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Comparative Biochemical and Genetic Analysis of Two Species of Ghost Crabs, *Ocypode rotundata* and *Ocypode ceratophthalmus* from the Coast of Pakistan

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ABSTRACT

The present study focused on the taxonomic, biochemical, and molecular redescription of the genus *Ocypode* (Family Ocypodidae) from two localities of the Karachi, Sindh coast (Sandspit and Sonari) and one locality of Miani Hor, Balochistan (Sonmiani). The species belonging to the genus *Ocypode* are commonly known as ghost crabs. Previously, there was no such research conducted on biochemical and molecular studies. Polyacrylamide gel electrophoresis was used for the biochemical study to identify and quantify proteins and other enzymes by using seven markers: Coomassie brilliant blue (COM), creatine kinase (CK), carbonate dehydratase (CD), catalase (CAT), amylases (AMY), peroxidase (PXD) and octanol (OCT). The isozyme CD was revealed as a distinguishing marker between two species with significant differences (P=0.00), deviating from the hardy weinberg (HW) equilibrium. The fixation index (FST) was higher (53.3%) between the two species indicating species are distinct from each. The molecular study of the combined data set revealed both species belong to a highly supportive monophyletic clade with 100 bootstrap values by using maximum likelihood (ML), maximum parsimony (MP), and bayesian interference (BI). Genetic distance also showed a higher level of variation between the two species (5.5%). The current study revealed a significant finding regarding *Ocypode rotundata* with a higher intra-specific variation in isozyme (37% polymorphic loci) and molecular data (genetic distance = 0.7%), which needs further analysis on *Ocypode rotundata*. It is highly recommended that targeted species be collected from different localities of Pakistan coastline and more molecular markers be used like (28S, ITS-1 and ITS-2) to resolve this issue.

INTRODUCTION

The Pakistan coastline is enriched with crab populations; most of the coastal areas provide habitat to crabs belong the family Ocypodidae Rafinesque, 1915 (Crustacea: Arthropoda) (Saher, 2008; Shih *et al*., 2016). Species belonging to this family inhabit sandy and muddy coastal beaches by constructing burrows of variable size and shape (Barass, 1963; Strachen *et al*., 1999; Vecchioni *et al*., 2019). This family is broadly distributed in tropical and some temperate coastal regions. They are dominant throughout the sandy and muddy coastal areas of Pakistan. These crabs always fascinate and attract visitors with their beautiful colors and behavior.

Although the family Ocypodidae is one of the largest studied groups of Brachyura in the world, most attention and detailed study have been conducted on its morphological, and taxonomic analysis by many scientists (Rosenberg, 2001). Sakai and Türkay (2013) described the comprehensive morphological study of taxonomic revision

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of ghost crab throughout the world, divided the family into two subfamilies Ucinae Dana, 1851 and Ocypodinae Rafinesque, 1815 (including two Genera Ocypode Weber, 1795; Hoplocypode Sakai and Turkay, 2013) reporting 22 species of ghost crabs. In comparison, Shih *et al*. (2016) described the complete phylogenetic tree of the family Ocypodidae using the mitochondrial analysis based on COI and 16S ribosomal genes. Based on the sequences, Shih *et al*. (2016) rearranged the family Ocypodidae. They proposed three sub-families Ocypodinae (including three genera as *Ocypode* Weber 1795, *Uca* Leach, 1814, and *Afruca* Crane, 1975), (Gelasiminae Miers, 1886 and Ucidinae Števčić, 2005).

The four species of ghost crabs *O. ceratophthalmus* Pallas 1772, *O. gaudichaudii* H. Milne Edwards and Lucas, 1843, *O. macleayana* Hess, 1865, and *O. rotundata* Miers 1882 were reported from the coastal areas of Pakistan (Hashmi, 1962, 1963, 1968; Tirmizi and Kazmi, 1986; Yousuf *et al*., 2007). Later, detailed description and evidence were provided by Sakai and Türkay (2013) that *O. gaudichaudii* is abundantly found in Eastern Pacific regions, whereas *O. macleayana* is the synonym of *O. ceratophthalmus* and *O. rotundata*. Therefore, it is considered that there are only two confirmed species found along the coast of Pakistan (*O. ceratophthalmus* and *O. rotundata*). The occurrence of *O. ceratophthalmus* is widely distributed throughout the Indo-West Pacific region from Eastern Australia to the Eastern and Southern part of Africa Whereas, *O. rotundata* is limited to India, Pakistan, Persian Gulf, and Gulf of Oman (Sakai and Türkay, 2013; Shih *et al*., 2016).

Isozyme study is a valuable technique to identify the species and distinguish sub-species by using the polyacrylamide gel electrophoresis (PAGE) (Snider, 1973; Aly *et al*., 2013). This technique is widely used for systematic study in plants, fungi, and invertebrates, including marine crustaceans (Thrope *et al*., 2000; Weber *et al*., 2003; Naz *et al*., 2017; Odhano *et al*., 2018). The electrophoretic patterns provide the irregular frequency and physicochemical properties of proteins or gene products. Previously, various isozyme types have been used for the interspecific and intraspecific variation, such as carbonic dehydratase (CD), catalase (CAT), amylase (AMY) extracted from muscle tissues of marine crabs such as Portunid crabs, fiddler crabs, and porcellanid crabs have been observed from the coastal waters of Pakistan (Saher *et al*., 2016; Naz *et al*., 2017; Odhano *et al*., 2018). However, no previous data is available on isozyme analysis of ghost crabs from the coastal waters of Pakistan.

No molecular data exists for these two species from Pakistan coastal areas to date. The current study reveals that wide distribution of *O. rotundata* throughout the coastal areas of Pakistan, whereas the distribution of *O. ceratophthalmus* is disjunct between a few coastlines of Pakistan. This study suggests that the gene flow is present in *O. rotundata* and absent in *O. ceratophthalmus* between the coastal regions of Pakistan. Due to the absence of gene flow, there are favorable chances of allopatric speciation or yet cryptic species emergence from *O. ceratophthalmus*.

The species samples were collected and analyzed genetically for sequencing the fragments of mitochondrial DNA (mtDNA) Cytochrome oxidase I 16S rRNA and 12S rRNA to test the hypothesis and investigate the patterns of molecular diversity from two coastal regions of Pakistan. Furthermore, samples from fiddler crabs (*Austruca iranica* and *Austruca sindensis*) were also selected as closely related species. The data set of COI, 16S rRNA and 12S rRNA of selected species were taken from GenBank and analyzed.

Previously, Tirmizi and Ghani (1996) reported two species of ghost crabs. Whereas Yousuf *et al*. (2007) reported four species of ghost crabs from Pakistani coastal waters. To remove the ambiguity and provide latest data using the latest techniques. Therefore, the present research was conducted based on the assessment of genetic variation within and between the species of ghost crabs using isozyme electrophoresis and molecular analysis along the coast of Pakistan.

MATERIALS AND METHODS

Site selection and sampling activities

The species belonging to the genus *Ocypode* were sampled for study from three sandy coastal regions of Pakistan, Sandspit (24°49′41.0^N 66°56′26.0″ E), Sonari (24°53′00.0^N 66°42′00.0″E), and Sonmiani (25°26′ 00.0^N 66°35′00.0″E) from 2015 to 2017 by random method at dusk time. The Sandspit and Sonari sites are in Sindh Province in Karachi city with 27.5 km distance, whereas Sonmiani is in Balochistan Province of Miani Hor area, which is approximately 90 km at a distance from Karachi. Twenty (20) specimens were randomly collected from each selected site for isozyme and molecular study. All the collected samples were placed in labeled polythene bags in the icebox, carried to the research laboratory, and kept at -20 °C.

Morphological analysis

The collected specimens were sorted as per their morphological characters and identified based on available literature (Sakai and Türkay, 2013; Shih *et al*., 2016). The ghost crab species were analysed morphologically by using the identification keys (Sakai and Turkay, 2013; Shih *et al*., 2016). Three distinguishing characters observed between

the species carapace structure and stridulating ridge and gonopod structure. The carapace of *O. ceratophthalmus* possesses red spots and stridulating ridge also have showed different number of cirri in both species. The identified ghost crab species were placed in marked polythene bags prior to tissue extraction for isozyme electrophoresis and DNA analysis.

PAGE analysis

The frozen muscle tissue was extracted from each specimen for isozyme study (PAGE-Native), and the same specimen was used for DNA extraction for correlation and accurate molecular measurements. Approximately 200 to 500 mg of frozen muscle tissue was extracted from enlarged cheliped for the electrophoretic study. Extracted tissue was soaked in 1.00 ml Tris-Citrate buffer (pH: 8.8) and minced with the help of hand homogenizer. The homogenate was then centrifuged at 13500 rpm for 15 minutes to settle the solid tissue particles and separate the enzymes from debris. The supernatant was poured into labeled Eppendorf tubes (1.5 ml) stored at -20°C for electrophoretic analysis. Vertical slab gel electrophoresis (PAGE-Native) was used for the isozyme electrophoretic study. A discontinuous buffer system was used, and five enzymes and a general protein were examined (Naz *et al*., 2017; Odhano *et al*., 2018).

For the staining of gel, the available literature (Murphy *et al*., 1996) was used with few modifications. The staining revealed the banding patterns representing the enzyme loci. The banding patterns were recorded through photographs. Shaklee *et al*. (1990) proposed the enzyme nomenclature system. Therefore, all the banding patterns were documented and assigned the nomenclature as described by Shaklee *et al*. (1990). The banding pattern and zymograms are inferred according to the expected phenotypes, which follow the patterns of co-dominant inheritance (Sin and Jonesf, 1983). All the alleles were designated with English letters according to enzyme name with a number such as *CD*-1, CAT-1* (Table I).

PCR amplification

The tissue sample was taken from muscles of enlarged cheliped with a scalpel form the same specimen which was used for isozyme study. About 25 mg of muscles tissue was collected and placed into a 1.5ml micro-centrifuge tube for DNA extraction. DNA was extracted through DNeasy Blood and Tissue Extraction Kit (Qiagen). All the work was performed at room temperature (15-25°C), and the complete protocol for DNA extraction was followed by DNeasy Blood and Tissue Extraction Kit (Qiagen).

The universal primers were selected for the gene amplifications. The genes were selected for PCR amplification and sequencing from previous literature for species identification and can best describe the taxonomic diversity of crabs of the family Ocypodidae (Shih et al., 2016). A total of three short fragments of genes were selected for amplification from mitochondrial DNA. The details of genes, size of the gene fragment, primers used, and their sources are given in Table II. The extracted DNA (1µl) was poured into a 24-µl reaction volume containing 9.5 µl double-distilled/sterile water, 12.5 µl of PCR master mix (EmeraldAmp Max PCR master mix), 1µl forward primer, and 1µl reverse primer. The thermal cycle maintained at 35 cycles of denaturation at 98°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s, followed by an incubation step at 72°C for 7 min.

Table I. Enzyme nomenclature system of Alleles, and buffer systems utilized in the genetic analyses buffer: discontinuous Tris-citrate (pH 8.7) buffer system.

Enzyme	Abbre-viation	Structure	No. of loci observed
Amylase	AMY*	Monomer	3
Octanol dehydrogenase	OCT*	Dimer	4
Peroxidase	PXD*	Uncertain	3
Catalase	CAT*	Tetramer	5
Carbonate dehydratase	CD*	Monomer	5
Creatine kinase	CK*	Dimer	5
Commassie (General protein)	COM*	Uncertain	7

Subsequently, the 5µl of PCR product was separated in 1% TAE Agarose Gel Electrophoresis (BioRad Power Pac Basic) at 70-80V for 30 min. The gel was stained in (Syber Safe DNA Gel Stain), and bands were visualized by ultraviolet (Illuminator (Taiwan)) transilluminator. The visible bands were clear with expected length, photographs were taken for record and gel was discorded.

Sequence of PCR products

The PCR products were sequenced from School of Life Sciences (SOLS) Core Laboratories (DNA Laboratory), ASU Tempe Campus.

Isozyme data analysis

The genetic variation was estimated by observing the banding pattern difference between two species of the genus *Ocypode*. The POPGENE ver. 1.31 program (Yeh *et al*., 1999) was used for statistical analysis of genetic data for isozymes alleles. The following analysis was carried out using the banding patterns: Percentage of polymorphic loci, frequency of genotypes, frequency of alleles, Hardy-Weinberg (HW) equilibrium test, fixation

Table II. Descriptive analysis of allele frequency between two populations of *O. ceratophthalmus* and *O. rotundata*.

Locus	Allele	Allele frequency	
		O. ceratophthalmus	*O. rotundata*
Amy-1*	*A*	1.0000	0.5000
	B		0.5000
Amy-2*	*A*	1.0000	1.0000
	B		
Amy-3*	*A*	1.0000	1.0000
	B		
Oct-1*	*A*	0.7000	0.0455
	B	0.3000	0.9545
Oct-2*	*A*		0.3182
	B	1.0000	0.4545
	C		0.2273
Oct-3*	*A*		0.7237
	B	1.0000	0.2727
Oct-4*	*A*	1.0000	1.0000
	B		
Pxd-1*	*A*	0.5000	0.9545
	B	0.5000	0.0455
Pxd-2*	*A*		0.9091
	B	1.0000	0.0909
Pxd-3*	*A*		0.4545
	B	1.0000	0.5455
Cat-1*	*A*		0.9091
	B	1.0000	0.0909
Cat-2*	*A*	1.0000	1.0000
	B		
Cat-3*	*A*	0.5000	
	B	0.5000	1.0000
Cat-4*	*A*		1.0000
	B	1.0000	
Cat-5*	*A*	0.5000	
	B	0.5000	1.0000
Cd-1*	*A*	1.0000	
	B		1.0000
Cd-2*	*A*	1.0000	
	B		1.0000
Cd-3*	*A*	1.0000	
	B		1.0000
Cd-4*	*A*	1.0000	
	B		0.5000
			0.5000

Table continued on next column.....

Locus	Allele	Allele frequency	
		O. ceratophthalmus	*O. rotundata*
Cd-5*	*A*	1.0000	
	B		1.0000
Ck-1*	*A*	1.0000	0.5000
	B		0.5000
Ck-2*	*A*		0.5000
	B	1.0000	0.5000
Ck-3*	*A*		1.0000
	B	1.0000	
Ck-4*	*A*		1.0000
	B	1.0000	
Ck-5*	*A*	0.5000	1.0000
	B	0.5000	
Com-1*	*A*	1.0000	0.5000
	B		0.5000
Com-2*	*A*	1.0000	
	B		1.0000
Com-3*	*A*		1.0000
	B	1.0000	
Com-4*	*A*		1.0000
	B	1.0000	
Com-5*	*A*	0.8000	
	B	0.2000	1.0000
Com-6*	*A*	0.5000	1.0000
	B	0.5000	
Com-7*	*A*	1.0000	
	B		1.0000
Percent of polymorphic loci		21.88%	37.50%
Mean number of alleles per locus		1.2188±0.4200	1.4062±0.5599
Mean Shannon information index		0.143±0.276	0.211±0.319
Mean observed heterozygosity		0.1875±0.3765	0.2301±0.4059
Mean expected heterozygosity		0.1066±0.2072	0.1485±02192
Mean Nei's (1973) expected heterozygosity		0.1012±0.1968	0.1418±0.2192
Genetic distance between two species		*D*=0.987	
Genetic Identity between two species		*I* =0.372	

A list of abbreviations is given in Table I.

index (FST), number of alleles per locus, observed homozygosity, observed heterozygosity (H_o), expected heterozygosity (H_e) (Nei, 1973), and Shanon information index. The genetic distance *D* was also calculated and dendrogram was constructed using the UPGMA (unweighted pair group method with arithmetic mean) (Sneath and Sokal, 1973). The frequency of genotypes was verified through Hardy-Weinberg equilibrium, followed by Leven (1949). The chi-square test ($\chi 2$) was performed, and the percentage of polymorphic loci was calculated at each locus for each population. Those loci were selected as polymorphic, which express more than one allele, while others were designated as monomorphic at 0.95 standards whose allele's frequency does not exceed 0.95. The expected heterozygosity of the mean was calculated at each locus through Nei (1973) randomly mating heterozygosity and Nei (1978) unbiased heterozygosity. For two species from three regions of coastal areas of Pakistan, unbiased genetic identity and genetic distance were also calculated, and with the help of Nei's genetic distance, a dendrogram was constructed using the UPGMA. The fixation index (FST) was also calculated to observe the genetic differentiation between and within the populations (Hartl and Clark, 1997).

DNA data analysis

The chromatograms were manually read and analyzed using the software Geneious v. 7.2 (http://www.geneious.com). Forward and reverse sequences were merged using the software Geneious (Version 7.2) to obtain consensus sequences of selected genes and the annotation and trimming of 16S, 12S, and COI. The final sequences were aligned in MUSCLE using default parameters and analyzed using software MEGA v.7 (Kumar *et al*., 2016) to identify the evolutionary model and gene diversity within and among the species. The software MEGA is also used to translate COI sequences into amino acids to test whether the presence of frameshifts or stop codons. If frameshifts or stop codons are present, it indicates sequencing errors or pseudogenes presences; such phenomenon is widely observed in crustaceans (Buhay, 2009). The combined sequences were analyzed with the maximum likelihood (ML), maximum parsimony (MP) and bayesian inferences (BI) methods using MEGA 7, PAUP, and MrBayes, respectively. The ML and MP analysis was achieved using an ML heuristic search method, setting the parameters to the values calculated by nearest-neighbor-interchange (NNI), No. of nucleotide difference for distance and ML initial tree Default-NJ/BioNJ applied. The bootstrap analyses were evaluated in ML and MP with 2000 replications and 107 replications for Mrbayes. The best fit model was obtained from MEGA 7 and subsequently applied to all tree construction methodologies (Table V). For nucleotide divergence and genetic distance matrix, it was using Kimura 2 Model in PAUP*. These phylogenetic methods were analyzed to assess the strength of the phylogeny.

All the aligned nucleotide sequencing representing all three genes merged with the help of Sequence Matrix (version 1.8), which gave a nexus format file. The combined data set was analyzed for Bayesian analysis through Mr Bayes v. 3.2.2 (Ronquist *et al*., 2011). The following parameters were used in software to get the best results: The search runs with four chains for 10 million generations and four independent runs with trees sampled every 1000 generations. The adequate sample size for convergence of chains was determined with recommended >200, and the first 25% of trees were discarded after the burning (Ronquist *et al*., 2011). The final tree was visualized with the software FigTree v. 1.4.3, while the maximum likelihood and maximum parsimony analysis were carried out through MEGA ver. 7 (Kumar *et al*., 2016) and PAUP*, respectively, with 2000 bootstraps.

RESULTS

Morphological analysis

During the species identification of ghost crabs three distinguishing characters found species-specific carapace structure, gonopod shape and stridulating ridge (Figs. 1 and 2). The stridulating ridge was composed of 10-15 tubercles with striae in *O. rotundata* whereas in *O. ceratophthamus* the stridulating ride was composed of 10-11 interspaced tubercles and 20-30 closely spaced striae. The colour pattern of ghost crabs found also different. The colour pattern can also be considered as distinguishing character among both species of ghost crabs. The carapace of *O. ceratophthalmus* is light maroon along with maroon-coloured spots found on its posterior region. Whereas the carapace of *O. rotundata* is pale yellow in colour and not visible spots can be observed. Importantly, *O. ceratophthalmus* (Ocypodidae) exhibits rhythmic changes in color change daily such as it is brighter in color during the daytime and during nighttime it is darker, significantly increasing camouflage on sandy substrates (Castro *et al*., 2015).

O. ceratophthalmus

Size of the crab ranges from 2.1 to 3.5 inches carapace width (CW) (Fig. 1A). Cornea located at the base of the eyestalk; away from the cornea the eyestalks extend to form horns or stylophthalmous (stylus). The outer margins of the exorbital corner laterally focused and exorbital positions generally trilateral and extended horizontally in large specimens. Enlarged cheliped possesses

Fig. 1. Posterior and anterior view of *O. ceratophthalmus* (A) and *O. rotundata* (B).

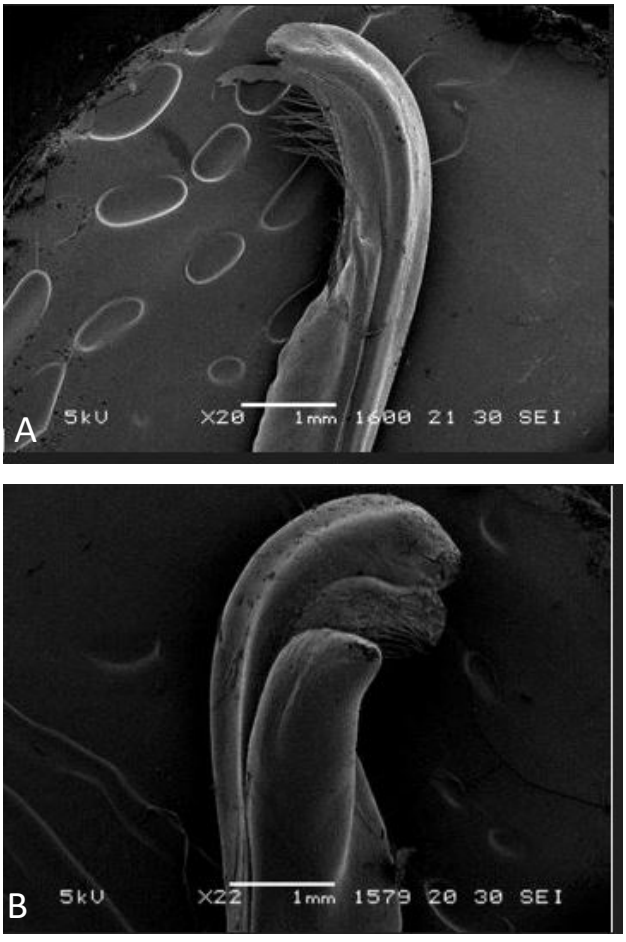

Fig. 2. Gonopod structure of male ghost crab *O. ceratophthalmus* (A) and *O. rotundata* (B) through the electron microscope.

approximately 10-11 inter-spaced tubercles on dorsal side of palm, 8 thick striae in the mid of palm and 20-30 close spaced striae on ventral third to form a stridulating ridge. Minor cheliped tapering to sharp distal end. Parapodium 2 and 3 having setae on dorsal half of anterior surface, possessing only one in female and two in male middle lines of setae. Slender shaped gonopods with bearing palp (Fig. 2A). Sternite settled around operculum in the direction of genital opening, no noticeable lateral border. Carapace light maroon with shades of pale having dark, maroon-coloured spots found on the posterior region of carapace (Fig. 1A). The abdominal region is completely dark maroon. Eyes are pale with horned straight stalks dark maroon. All the five pairs of legs including both chela along with the ambulatory legs are pale yellow on their lateral sides while their ventral sides are purely dark maroon.

O. rotundata

These species are relatively bigger in size than *O. ceratophthalmus* (max: 4.1 inches CW) (Fig. 1B). Eyestalks prolonged distally beyond cornea in a stylus. Exorbital angles rounded. Only 10-15 tubercles with striae form stridulating ridge. Small cheliped narrower at the point of distal end. Two rows of setae on anterior surface at the centre of P2 propodus present. P3-5 porpodi smooth devoid of setae. Gonopod 1 thickened, curled sideways from distal end, with separate palp (Fig. 2B). The female genital operculum is rounded distally, leading medially in button shape. Vaginal opening focused lengthwise. Carapace is pale yellow in colour; eyes are white with light maroon eye stalk (horned eyes) (Fig. 1B). All the appendages including both chela found pale yellow in colour.

Isozyme analysis

A total of five isozymes with two general proteins using different dyes (Amido Black and Coomassie Brilliant Blue R-250) were examined for the genetic variability between two species of the genus *Ocypode* along the coasts of Pakistan (Sandspit, Sonari, and Sonmiani). The current study yielded 32 bands using the 10% vertical slab gel (PAGE-Native). The *O. rotundata* showed relatively higher percentage values of polymorphic loci 12 loci out of 32 were polymorphic (37.5%), the mean number of alleles per locus was 1.406±0.559, Shannon information index was 0.211±0.319, mean observed heterozygosity was 0.2301±0.4059, and moreover, the mean expected heterozygosity (Nei, 1973) was 0.1485±02192 when compared with *O. ceratophthalmus* (Table III). The overall allele frequency among all the populations of *O. rotundata* clarified allele B with a higher (18/32 loci)

frequency as compared to allele A (14 loci). Total 20/32 loci showed a significant value (P<0.05); among them, carbonate dehydratase enzymes appeared as the most specific enzyme between two species of 5/5 loci with a significant value (P=0.00), confirming the differences between two species of the genus *Ocypode* deviating from Hardy-Weinberg Equilibrium. While the other isozyme also showed a partial significant difference, i.e., amylase (2/3 loci), octanol (2/4 loci), peroxidase (1/3), and catalase (3/5) loci, partially deviating the HW-equilibrium. Wright's (1978) Fixation Index (FIS=-0.72) revealed significant intraspecific variation that may be the cause of higher gene flow among the same species (*O. rotundata*). The fixation index (F-Statistics) was also tested for genetic structure and gene flow among the two species *O. rotundata* and *O. ceratophthalmus*. The average FST value across all the loci was negative (FST = -0.69), whereas, in contrast the enzyme carbonate dehydratase enzyme value was positive (FST=1.00) meaning that this enzyme can be used as key isozyme for separating the species of ghost crabs. Nei's unbiased genetic distance (*D=0.99*) and dendrogram revealed two selected species as genetically isolated and less similar (*I=0. 37*).

Table III. The level of genetic differentiation through Wright's (1943) fixation index and Chi-Square test for HW-Equilibrium.

Locus	Sample size	Chi-	P value	Fis	Fit	Fst	Nm*
Amy-1	40	02.36	0.124	-1	-0.33	0.33	0.50
Amy-2	40	00.00	0.000	****	****	0	****
Amy-3	40	00.00	0.000	****	****	0	****
Oct-1	40	01.88	0.169	-0.36	0.26	0.46	0.30
Oct-2	40	02.43	0.487	-0.42	-0.05	0.26	0.71
Oct-3	40	08.14	0.000	0.08	0.61	0.57	0.19
Oct-4	40	00.00	0.000	****	****	0	****
Pxd-1	40	02.34	0.124	-0.86	-0.38	0.26	0.71
Pxd-2	40	22.05	0.000	1.00	1.00	0.83	0.05
Pxd-3	40	01.81	0.177	-0.83	-0.29	0.29	0.60
CAT-1	40	22.05	0.000	1.00	1.00	0.83	0.05
CAT-2	40	00.00	0.000	****	****	0	****
CAT-3	40	01.81	0.177	-1.00	-0.33	0.33	0.50
CAT-4	40	22.05	0.000	****	1.00	1.00	0.00
CAT-5	40	1.814	0.177	-1.00	-0.33	0.33	0.50
CD-1	40	22.05	0.000	****	1.00	1.00	0.00
CD-2	40	22.05	0.000	****	1.00	1.00	0.00
CD-3	40	22.05	0.000	****	1.00	1.00	0.00
CD-4	40	41.58	0.000	-1.00	0.20	0.60	0.17
CD-5	40	22.04	0.000	****	1.00	1.00	0.00
GP-1	40	2.365	0.124	-1.00	-0.33	0.33	0.50
GP-2	40	2.365	0.124	-1.00	-0.33	0.33	0.50
GP-3	40	22.05	0.000	****	1	1	0
GP-4	40	22.05	0.000	****	1	1	0
GP-5	40	01.81	0.177	-1.00	-0.33	0.33	0.50
COM-1	40	2.365	0.124	-1.00	-0.33	0.33	0.50
COM-2	40	22.05	0.000	****	1.00	1.00	0.00
COM-3	40	22.05	0.000	****	1.00	1.00	0.00
COM-4	40	22.05	0.000	****	1.00	1.00	0.00
COM-5	40	08.14	0.000	-0.25	0.58	0.67	0.13
COM-6	40	01.81	0.770	-1.00	-0.33	0.33	0.50
COM-7	40	22.05	0.000	****	1.00	1.00	0.00
Mean	40			-0.72	0.47	0.69	0.11

* Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

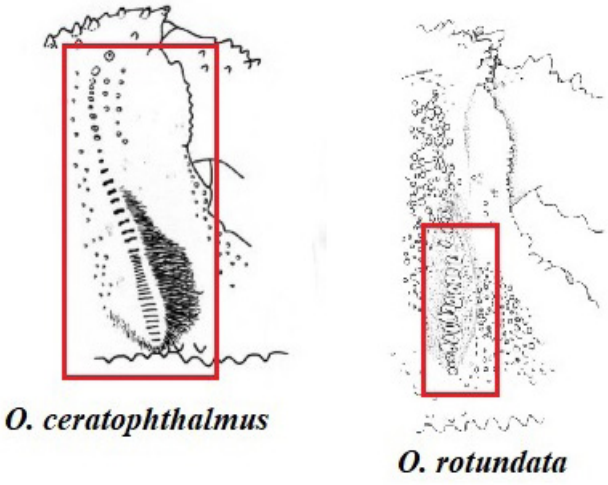

Fig. 3. Stridulating ridge of male ghost crabs *O. ceratophthalmus* and *O. rotundata* (modified from Sakai and Türkay, 2013)

DNA analysis

A total of three gene (16S, 12S, and COI) short segments for two species were sequenced and aligned, including the outgroup (Table IV) from the 14 specimen and two outgroup sequences (taken from GenBank data). A monophyletic phylogenetic tree with a high value of bootstrap support (ML, MP, and BI) was observed. The phylogenetic tree revealed two clades one for ghost crabs and other outgroup. The clade belonging to ghost crabs was further divided into two subclades *O. rotundata* and *O. ceratophthalmus,* respectively (Fig. 4).

Table IV. The selected genes, fragment size, primer name, and reference used for the analysis.

Selected genes	Fragment size (bp)	Primer name	Primer sequence 5'- 3'	Source of purchase	Reference
16S	~540-550	16Sar-F	CGCCTGTTTATCAAAAACAT	IDT, USA	Shih *et al.,* 2016
		16Sbr-R	CCGGTCTGAACTCAGATCACGT		
12S	~440-450	12Sai-F	AAACTAGGATTAGATACCCTATTAT	IDT, USA	Shih *et al.,* 2016
		12Sbi-R	AAGAGCGACGGGCGATGTGT		
COI	~650-700	LCO1490	GGTCAACAAATCATAAAGATATTGG	IDT, USA	Folmer *et al.* 1994
		HC02198	TAAACTTCAGGGTGACCAAAAAATCA		

Table V. Description of molecular markers including variable sites, best-fit models for ML, MP, and BI selected through Bayesian Information Criterion (BIC) criterion and were corrected by Akaike Information Criterion (AICc), Nucleotide content in percent, Total haplotypes observed, overall mean distance, intraspecific and interspecific distance through Kimura 2 Parameter in two species of genus Ocypode along the coast of Pakistan.

	Molecular markers		
	16S	**12S**	**COI**
Total sequences	10	11	10
Total variable sites	42	112	105
Informative sites	40	86	80
Model	TN93+G	T92+I	TN93+G
Nucleotide contents			
A%	33.4	35.9	28.2
T%	35	37.1	34.1
C%	11.0	16.8	21.4
G%	20.6	10.2	16.2
AT%	68.4	73.94	62.3
GC%	31.6	26.06	37.7
Total Haplotypes	05	07	09
Overall distance	10.1%	18.2%	16.6%
Distance b/w two species	8.6%	1.19%	16.2
O. ceratophthalmus	0.3%	0.2%	0.5%
O. rotundata	0.2%	1.3%	1.8%
Overall mean distance			5.5%
Mean distance between the two species			9.8%
Mean distance in *O. ceratophthalmus*			0.2%
Mean distance within *O. rotundata*			0.7%

TN93, Tamura-Nei; T92, Tamura 3-parameter; +G, discrete Gamma distribution; +I, Assuming that a certain fraction of sites is evolutionarily invariable.

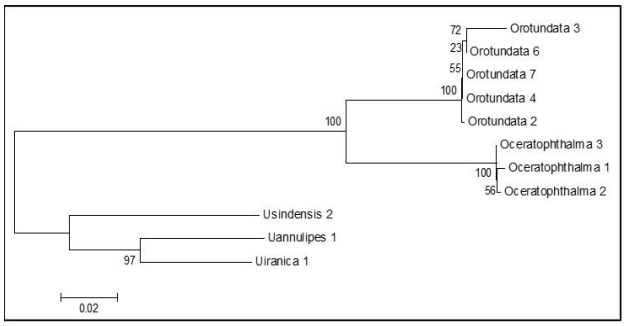

Fig. 4. A Bayesian inference (BI) tree of the Ocypode species from the Sandspit, Karachi, and outgroups of the combined 12S, 16S, and COI genes (1994) bp. Probability values at nodes represent bootstrap support for Bayesian inferences.

A~520 bp segment of the 16S rRNA gene was analysed (excluding primers) of two ghost crab species amplified and aligned together. A total of 42 variable sites found in each sample, among which 40 sites were parsimony informative sites. AT-rich content (68.4%) observed in 16S rRNA gene (A= 33.4%, T=35%, G=20.6%=, C=11.0%). For 12 rRNA gene, yield approximately 304 bp segment. A total of 112 positions were found variable, among which 86 were parsimony-informative positions. Eleven observed haplotypes from the 14 samples showed AT-rich content (73.94%). Whereas COI gene with approximately ~ 680 bp segment was analyzed, amplified, and aligned; total 105 positions found variable and 80 positions parsimony informative. Fifteen haplotypes were observed in COI sequences by using DnaSP (ver. 5). The COI segment observed with AT-rich content (62.30%) (T=34.1%, A=28.2%, G=16.2% and C=21.4%) (Table V).

The best-fitting model for sequences evolution with the lowest Bayesian Information Criterion (BIC) scores describe the best-selected substitution pattern, and each model was corrected by Akaike Information Criterion (AICc) value. The model was determined by MEGA (ver. 6), and Mr Model test (ver. 2.2) for each gene (Table

V) was applied to construct a dendrogram through ML, MP, and BI analysis by using MEGA (ver. 6), PAUP*, and MrBayes, respectively. Each tree showed the same topological structure with maximum bootstrap support (> 90%). Based on four gene sequences of the genus Ocypode (*O. rotundata* and *O. ceratophthalmus*) is monophyletic with high BI, ML, and NJ support. Within this complex, two sub-clades of both species were observed with high BI, ML, and NJ support.

A relatively higher level of intra-specific divergence within the *O. rotundata* species was observed in 12S (13 base pair difference and 4.42% nucleotide divergence) (Table VI) and COI (20 base pair difference and 3.26% nucleotide divergence) (Table VIII). For the 16S gene, the genetic distance and divergence showed little variation among the sequences of the same species (≤2) bp difference (Table VII). Whereas interspecific nucleotide differences (12S≤76, 16S≤87, and COI≤123) bp and nucleotide divergence (12S≤32%, 16S≤19% and COI≤23%) were observed (Table VIII).

Table VI. shows the nucleotide percent pairwise divergence (K2P) matrix (lower-left) and number of base pair differences (upper right) between *Ocypode* species based on 12S rRNA gene between haplotypes.

S.	OC1	OC2	OR1	OR2	OR3	OR4	OR5	AA	AI	AS
1	-	1	30	28	39	29	29	73	75	69
2	0.33	-	31	29	40	30	30	73	75	69
3	10.71	11.10	-	2	9	3	4	69	66	69
4	9.97	10.36	0.66	-	11	1	2	67	64	67
5	14.33	14.75	3.03	3.72	-	10	13	74	71	76
6	10.36	10.76	0.99	0.33	3.37	-	3	66	63	66
7	10.34	10.73	1.33	0.66	4.42	0.99	-	67	64	67
8	29.65	29.65	27.49	26.58	30.00	26.08	26.64	-	21	45
9	30.67	30.67	26.03	25.13	28.47	24.64	25.18	7.27	-	38
10	27.73	27.73	27.67	26.75	31.29	26.24	26.82	16.92	14.01	-

OC, *O. ceratophthalmus*; OR, *O. rotundata*; AA, Au.

Table VII. Shows the nucleotide percent pairwise divergence (K2P) matrix (lower-left) and number of base pair differences (upper right) between *Ocypode* species based on 16S rRNA gene between haplotypes.

S.	OR1	OR2	OR3	OC1	OC2	AA	AI	AS
1	-	2	1	38	40	77	76	79
2	0.39	-	1	40	42	78	77	79
3	0.19	0.19	-	39	41	77	76	79
4	7.70	8.14	7.92	-	2	78	83	79
5	8.14	8.58	8.36	0.39	-	78	83	79
6	16.62	16.87	16.62	16.74	16.74	-	37	47
7	16.34	16.59	16.34	17.99	17.99	7.55	-	49
8	17.17	17.17	17.17	17.02	17.02	9.66	10.12	-

OC, *O. ceratophthalmus*; OR, *O. rotundata*; AA, Au.

Table VIII. shows the nucleotide percent pairwise divergence (K2P) matrix (lower-left) and number of base pair differences (upper right) between *Ocypode* species based on COI rRNA gene between haplotypes.

S.	OR1	OR2	OR3	OC1	OC2	OC3	AA	AI	AS
1	-	20	19	93	91	92	123	122	121
2	3.26	-	3	78	76	77	114	113	110
3	3.09	0.48	-	79	77	78	114	111	110
4	16.75	13.84	14.03	-	4	3	107	102	111
5	16.33	13.44	13.62	0.64	-	3	106	100	109
6	16.56	13.65	13.84	0.48	0.48	-	107	101	111
7	22.91	21.01	20.99	19.53	19.32	19.52	-	82	68
8	22.59	20.69	20.27	18.51	18.09	18.28	14.59	-	82
9	22.53	20.20	20.19	20.46	20.02	20.44	11.95	14.58	-

OC, *O. ceratophthalmus;* OR, *O. rotundata;* AA, Au.

The combined data set created comprised of total 1994 bp (characters). The alignment comprised 1069 conserved sites and 246 total variable sites; 186 positions were parsimony informative. The alignment showed AT-rich contents (in average: A=28.8%, T=28.9%, G=21.8%, C=20.5%). A phylogenetic tree for the combined data set is shown (Fig. 5) with supported values of ML, MP, and BI analyses besides the nodes. The results show that both species formed a well-supported and distinct clade at the species level.

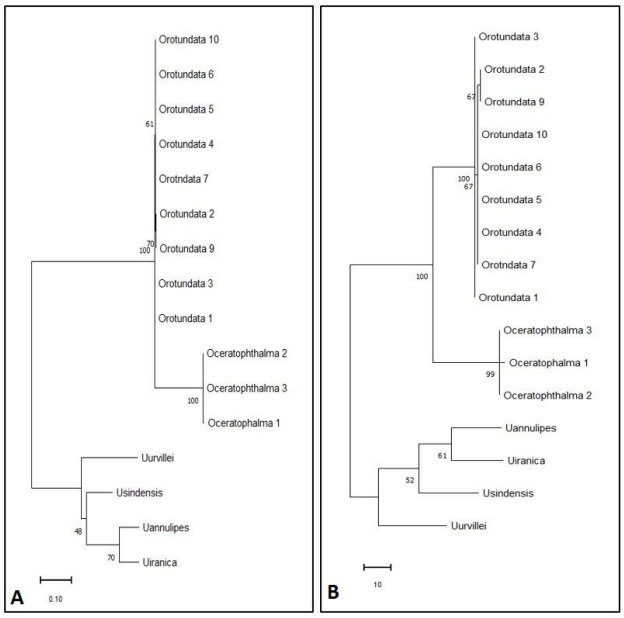

Fig. 5. A combined data set tree (12S, 16S, COI) obtained using Maximum Likelihood (A) and Maximum Parsimony (A) for Genus Ocypode from Sandspit area.

For nucleotide divergence, both species of ghost crabs showed genetically distinct nucleotide divergence of (5.5%). Although, intra-specific variation has been observed higher at a certain degree in *O. rotundata* species (6.68%) and 0.64% observed in *O. ceratophthalmus*. The mean distance within the same species was (0.2%, 0.7%) in *O. ceratophthalmus* and *O. rotundata*, respectively. The highest interspecific nucleotide divergence was 16.66%, while the mean distance between the species was (9.8%) Table V.

DISCUSSION

Morphological analysis

The species identification of ghost crabs always remained confusing during the morphological analysis (Sakai and Turkay, 2013; Shih *et al*., 2016). Total two species were identified through morphological analysis. Although few characters are considered as distinguishing features among the species of ghost crabs such as stridulating ride, carapace structure, body colour and gonopod shape in males. Tirmizi and Ghani (1996) reported these two species from the Pakistani coastal waters. Whereas Yousuf *et al*. (2007) described the four species of ghost crabs from the Sonmiani Bay. Sakai and Turkay (2013) denied the possible presence of reported species as they were reported from other parts of world. Therefore, the current study was planned on the basis of Isozyme electrophoresis and DNA analysis for the confirmation and identification of ghost crab species from Pakistani coastal waters.

Isozyme analysis

The genetic variation of two species of the genus *Ocypode* along the sandspit coastal area is measured at the species level. We found lower genetic variation (0.101 and 0.141) in *O. ceratophthalmus* and *O. rotundata*. Similar results can also be observed from the previous studies on the family Ocypodidae, such as *U. arcuta* (0.232) (Huang and Shih, 1995); *U. rosea* (0.071), *U. forciptata* (0.025), *U. vocans* (0.023), *U. triangularis* (0.031), and *Uca lactea* (0.111) (Suzawa *et al*., 1993); *U. musica* (0.097); *U. princeps* (0.028); *U. speciosa* (0.031) and *U. spinicarpa* (0.029) likewise, other group means of decapods (0.07) (Hedgecock *et al*., 1982) and other crustacean species such as coconut crab (0.018); Penaeid shrimps (0.006-0.03) (Lavery and Fielder 1993; Mulley and Latter, 1980); Norway lobsters (0.180 –0.187) (Stamatis *et al*., 2006); Portunid crabs (0.015 –0.0225) (Saher *et al*., 2016) and invertebrates (0.110) (Nevo, 1978). This value of genetic variation can cause abundant gene flow or by randomly matting individuals within the site (Huang and Shih, 1995; Wright, 1946, 1978). The intra-specific genetic diversity was analysed by polymorphism, the average allele frequency, an adequate number of alleles, and average expected heterozygosity (*He*). Observed heterozygosity (*Ho*) calculated directly from observed genotype frequencies resulted from all the species *Ho* is higher than expected heterozygosity (*He*) (0.187 and 0.230) in *O. ceratophthalmus* and *O. rotundata*, respectively. These results show that observed heterozygosity and expected heterozygosity are not equal; that statement qualifies that these two species are not in HW equilibrium. The observed heterozygosity (*Ho*) is less valuable for the genetic diversity comparison because it is affected by many environmental factors such as (genetic drift, mutation, gene flow, and natural selection) which may violate the HWE assumptions (Berg and Hamrick, 1997).

The mean number of alleles per locus was relatively higher in *O. rotundata* (1.41±0.56) than *in O. ceratophthalmus* (1.22±0.42). For each species diversity, allele frequency and heterozygosity can significantly influence the number of alleles per locus; if the frequency and heterozygosity is a higher number of alleles per locus, it can be informative for establishing the collective strategy to measure the diversity.

The fixation index was analysed to observe the proportional differentiation from HW-Equilibrium (Berg and Hamrick, 1997). The fixation index value (Table V) indicates how much two species are isolated from each other with a more significant difference in allele frequency that they do not share any allele or breed and are completely isolated from one another. The expected heterozygosity 2pq symbolized with *He* can be compared with *Ho,* which denotes the proportion of heterozygotes in a population. If expected heterozygosity and observed frequency are not equal ($He \neq Ho$), then the population is not in the HW equilibrium, which can be further measured by fixation index (FST). Therefore, the current study showed that *He and Ho* are not equal other than data further analyzed with fixation index. The current study shows that the level of genetic differentiation value between both species of ghost crabs using multiple enzyme systems is very high, FST = 0.69 (69%), this is higher than the previous studies of the crab species, i.e., *U. arcuata* (8.5%) (Huang and Shih, 1995); horseshoe crab (Limulus, 7.6%) and Drophilla equinoxialis (10.9%) (Nei, 1975).

DNA analysis

The current study represents the multi-locus phylogeny of ghost crabs from the genus *Ocypode*. Yousuf *et al*. (2007) surveyed various coasts of Pakistan to describe the taxonomic structure of ghost crabs. They described a total of four species of ghost crabs (*O. rotundata*, *O. ceratophthalmus*, *O. macleayana*, and *O. guadichaudi*), but after detailed morphological taxonomy provided by Sakai and Turkay (2013) arguing that Yousuf *et al*. (2007) has described the pictures of same species with different names meaning that they only reported one species *O. rotundata*. Hashmi (1962, 1963, 1968) and Tirmizi and Kazmi (1986) first recorded the *O. rotundata* and *O. ceratophthalmus* from the Manora island and sandspit beach. After this, no valuable attention has been paid to this genus. The current study is based on a re-description of two species, *O. rotundata* and *O. ceratophthalmus*, along the coast of Karachi in detail. During the current study, *O. rotundata* and *O. ceratophthalmus* were recorded from two localities of Karachi (Sandspit and Sonari). Both species showed a sympatric relationship, but *O. ceratophthalmus* remains much lower in abundance comparatively. A relatively higher level of intra-specific divergence within the *O. rotundata* species suggests that there might be a promising gene flow within this species just because it is widely distributed across the coastal belt of Sandspit.

The phylogenetic analysis with three different methodologies showed that both species of ghost crabs could be well differentiated at the species level yielding only one monophyletic clade with two species of ghost crabs. The inter-specific mean divergence had at least (16S=8.6%, 12S=1.19%, and COI=16.2%) between *O. ceratophthalmus* and *O. rotundata*, which can be considered adequate for inter-specific taxonomic identification. These results can be followed by other previous studies on inter-specific species identification among the species of crabs belonging to the family Ocypodidae and concluded an even lower percentage of differences to be valid such as 2.79% between *Uca splendida* and *U. cressipes* (Shih *et al*., 2012), 5.32% between *Uca jocelynae* and *U. neocultrimana* (Shih *et al*., 2010). Even other brachyuran crabs showed a lower percentage of divergences, such as 3.5% between species belonging to the family Galatheidae (Macpherson and Machordom, 2005), 3.62% between *Mictyris guinotae* and *M. brevidactylus* (Davie *et al*., 2010), 4.43% between *Scopimera ryukyuensis* and *S. globose* (Wong *et al*., 2010) and 4.74% between *Helice tridens* and *H. latimera* clade (Shih and Suzuki, 2008). Burton and Davie (2007) suggested that at least 2% divergence is sufficient between two lobster species, subsequently supported by allozyme electrophoresis.

Although, intra-specific variation has been observed higher at a certain degree in *O. rotundata* species (12S=1.3%, and COI=1.8%) which needs further analysis by comparing other populations of the same species. Yeo *et al*. (2008) suggested that the difference between two species, at least ~1% bp in 16S rRNA, can be considered a significant difference between closely related species. If this criterion is considered within *O. rotundata* species, the relationship in phylogenetic tree species does not support because the current study shows only a 0.2% bp difference, which does not support distinguishing any hidden species. However, COI is considered more variable than 16S rRNA (Schubart *et al*., 1998; Shih and Suzuki, 2008).

CONCLUSION

The current study concludes that there are only two species of ghost crabs *O. ceratophthamus* and *O. rotundata* from Pakistani coastal waters. During present study no other species were recorded from the Pakistani coastal waters. The genetic differentiation (69%) and Nei's genetic distance (D=0.98) between two species are very

high, and both species can easily be distinguished from each other morphologically by size, color, and stridulating ridge. Besides this, intraspecific variations were relatively higher within the *O. rotundata* species. Such patter was also observed through DNA analysis in which the current study revealed the average divergence ratio within the *O. rotundata* species was 1.8% which is at the borderline to distinguish the species. More study is needed to analyze the *O. rotundata* different locations to resolve such higher intraspecific variation.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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