cd-1	**a*	1.000	1.000	1.000	1.000	1.000	1.000
Cd-2	**a*		0.200	1.000	1.000	1.000	
	**b*	1.000	0.500				1.000
	**c*		0.300				
Cd-3	**a*	-	-	0.818	0.863	0.863	1.000
	**b*			0.181	0.136	0.136	
Cat-1	**a*	1.000	0.721	1.000	1.000	1.000	
	**b*		0.389				1.000
Cat-2	**a*			0.318	0.227	0.227	1.000
	**b*	1.000	1.000	0.634	0.727	0.727	
	**c*			0.045	0.045	0.045	
Cat-3	**a*			1.000	1.000	1.000	1.000
	**b*	1.000	1.000				
Ck-1	**a*	1.000	1.000	1.000	1.000	1.000	1.000
Ck-2	**a*	1.000	1.000	1.000	1.000	0.916	1.000
	** b*					0.083	
Ck-3	**a*			1.000	1.000	1.000	1.000
	**b*	1.000	1.000				
Per-1	**a*	1.000	1.000	1.000	1.000	1.000	1.000
Per-2	**a*			1.000	1.000	0.833	1.000
	**b*	1.000	1.000				
	**c*					0.166	
Odh-1	**a*	1.000	1.000	1.000	1.000	1.000	1.000
Odh-2	**a*	0.312	1.000	0.285	0.214	0.666	1.000
	**b*	0.687		0.714	0.785	0.333	
G6pdh	**a*	1.000	1.000	1.000	1.000	1.000	1.000
Gp-1	**a*						1.000
	**b*	1.000	1.000				
	**c*			1.000	1.000	1.000	
Gp-2	**a*						1.000
	**b*	1.000	1.000				
	**c*			1.000	1.000	1.000	
Gp-3	**a*	1.000	1.000	1.000	1.000	0.916	1.000
	**b*					0.0834	
Gp-4	**a*	1.000	0.700	1.000	1.000	1.000	1.000
	**b*		0.300				
Gp-5	**a*	1.000	0.888	1.000	1.000	1.000	1.000
	**b*		0.111				
Gp-6	**a*	1.000	1.000	1.000	1.000	1.000	0.937
	**b*						0.062
Gp-7	**a*	0.700	0.700	1.000	1.000	1.000	1.000
	**b*	0.300	0.300				
GP-8	**a*	0.500	0.500	1.000	1.000	1.000	-
	**b*	0.500	0.500				
N		20	19	24	20	12	16
%P		29.17	29	16.67	12.5	25	25
Ho		0.091	0.137	0.076	0.051	0.097	0.038
He		0.118	0.140	0.059	0.043	0.075	0.034

Table 3 — Summary of *F* statistics among different populations of *N. dotilliformis* and *O. indica* along the coastal areas of Pakistan. KC: Korangi Creek; BHM: Bhambore; DBJ: Dhabji; and SB: Sonmiani Bay

Species/Population	Sample	F_{IS}	F_{IT}	F_{ST}
O. indica (KC/BHM)	37	0.0471	0.1723	0.1305
N. dotilliformis (KC/BHM)	32	-0.354	-0.125	0.170
N. dotilliformis (DBJ/BHM)	40	-0.314	-0.302	0.009

Table 4 — Nei's Unbiased measures of genetic similarities (above the diagonal) and genetic distance (below the diagonal) among species of family Camptandriidae. KC: Korangi Creek; BHM: Bhambore; DBJ: Dhabji; and SB: Sonmiani Bay

Sr. No.	Species/ Population	*O. indica* (KC)	*O. indica* (BHM)	*N. dotilliformis* (DBJ)	*N. dotilliformis* (BHM)	*N. dotilliformis* (KC)	*M. arabicum* (SB)
1	*O. indica* (KC)		0.964	0.564	0.560	0.535	0.636
2	*O. indica* (BHM)	0.036		0.570	0.571	0.540	0.612
3	*N. dotilliformis* (DBJ)	0.572	0.560		1.000	0.980	0.680
4	*N. dotilliformis* (KC)	0.578	0.558	-0.000		0.979	0.670
5	*N. dotilliformis* (BHM)	0.624	0.615	0.019	0.021		0.678
6	*M. arabicum* (SB)	0.451	0.489	0.385	0.399	0.387	

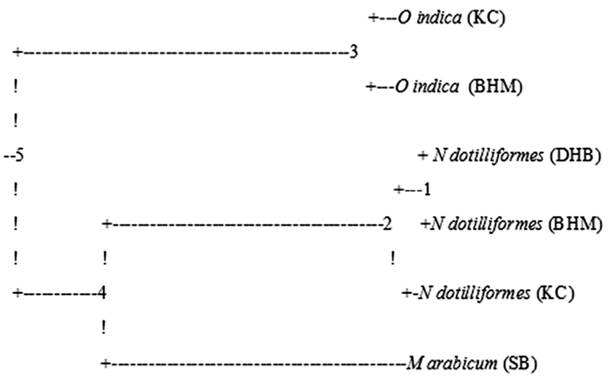

Fig. 1 — Dendrogram of UPGMA analysis of genetic distances based on isozyme and protein data, showing relationships among three species of family Camptandriidae from the intertidal areas of Pakistan

divergence. These semiterrestrial crabs have pelagic larvae like others that disperse through water currents. This might help continuous distribution throughout the coastline as exemplified by the low values of genetic distances and absence of any apparent structuring within the species. Current patterns of the oceans appear to be barrier-free, and planktonic dispersal minimizes the chance of heterozygosity. These results were in close agreement with Avise[35], which shows that for a continuous population, *I*-values ranged between 0.9 to 1.0. Selander *et al.*[36] showed that in the horseshoe crab of genus *Limulus*, the genetic similarity between the different populations were *I* = 0.99. Similarly, Ayala *et al.*[37], calculated the low genetic similarity (*I* = 0.97) between conspecific populations of *Drosophila willistoni* group.

The genetic relationships calculated by a UPGMA dendrogram using Nei's genetic distance[25] revealed three isolated species (*O. indica*, *N. dotilliformis* and *M. arabicum*). The three groups are in turn separated into two main clusters. First included *N. dotilliformis* and *M. arabicum*, while the other includes *O. indica.* Interspecific genetic identity values among the species of Camptandriid crabs fall within the range of 0.627 – 0.753 (mean = 0.693). The genetic relationship (*I)* value suggested that *N. dotilliformis* and *M. arabicum* were separated at an identity value of 0.621; while *I* value of 0.53 were found between *N. dotilliformis* and *O. indica*, indicating further divergence. The genetic similarity values obtained between studied species are similar to those reported in earlier studies of crustacean fauna. Salmon *et al.*[38] and Ayala *et al.*[37], summarized the genetic identity between conspecific populations as *I* = 0.97, about 0.80 between subspecies, 0.56 for sibling species, and 0.35 for non-sibling species. In the previous study on Ocypodoid crabs, Salmon *et al.*[38] obtained the a low *I*-value of 0.50 for *Uca speciosa* and *U. spinicarpa* which is indicative of further divergence, similar to the current study. Further advanced studies, such as mtDNA analyses, are needed to define the taxonomical position of these species clearly.

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Conflict of Interest

There is no competing or conflicts of interest to influence the work reported in this paper.

Author Contributions

NUS: Formal analysis, review & editing, funding and supervision; UA: Field collection, laboratory performance, and writing original draft; and MK: Review, editing and supervision.

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