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Morphological identification, molecular bar-coding and nutritional value of *Siphonaria* species from Sindh Coast, Pakistan

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ABSTRACT

The current study was designed to establish the specie identification of the predominant *Siphonaria* species (*S. asghar, S. belcheri* and *S. kurracheensis*) via PCR based amplification of the two mitochondrial housekeeping genes i.e. 16S rDNA, COI, and one nuclear H3 gene. The nutritional significance of the *Siphonaria* sp. is also highlighted via amino acid profiling. Sampling was conducted at Buleji, the major coastal site of Karachi which harbours a huge and varied number of gastropods. The body muscle was dissected out of the shell and used for molecular identification and biochemical analysis (Amino acid profiling). Macro/micro morphology of the shells was also studied by scanning electron microscopy and energy dispersive X-ray spectrometry. The analysis of nutritional status of the three *Siphonaria sp.* revealed quite similar profiles among the three *Siphonaria sp* with the presence of nine essential and nine non-essential amino acids. Our results successfully established the method of molecular identification of *Siphonaria sp*. The study also showed that *Siphonaria sp.* are nutritionally rich and thus can be consumed as food or feed as valuable marine resource to overcome the malnutrition problem in the developing countries.

Keywords: Amino acids, Gastropods, Phylogeny, *Siphonaria*

INTRODUCTION

Gastropods are the most abundant class representing phylum Mollusca. This class represents marine, fresh water and terrestrial snails and slugs which are very successful in the period of evolution since they managed to make adaptations to different environmental conditions of dry land. Marine snails are considered as conspicuous invertebrates in terms of being rich in significant therapeutic proteins and peptides. Many of them have been isolated and purified and subjected to extensive studies for their antimicrobial, antifungal, antiviral properties and being used as adjuvant to boost immune response in cancer therapy (De Smet *et al*., 2011; Antonova *et al*., 2014; Cheung *et al*., 2014; Gesheva *et al*., 2015; Dolashka *et al*., 2016; Mora *et al*., 2019; Dolashki *et al*., 2019). The rising significance of such proteins and peptides in terms of their therapeutic efficacy has led to their use in immunotherapy (Arancibia *et al*., 2014; Coates and Nairn, 2014). Besides the medicinal importance, gastropods are also used as excellent biomarker in number of environmental studies for monitoring marine pollution (Tan, 2000; Tewari *et al*., 2002; Claremont *et al*., 2012).

Pakistan's coastline has a biologically rich environment which harbors highly diverse flora and fauna. Among the invertebrate fauna, gastropods dominate the intertidal zones of sandy, rocky and muddy shores along the Coasts of Sindh and Baluchistan provinces of Pakistan. Mubarak Village, Manora channel, Manora rocky ledge, Old and New Korangi fish harbor, Cape Monze, Pacha and Somiani are the reported Sites in Pakistan that has been extensively studied for species diversity and biomass of gastropods (Ahmed and Hameed, 1999; Hameed and Ahmed, 2000; Nasreen *et al*., 2000; Rahman and Barkati, 2012; Afsar *et al*., 2012). Pakistan's coastline extending from Sir Creek (southeast) to Jiwani (northwest) is around 1045 km long, bordering the Arabian Sea. Most of the

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coastal zones of Pakistan like Buleji, West wharf and Manora Island are characterized by rocky, sandy and muddy profiles (Ali *et al*., 2011). Tides along the coast of Pakistan are semidiurnal, which varies from range of 1.8 m to 3.2 m. The Karachi port has a tidal range of about 2.3 m (Khan *et al*., 2002).

The present study on three major species of the genus *Siphonaria* (*S. asghar, S. belcheri* and *S. kurracheensis*), is an extension of our earlier contribution which presented data on morphological identification, biochemical profiles and occurrence of hemocyanin in the *Siphonaria sp.* for the very first time from Pakistan (Ali *et al*., 2011). Sampling was conducted at Buleji, which is in southwest of Karachi. It is situated 20 km from the center of Karachi city between Hawks Bay and Paradise Point bordering Arabian Sea (Fig. 1) with Latitude 24^{o} 50′ N and Longitude 66^{o} 48′ E of Karachi near the fishing village of Buleji. One part of this ledge is exposed to high wave action since it faces the open ocean. The other part of the ledge, which lies on the west, is exposed to less wave action and is thus protected. The coast offers variety of habitats including exposed and sheltered rocks crevices, boulders, pebbles, muddy and sandy portions and tide pools of varying sizes (with sandy, muddy and rocky bottoms). The overall infrastructure of the ledge is rocky with few sandy pockets. The intertidal zone of rocky shore is mainly composed of sedimentary rocks. Thus, it has been extensively studied for being diverse habitat of various species and for their biomass (Ahmed and Hameed, 1999; Hameed and Ahmed, 2000).

The family *Siphonariidae* (false limpets) are regarded as Pulmonates, represents around 60 species worldwide. Most of these species are found in the Indo-Pacific region, especially in the southern hemisphere (Hubendick, 1946; White *et al*., 2011). *Siphonaria* scrap off algae and lichens attached to rocks using their hard rasp like radula (Aliakrinskaia, 2005). The morphological appearance of *S. ashgar, S. belcheri* and *S. kurracheensis* is quite different. Comparing the shell structure, shell of *S. ashgar* is thick and conical, *S. belcheri* is characterized with thick but slightly conical shell, whereas the shell of *S. kurracheensis* is thick and flattened. The three shells of *S. ashgar*, *S. belcheri* and *S. kurracheensis* are found adhered to rocks distributed at about 3 meters, 1-2 meters and 0.5 meter along the tides, respectively (Bano *et al*., 2011). Predominant species of Genus *Siphonaria* including *S. javanica, S. lecanium, S. asghar, S. belcheri, S. kurracheensis* has been reported previously from Pakistan and mainly studied for their morphological identification and biomass (Melvil and Standen, 1901; Bano *et al*., 2011). Moreover, the biochemical characterization (Ali *et al*., 2011) and fatty acid profiling (Bano *et al*., 2014) of three major species i.e. *S. asghar, S. belcheri, S. kurracheensis* is also reported by us but its identification at molecular level and amino acid profile is not yet established.

MATERIALS AND METHODS

Sampling

The sampling was conducted by transect line and quadrate method at Buleji (Fig. 1). The three transect lines, A, B and C was placed in the center of high, mid and low tidal zone, respectively. A total of three quadrates (50 x 50 cm) were taken from each transect line. All the gastropods within each quadrate were removed and placed in pre-labeled plastic bags and brought to the laboratory. In the laboratory, animals were washed with tap water, counted, weighed wet and tissue dried at 70 **°C**. The gastropods were identified with the help of literature described elsewhere (Khan and Dastagir, 1970; Dance, 1974; Bosch *et al*., 1995; Ali *et al*., 2011; Bano *et al*., 2014).

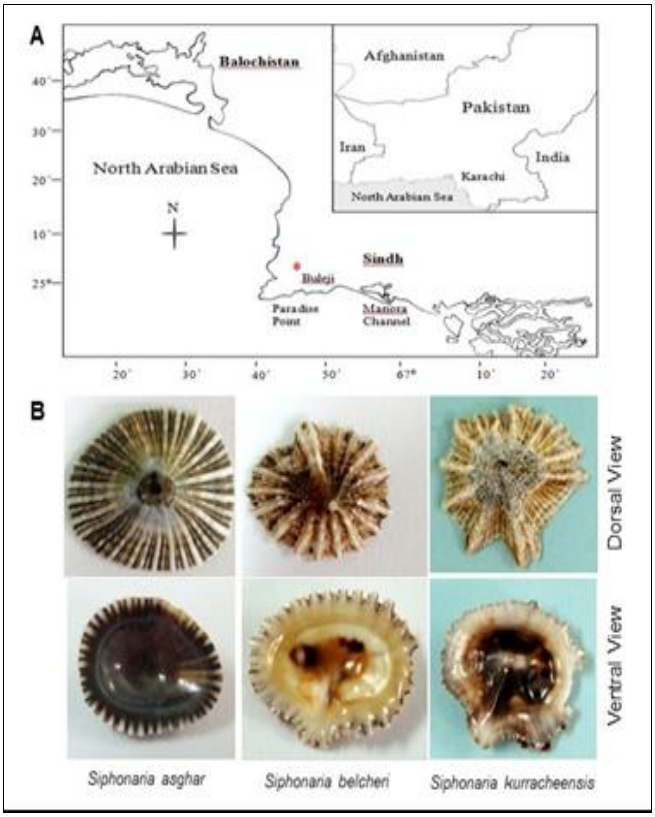

Fig.1: A, Map showing the study area marked with asterisk. B, Dorsal and ventral view of the shells of *Siphonaria* species collected from Buleji site Karachi Pakistan (Ali et al., 2011; 2018).

Scanning electron microscopy (SEM)

Cross-sections were obtained by cutting the shells with a razor blade. After gold coating (JEOL JFC 1500 fine coater) up to 300 Å, samples were observed with a JEOL JSM-6380A (Jeol, Japan), analytical SEM operating at 20 kV at the Centralized Science Laboratory (CSL), University of Karachi. Images were observed via SEM main menu. Elemental characterization, was achieved by energy dispersive X-ray spectrometry (EDS) mode (9335 emission peaks per second). Scanning was performed under high vacuum. EDS Analysis Station program associated with EDS-SEM was used to analyze the data (Rech *et al*., 2011).

Genomic DNA extraction, PCR amplification and RFLP analysis

To extract the genomic DNA from the specimen, tissue was dissected from the shells and crushed in liquid nitrogen using mortar and pestle. The crushed tissue was stored at -20 °C in pre-labeled 1.0 ml eppendorf tubes for future use. DNA purification kit (Promega, USA) was used for the extraction of genomic DNA from tissue following the protocol provided with the Kit. Purity, concentration and yield of genomic DNA isolated were estimated by Nano-Drop (ND 2000, Thermo scientific, USA).

Fragments of two mitochondrial genes (COI and 16S rDNA) and one nuclear Histone (H_3) genes were amplified successfully with primer combinations of (1) COI-1490F/COI-2198R, (2) 16S-H/16S-R, and (3) H3A-F/H3A-R as ideally described in Nakano *et al*. (2009) and Dayrat *et al*. (2011). The set of primers were synthesized and obtained from Eurofins (Operon, USA). PCR was performed in Master cycler *ProS*, (Eppendorf Germany) using PCR amplification kit (KAPA Biosystems, USA). Details for the sequence information of primers, product sizes of the amplified gene, PCR reaction mix, and thermal profiles are summarized in Table I.

The integrity of PCR products was analyzed via agarose gel electrophoresis along with 1 kb and Ultralow DNA ladder (Fermentas, USA). 2 % agarose gel was run for 45 min at 100 volts and the ethidium bromide stained bands were visualized on UV transilluminator (UVP, UK). RFLP was performed with 20 µl total reaction mix using restriction enzyme *Alu*-I (Promega, USA). Prior the reaction, virtual cutting site was analyzed via Restriction Mapper V3 (Available On-line) to see the restriction sites in 16S rDNA, COI genes and H3 genes. The reaction mixture was incubated at 37 **°C** for 4 h following the protocol provided by the manufacturer. 6x loading dye (Fermentas, USA) were added to the reaction mixtures, separated and visualized on 2 % agarose gel (Ali *et al*., 2018).

DNA sequencing and phylogeny

Amplified products were purified using DNA purification kit (Promega, USA) and were sequenced on Genetic Analyzer (Model - 3130, Applied Bio Systems, USA) at the Centralized Science Laboratory (CSL), University of Karachi. Alignments were obtained via software Sequencing Analysis v512. Prior to phylogenetic analysis, nucleotide database searching was made via nBLAST server at NCBI to get the percent similarity with the sequence of 16S rDNA, COI and Histone gene from other Siphonaria species reported so far in the NCBI Genbank. CLC Main Workbench version 6.5 was used to obtain multiple sequence alignments. Phylogenetic analyses of datasets were analyzed via online tool PhyML to observe the differences among the other Siphonaria species (Dereeper *et al*., 2008; Guindon *et al*., 2010).

Amino acid analysis

Acid hydrolysis of the samples was performed by the method described by Horwitz and Latimer (2012) with minor modifications. Briefly, dry and meshed samples (~1 mg/ml) were subjected for digestion in separate tubes under vacuum and treated with hydrochloric acid-phenol solution (6 N) for 18-24 h at 110 °C. The hydrolyzed samples were first washed with water followed with evaporation to complete dryness via rotary evaporator (BÜCHI, Switzerland). Final volume was made up to 10 ml with deionized water, syringe filtered (0.22 µm, PVDF, Millipore USA) and directly diluted in buffer-A, prior to injection (20 µl) into the amino acid analyzer. Analysis was performed on the amino acid analyzer (10A, Shimadzu Japan) equipped with Shim-Pack Amino-Na column (4.6 mm, I.D x 100 mm) packed with strong acidic cation exchanger resin (styrene divinyl benzene copolymer with sulphonic groups). Sample was injected by the auto-injector SIL-10ADVP. The mobile phase comprises of buffer-A (0.2 N sodium citrate), buffer-B (0.6 N sodium citrate and 0.2 M boric acid), and buffer-C (0.2 M NaOH). Ammonia trap column (Shim-pack ISC-30/SO504 Na) was used prior to column elution. Fluorescence detector (RF-10A XL) was adjusted at Ex=350 nm and Em=450 nm for measurement. The column oven temperature was set at 60°C. This system uses the detection by post-column fluorescence derivetization with *o*-phthalaldehyde (OPA)/N-acetylcysteine as the reaction reagent.

Table I. List of the PCR primers and conditions used for the amplification of genes for molecular identification of *Siphonaria* species

Gene region	Specie	GenBank Acc. No	Amplicon Size (bp)	Primers Name	Sequences 5' → 3'	PCR program	References
16S rDNA	*Siphonaria* ***asghar***	KM492937	462	16S-H	CGCCTGTTTATCAAAAACAT	94 °C 5 min, (94 °C 40 sec,52 °C 1 min,72 °C 5 min) x30, 72 °C 10 min	Dayrat *et al*., 2011
	Siphonaria belcheri	KM492938	442				
	Siphonaria kurracheensis	KM492939	448	16S-R	CCGGTCTGAACTCAGATCACGT		
COI	*Siphonaria asghar*	NS	681	COI-1490F	GGTCAACAAATCATAAAGATATTGG	94 °C 3 min, (94 °C 45 sec, 50 °C 90 sec, 72 °C 120 sec) x30, 72 °C 5 min	Nakano *et al*., 2009
	Siphonaria belcheri	NS	680				
	Siphonaria kurracheensis	NS	682	COI-2198R	TAAACTTCAGGGTGACCAAAAAATCA		
Histone H3	*Siphonaria asghar*	NS	344	H3AF	ATGGCTCGTACCAAGCAGACVGC	94 °C 3 min, (94 °C 45 sec, 54 °C 90 sec, 72 °C 120 sec) x30, 72 °C 5 min	Nakano *et al*., 2009
	Siphonaria belcheri	NS	345				
	Siphonaria kurracheensis	NS	319	H3AR	ATATCCTTRGGATRATRGTGAC		

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NS = Not submitted

RESULTS

Shells macro and micro-morphology

Morphologically, three major collected species of *Siphonaria* i.e. *S. ashgar, S. belcheri* and *S. kurracheensis* were found to be different. Comparing the macro structures, shells of *S. ashgar* is quite thick and conical, *S. belcheri* is characterized with thick but slightly conical shell, whereas the shell of *S. kurracheensis* is thick and flattened (Fig. 1). On the other hand, elemental composition determined via EDS showed the presence of more or less similar combination of elements, though with minor differences observed in case of elements like Fe, S, Ti and Au. An intense calcium peak was observed in *S. asghar* and *S. belcheri*, whereas the shell of *S. kurracheensis* was found with a prominent peak of Si. Other minor peaks were also found corresponding to low abundant elements i.e. Mg, Na, Al, Si, Fe, K, S, Ti and Au (Fig., 2; Table II). Distinct macro/micro-morphologies, may also be complemented by our previously reported highly variable biochemical compositions (Ali *et al*., 2011) and fatty acid profiling (Bano *et al*., 2014) of the three major *Siphonaria* species.

Table II. Elemental composition of the shell material determined using energy-dispersive X-ray spectroscopy (SEM-EDS).

Element	*Siphonaria asghar*	*Siphonaria belcheri* (% mass)	*Siphonaria kurracheensis*
C	18.84	26.12	34.67
O	49.82	44.795	33.20
Na	0.65	0.79	-
Mg	0.86	1.43	1.48
Al	1.32	3.82	5.26
Si	1.9	6.89	10.78
Ca	26.64	13.04	6.28
K	0.43	1.125	1.67
Fe	-	1.97	4.56
S	-	1.51	-
Ti	-	-	0.37
Au	-	-	9.02

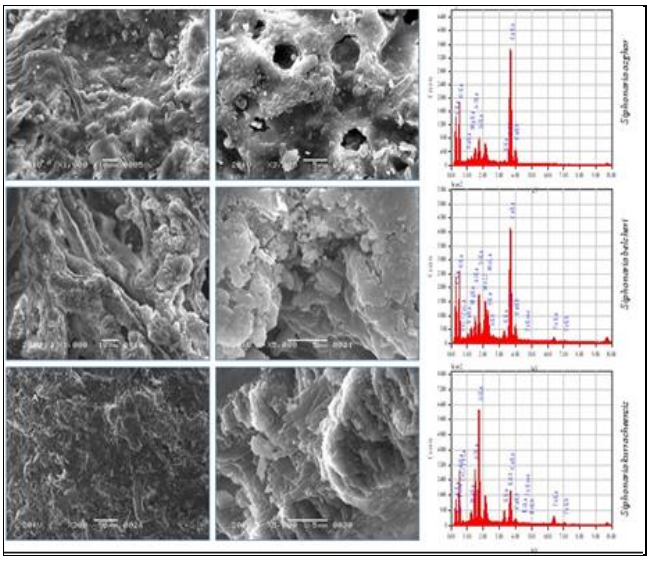

Fig. 2: SEM and EDX spectra obtained from the shell of three *Siphonaria* sp. showing calcium as the main inorganic component, while differential and to a lesser extent Na, Mg, Al and Si peaks. See "Materials and Methods" for detail.

PCR amplification, RFLP and DNA sequencing

PCR amplification of the three molecular marker genes (COI, 16S rDNA and Histone H_3) was performed with great success (Table I). Restriction patterns of all three *Siphonaria* species displayed different restriction pattern owing to the unique restriction sites in each case (Fig. 3-5). Moreover, the macro/micro-morphological differences were further verified by DNA sequencing and phylogenetic analysis of 16S rDNA and COI genes (Fig. 6). The partial sequences obtained for the marker genes were deposited in gene databank and were assigned with the accession numbers by the NCBI GenBank (Table I).

The length of the 16S rDNA was found to be 462 bp in *S. asghar*, 442 bp in *S. belcheri* and 448 bp in *S. kurracheensis* (Fig. 3A). Partial sequence lengths of COI gene were 681 bp for *S. asghar* and 682 bp for *S. kurracheensis* (Fig. 4A). The amplified Histone H3 gene of *S. asghar* was 344 bp, 345 bp in *S. belcheri* and 319 bp in *S. kurracheensis* (Fig. 5A). Moreover, different restriction patterns were obtained in case of 16S rDNA, COI and Histone genes reflecting the presence of unique restriction sites in each case (Fig. 3B, 4B & 5B). However, the enzyme *Alu-I,* couldn't digest the COI gene of *Siphonaria kurracheensis*, showing that it might lack the restriction site for this restriction enzyme supporting the genetic differences among the species (Fig. 4B). As per phylogenetic analysis of 16S rDNA, *S. asghar* was found to share same clade with *S. concimma, S. capensis* and *S. pectinata* (92 %, 88 %, 87 % identity respectively). On the other hand, *S. belcheri* and *S. kurracheensis* were found to lie in the same clade and showed 88% identity in NCBI Blast. The average mean character distance for 16S rDNA varied between 0.06 to 0.9, whereas in case of COI, the average mean character distance was found to lie between 0.6 to 1 (Fig., 6).

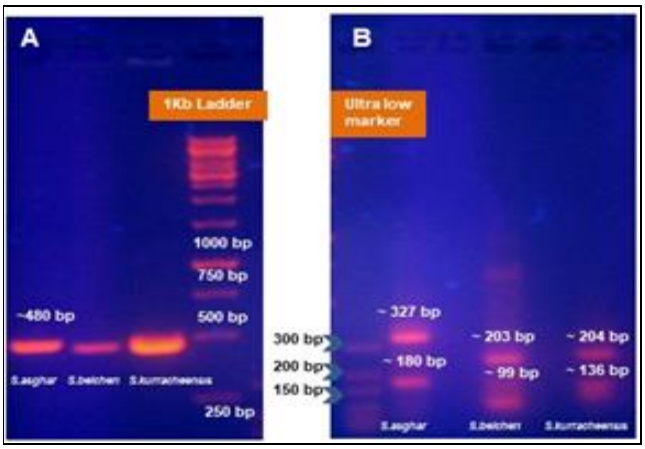

Fig. 3: PCR based amplification of 16S rDNA (A) and the restriction pattern analysis (B) of the three major species of genus *Siphonaria* collected from Buleji site Karachi Pakistan.

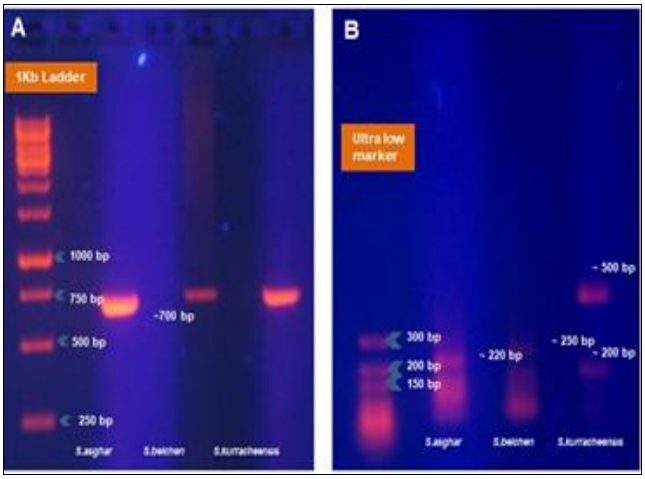

Fig. 4: PCR based amplification of COI (A) and the restriction pattern analysis (B) of the three major species of genus *Siphonaria* collected from Buleji site Karachi Pakistan.

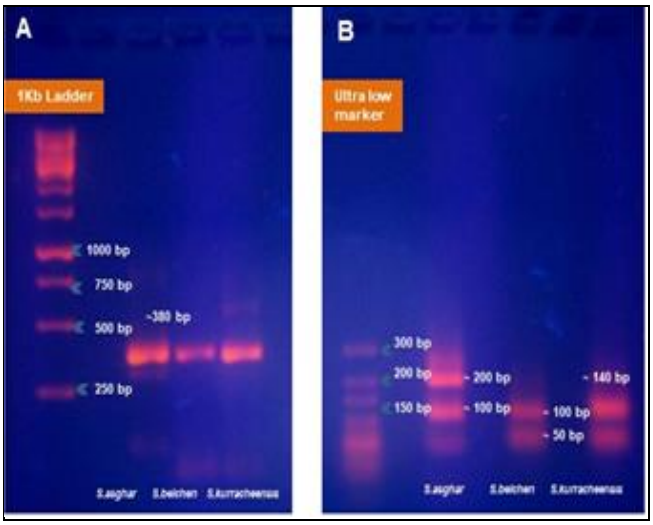

Fig. 5: PCR based amplification of Histone H3 (A) and the restriction pattern analysis (B) of the three major species of genus *Siphonaria* collected from Buleji site Karachi Pakistan.

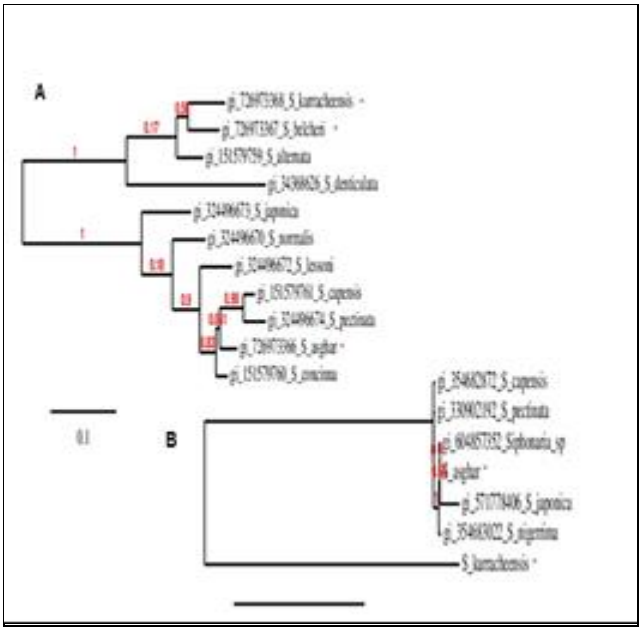

Fig. 6: Phylogeny of out groups based on analysis of 16S rDNA (A) and COI (B). * Indicates the three *Siphonaria* species subjected for molecular identification in the present study. See Table I and "Materials and Methods" for detail.

Amino acid analysis

Amino acid analysis of the three *Siphonaria* species exhibited the presence of 18 amino acids including both essential and non-essential amino acids (Table III). Among them, nine were found to be essential amino acids and nine were non-essential amino acids. The total amino acid composition of the body muscles (including essential and non-essential amino acids) was found to be 529.4, 640.5 and 673.8 mg/g in *S. asghar, S. belcheri* and *S. kurracheensis*, respectively.

Table III. Amino acid composition analysis of the three major *Siphonaria* species.

Amino acids	*Siphonaria asghar*	*Siphonaria belcheri* (mg/g)	*Siphonaria kurracheensis*
Non-essential amino acids			
Aspartic acid	53	57.2	145.4
Serine	8.6	7.9	3.3
Glutamic acid	118.2	134.3	63.9
Glycine	43.3	52.5	89.1
Alanine	94.4	96.7	79.5
Cysteine	9.2	10.2	5
Tyrosine	13.5	28.4	19.1
Proline	In trace	In trace	31.7
Arginine	39	8.5	9.8
Essential amino acids			
Threonine	0.1	In trace	In trace
Valine	21.2	25.7	21.3
Methionine	8.4	13.6	8.9
Isoleucine	13.4	22.4	14.2
Leucine	43.4	72.6	45.9
Phenylalanine	15.8	30.1	19.8
Histidine	4.7	4.5	3.9
Tryptophan	39	72.3	74.6
Lysine	4.2	3.6	38.4
Total	529.4	640.5	673.8

DISCUSSION

In extension to our previous studies on the biomass, biochemical characterization, nutritional status of the three *Siphonaria* species (i.e. *S. asghar, S. belcheri* and *S. kurracheensis*), present studies demonstrate identification of these *Siphonaria* species at molecular level and their amino acid profiles highlighting its nutritional value as the dietary resource. The microstructures of

shells in this study were clearly resolved through the combination of SEM-EDX, and that represent valuable criteria for shell identification. *S. asghar* was found to possess C, O, K, Ca, Na, Si, Mg and Al in the shell microstructures (Fig. 2; Table II). The shell of *S. belcheri* comprises of C, O, Na, Mg, Al, Si, Ca, K, Fe and S. While *S. kurracheensis* shell microstructure showed the presence of similar elements with addition of Ti and Au. Molluscan shells are composed of typical biocomposites largely consisting of organic matrix associated with calcium carbonate crystals that exhibit unique structural properties. They represent an ideal tool of choice for studying biomineralization. Its complex architecture and the involvement of biological macromolecules offer maximum mechanical protection including the toughness, stiffness and tensile strength (de Paula and Silveira, 2009; Upadhyay *et al*., 2016). Thus, the protection against environmental stress and a mechanical barrier is provided by the complexity in the microstructure of shells.

The composition of a marine shell is mainly consisting of mixture of biomaterials and organic matrix which are ultimately produced by the mantle epithelial cells (Auzoux *et al*., 2010). Studies report that several layers compose a molluscan shell which is typically made up of organic periostracum, with outer and inner calcified layers called prismatic and nacre, respectively. These Calcified layers of shells in turn consist of calcite or aragonite that gives a distinguishing and characteristic microstructure for each shell. Therefore, the composition of organic matrices can even differ within in the same shell structure (Furuhashi *et al*., 2009).

Morphology based analysis of the species revealed a high level of homoplasy, but a large number of molecular studies based on the amplification of housekeeping genes like 16S, 18S, 28S, and COI have been extensively investigated for establishing the phylogenetic relationships among different taxons of mollusk (White *et al*., 2011). One of the major reasons for relying on the identification of gastropods at genetic level is the environmental factors like physical or ecologically driven barriers which are found to be involved in gene flow thus making the morphological identification controversial (Kurihara *et al*., 2006; Bird *et al*., 2011; Claremont *et al*., 2012, Silva *et al*., 2017). Previous studies on the identification of shells based on either genetics or morphology reported the fact that marine gastropods share common morphological characters. Above all, other factors like extreme temperature, desiccation stress, habitat, plasticity in shell morphology etc. also accounts for changes in the shell pattern, color and morphology making the morphological identification more difficult. The study based on the DNA bar-coding thus becomes very fruitful in such cases (Teske *et al*., 2007, Teske *et al*., 2011; Kumbhar and Rivonker, 2012). Months of work and detail analysis of Mollusc mitogenomes are required to study the evolutionary history of Mollusc (Ki *et al*., 2010) but it is more helpful in constructing the phylogenetic relationships of gastropods.

Importance of amino acids in food and feed cannot be neglected since they are not just the constituent of proteins but are also involved in several vital cellular processes. Marine gastropods have been found as good source of nutrients for human diet (Pereira *et al*., 2013). Over all, the amino acids profile of subjected gastropods species from the genus *Siphonaria* were found with eighteen amino acids. The three major species of the genus *Siphonaria* showed close similarity in their amino acid composition. Among the essential amino acids, tryptophan was found to be maximum i.e. 39, 72.3 and 74.6 mg/g in *S. asghar, S. belcheri* and *S. kurracheensis*, respectively. Threonine was found in negligible amount in the three *Siphonaria* species with trace concentrations of Proline. Moreover, glutamic acid was found with highest proportion among the non-essential ones i.e. 118.2, 134.3 and 63.9 mg/g in *S. asghar, S. belcheri* and *S. kurracheensis*, respectively. In comparison to other known gastropods, total amino acid composition (including both essential and non-essential amino acids) was reported 9.911 mg/g in *Babylonia spirata* (Periyasamy *et al*., 2011), whereas in *Bursa spinosa,* 50.01 % essential amino acids and 46.79 % non-essential amino acid was reported (Babu *et al*., 2010). Thus, Siphonaria species prove to be much better and cheap source of amino acids (i.e. 529.4, 640.5 and 673.8 mg/g in *S. asghar, S. belcheri* and *S. kurracheensis*, respectively Table III).

Despite the recent efforts for taxon sampling and identifying new species on the basis of morphology of the shells, several taxons are still uncertain in case of their taxonomical distribution. The genetic markers (16S rDNA, COI and Histone) chosen in this study very well supported the purpose of establishing the phylogenetic analysis and evaluation of the common ancestors of the *Siphonaria* species. The present study is the first contribution from Pakistan on the comparative molecular study of *Siphonaria* species, supporting the macro/micro-morphological identification. It would be worthwhile to extend the study in the direction of detail bioinformatics and evolutionary divergence in follow up studies.

CONCLUSION

The data from SEM-EDS analysis combined with molecular identification establishes a new insight and more reliable way of identifying the species at the morphological and molecular basis. Moreover, the selected gastropods subjected in the present study can be utilized economically as possible source of nutrition (considering amino acid composition) in the developing countries to overcome the shortage of food and/or feed.

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