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Variation of TMO-4C4 Nucleotide and Protein Sequences of *Plectropomus areolatus* (Grouper) Sample Collected from Yanbu Coast on the Red Sea in Saudi Arabia

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Abstract: *Plectropomus areolatus* (coral reef guide) fish sample collected from Yanbu coast on the Red Sea in Saudi Arabia was identified and morphologically characterized. PCR amplification of the sample DNA revealed the 194 bp fragment that represented the TMO-4C4 gene. Sequence alignment showed that the homology of TMO-4C4 gene and the reported accession sequences ranged from 97 to 98% maximum. *P. areolatus* sample showed 97% with the two *P. areolatus* accessions in GenBank, while the other four *Plectropomus* species; *P. oligacanthus*, *P. leopardus*, *P. maculates* and *P. laevis* revealed 98% similarity with Yanbu sample. Sequence alignment of TMO-4C4 gene was performed and total number of 14 nucleotide positional differences with base-pair substitutions was identified compared with *Plectropomus* GenBank species. Analysis of TMO-4C4 nucleotide sequences revealed 7 transversions interchanges, one transition from A to G and four from TDC. The phylogenetic tree represented the relationship between Yanbu samples with *Plectropomus* species in NCBI GenBank showed a remarkable consequence, whereas Yanbu sample was presented as a main root origin of all divided *Plectropomus* species clusters. Translation of TMO-4C4 gene in *P. areolatus* sample induced a polypeptide of 46 amino acids with a molecular mass of about 5421.02 kDa and isoelectric point (*pI*) of 4.19. Amino acid sequence comparison revealed 100 % genetic similarities with six available GenBank *Plectropomus* species. Phylogenetic relationships based on amino acid sequences divided all species into one main discrete cluster *P. areolatus* with accession AAY68548 and *P. areolatus* Yanbu sample occupied the first distinctive cluster separated from all and originated from *P. areolatus* (AAY68548). The results emphasized variations in TMO-4C4 Yanbu sample gene and its translated protein.

Key words: *Plectropomus areolatus* · TMO-4C4 Gene · Nucleotide Variation · Yanbu Coast on the Red Sea · Saudi Arabia.

INTRODUCTION

Fishes show an astonishing diversity of shapes, sizes and colors. The delimitation and recognition of fish species is not only of interest for taxonomy and systematists, but it is also a requirement in studies of natural history and ecology, fishery management, tracking the dispersal patterns of eggs and larvae, estimations of recruitment and spawn areas and authentication of food products [1]. Fish identification is traditionally based on morphological features. However, due to high diversity and morphological plasticity, in many cases, fish and their diverse developmental stages are difficult to identify by using morphological characteristics alone [2]. DNA-based identification techniques have been developed and proven to be analytically powerful [3]. As a standardized and universal method, DNA barcoding identification systems have been widely advocated to identify species and uncover biological diversity in these years [4]. Inter-specific genetic relationships among regional populations of two species of grouper (Plectropomus maculatus and Plectropomus leopardus) were examined by Santos *et al.* [5] using mitochondrial and nuclear markers. The mtDNA revealed contrasting regional inter-specific patterns whilst nuclear markers revealed contrasting patterns among markers, irrespective of region. In eastern Australia (EA) the species form a single mtDNA lineage, but the two

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species are reciprocally monophyletic in Western Australia (WA). This supports previous evidence for hybridization between these species on the east coast. WA P. leopardus forms a sister relationship with the EA P. leopardus-maculatus clade while WA P. maculatus is more basal and sister to the P. leopardus lineages, indicating mtDNA does not suffer from incomplete lineage sorting for these species. In contrast, one of three nuclear markers (locus 7-90TG) differentiated the species into two reciprocally monophyletic clades, with no evidence of hybridization or ancestral polymorphism. The remaining two nuclear markers (2-22 and ETS-2) did not separate these two species, while distinguishing other plectropomid species, suggesting incomplete lineage sorting at these nuclear loci. These results together with coalescence analyses suggest that P. leopardus females have hybridized historically with P. maculatus males and that P. maculatus mitochondria were displaced through introgressive hybridization and fixation in the P. maculatus founder population on the Great Barrier Reef. The contrasting regional patterns of mtDNA structure may be attributed to Quaternary sea-level changes and shelf width differences driving different reef configurations on each coast. These reef configurations have provided opportunities for local scale interaction and reproduction among species on the narrower EA continental shelves, but not on the broader WA continental shelves.

To elucidate the phylogenetic relationships among the species of *Haemulon*, Rocha *et al.* [6] obtained a combined total of 2639 base pairs from two mitochondrial genes (cytochrome b and cytochrome oxidase I) and two nuclear genes (TMO-4C4 and RAG2) from all nominal species. The results of Santos et al. [7] suggested a great diversification for the western Atlantic assemblage using mitochondrial (COI and 16S rRNA) and nuclear (Tmo-4C4) genes to evaluate the phylogenetic relationships among 15 genera of the western South Atlantic Sciaenidae, two freshwater genera and the northwest Pacific *Larimichthys crocea*. The phylogenetic relationships among the fishes in the perciform tribe Epinephelini (Serranidae) have long been poorly understood, in large part because of the numerous taxa that must be considered and the large, circumtropical distribution of the group. In Craig and Hastings [8] study, genetic data from two nuclear (Tmo-4C4 and histone H3) and two mitochondrial (16S and 12S) genes were gathered from 155 serranid and acanthomorph species as a means of developing a phylogenetic hypothesis using both maximum-likelihood and -parsimony criteria. DNA sequence data from both the mitochondrial and nuclear genomes and includes 32 species of the Centrarchidae are ecologically important components of the diverse fish communities that characterize North American freshwater ecosystems. Gene sequence data of Near et al. [9] were collected from a complete mtDNA protein coding gene (NADH subunit 2), nuclear DNA intron (S7 ribosomal protein intron 1) and a portion of a nuclear DNA protein-coding region (Tmo-4C4). Phylogenetic trees generated from analysis of the three-gene dataset were used to test alternative hypotheses of centrarchid relationships that were gathered from the literature. Despite recent progress on the higher-level relationships of the Cottoidei and its familial components, phylogenetic conflict and uncertainty remain within the Cottoidea. Smith and Busby [10] analyzed a dataset composed of 4518 molecular (mitochondrial 12S, tRNA-Val, 16S and cytochrome b and nuclear TMO-4c4, Histone H3 and 28S) and 72 morphological characters for 69 terminals to address cottoid intrarelationships. The resulting well-resolved phylogeny was used to produce a revised taxonomy that is consistent with the available molecular and morphological data and recognizes six families: Agonidae, Cottidae, Jordaniidae, Psychrolutidae, Rhamphocottidae and Scorpaenichthyidae. Phylogenetic relationships within tube blennies (Chaenopsinae) were reconstructed [11] using Bayesian, maximum parsimony and likelihood analyses of multiple molecular markers (mitochondrial DNA: COI; nuclear DNA: TMO-4C4, RAG1, Rhodopsin and Histone H3) and 148 morphological characters. This total-evidence based topology is well-resolved and congruent across analytical methods with strong support for the monophyly of the Chaenopsinae, all included genera and several internal nodes.

The aim of the present study was to determine and analyze the sequence of TMO-4C4 gene and its polypeptide protein domain in a new *Plectropomus areolatus* fish sample collected from Yanbu coast on Red Sea in Saudi Arabia.

MATERIALS AND METHODS

Plectropomus areolatus fish sample used in the present study was collected from it is natural habitat in Yanbu coast on the Red Sea in Saudi Arabia.

DNA Extraction of *Plectropomus Areolatus* Fish Sample: DNA was extracted from approximately 2-4 mm^3 of tissue sample using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Tissue was homogenized in a low molarity salt buffer and digested overnight with proteinase K [12]. DNA was precipitated in isopropanol with 20 mg/ml glycogen at room temperature and the pellet was obtained by centrifugation on 12,000 rpm followed by washing in 70% ethanol. DNA was then stored at 4°C in Puregene DNA hydration solution and aliquots of genomic DNA isolates were used as templates for polymerase chain reaction (PCR) to amplify double-stranded DNA product [13].

PCR Amplification of TMO-4c4 Gene: PCR-amplification reaction was used in a final volume of 25µl containing 10X PCR buffer (10 mM Tris-HCl, 50 mM $MgCl_2$, 2 mM dNTPs, 10 mM of each forward and reverse primers, 50 ng of template DNA and 5 U of Taq polymerase (Promega, USA). Reactions were performed in a thermocycler (Biometra, GmbH, Germany) and PCR was performed as one cycle of 94°C for 3 min (denaturation), 40 cycles of 94°C for 30 sec, 49°C for 1 min and 72°C for 1 min (annealing) and with a final extension of 5 min at 72°C. PCR amplified product was analyzed using 1.2% agarose gel electrophoresis in 1X TBE buffer by staining with 0.8 ìg/ìl ethidium bromide and visualized under UV light. The size of the TMO-4C4 fragment of 194 bp was estimated based on a 50 bp DNA ladder (Bioron, Germany).

Design Specific Primer for TMO-4C4 Gene: The TMO-4C4 gene was detected using two primers: forward (5' ACCCAGCTGGTGGCAGATGA 3') and reverse (5' ACCTCAGTTCCTGCGATACA 3') obtained from accession EF517750 of NCBI GenBank and procured from Bioron GmbH (Germany).

TMO-4C4 Gene Purification and Sequencing: PCR product of 194 bp was purified with the QIA quick PCR Purification Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. DNA was eluted in 20µl of sterile water. The TMO-4C4 fragment was sequenced on an Applied Biosystems automatic sequencer (ABI PRISM® 1200 DNA Sequencer, Bioron GmbH, Germany).

Sequence Alignment of TMO-4C4 Gene: Sequence was compared with sequences of representatives of the most related *Plectropomus areolatus* fish samples deposited in GenBank and sequencing-genome databases by using the BLAST search (http://www.ncbi.nlm.nih.gov/blast). Highly conserved residues have a black background, whereas partially conserved residues are shown with a gray shaded background. Numbering at the end of each line refers to the position in the alignment.

Phylogenetic Data Analysis: Genetic distances were obtained using Kimura's two-parameter model [14]. A phylogenetic tree and dendrogram were constructed using multiple alignment of the TMO-4C4 from *P. areolatus* by the neighbor-joining method [15] with the Geneious Pro 4.5.4 program.

RESULTS AND DISCUSSION

PCR Amplification and Sequence Analysis of *P. Areolatus* TMO-4C4 Gene: PCR amplifications revealed the fragment with expected size of 194 bp that represented the TMO-4C4 gene. The 194 bp fragment correspond to the partial sequence of TMO-4C4 gene isolated from *P. areolatus* was aligned and compared in the GenBank using the BLAST search. A total of eight TMO-4C gene partial sequences from different accessions of *Plectropomus* included different species were identified (Table 1). Sequence Blast alignment showed that the homology of TMO-4C4 gene and the reported accession sequences ranged from 97 to 98% maximum. It is interesting to note that *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia showed 97% with the two *P. areolatus* accessions in GenBank, while the other four *Plectropomus* species; *P. oligacanthus*, *P. leopardus*, *P. maculates* and *P. laevis* showed 98% similarity with Yanbu coast sample. Sequence alignment of the TMO-4C4 gene of *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia compared with other *Plectropomus* GenBank species revealed 14 positional differences in nucleotide sequences and base-pair substitutions colored by yellow as shown in Fig. 1. The analysis of TMO-4C4 nucleotide sequences revealed 7 transversions interchanges of purine Dpyrimidine nucleotide, one transition from purine (Adenine =A) to purine (Guanine =G) and four from pyrimidine (Thymine= T) to D pyrimidine (Cytosine =C) as presented in Table 2.

Table 1: Blast search of TMO-4C4 gene sequence identity between of *P. areolatus* collected from Yanbu coast on the Red Sea in Saudi Arabia compared with other TMO-4C4 sequences *Plectropomus* GenBank species.

Accession	Description	Identity %
EF517748	*Plectropomus oligacanthus* TMO-4C4 gene	98
AY949300	*Plectropomus oligocanthus* TMO4C4-like (TMO4C4) gene	98
EF517751	*Plectropomus maculatus* TMO-4C4 gene	98
EF517749	*Plectropomus laevis* TMO-4C4 gene	98
EF517747	*Plectropomus leopardus* TMO-4C4 gene	98
AY949211	*Plectropomus leopardus* TMO4C4 (TMO4C4) gene	98
EF517750	*Plectropomus areolatus* TMO-4C4 gene	97
AY949267	*Plectropomus areolatus* TMO4C4 (TMO4C4) gene	97

Table 2: Positional differences and base pair substitutions in nucleotide sequences between *P. areolatus* sample collected from Yanbu coast in Saudi Arabia and numerous species based on TMO-4C4 gene similarity.

Exist in the sample as		A	A	A	C	C	G	G	T	C	--
----------	Nucleotide	----------									
Exist in NCBI as:	sequence range	C	G	T	A	T	T	C	C	--	C
Change at nucleotide positions:	T=14	471	200	467	198	293	199	197	292	305	313
		469	--	--	--	302	196	--	358	--	--
		2	1	1	1	2	2	1	2	1	1

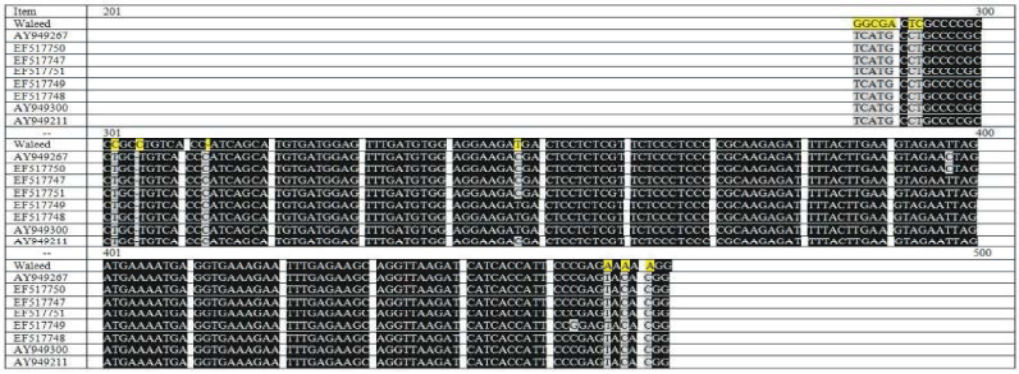

Fig. 1: Sequence alignment of 194 bp fragment of TMO-4C4 gene in *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia with other *Plectropomus* species existed in NCBI GenBank. Conserved nucleotides between my isolates and other sequences are boxed in black. Putative conserved between the different isolates with no identity with isolates are boxed in grey. The yellow box referred to the identity of all accessions except my isolates. Dashes correspond to gaps introduced to optimize the alignments.

Phylogenetic Analysis of TMO-4C4 Gene Sequence of *P. areolatus* Sample: The phylogenetic tree represented the relationship between *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia with other *Plectropomus* species in NCBI GenBank as shown in Fig. 2. The dendrogram showed a remarkable result, whereas *P. areolatus* Yanbu coast sample was presented as a main root origin of all divided *Plectropomus* species clusters.

Analysis of TMO-4C4 Amino Acid Sequence: Translation of the 194 bp fragment of TMO-4C4 gene in *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia induced a polypeptide of 46 amino acids with a molecular mass of about 5421.02 kDa and isoelectric point (*pI*) of 4.19 (Fig. 3). An amino acid sequence comparison using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST) revealed that the predicted protein is a TMO-4C4 as shown in Table (3). Moreover, amino acids alignment of TMO-4C4 gene in *P. areolatus* sample collected from Yanbu coast revealed 100 % genetic similarities with six existing GenBank *Plectropomus* species (Table 3). Phylogenetic relationships between TMO-4C amino acids of *P. areolatus* sample with other *Plectropomus* species in NCBI GenBank are shown in Fig. 4.

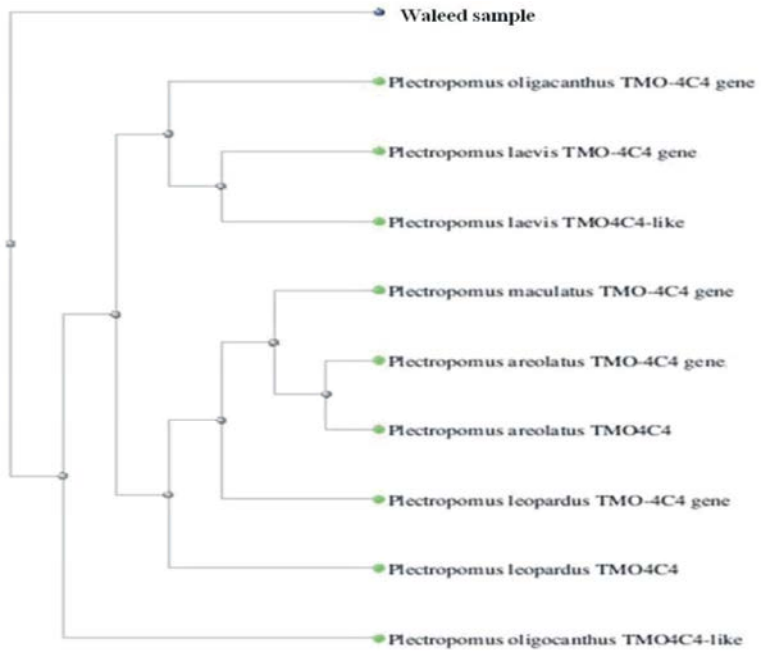

Fig. 2: Phylogenetic relationships between TMO-4C4 gene sequence identity of *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia compared with other *Plectropomus* TMO-4C4 sequences in GenBank.

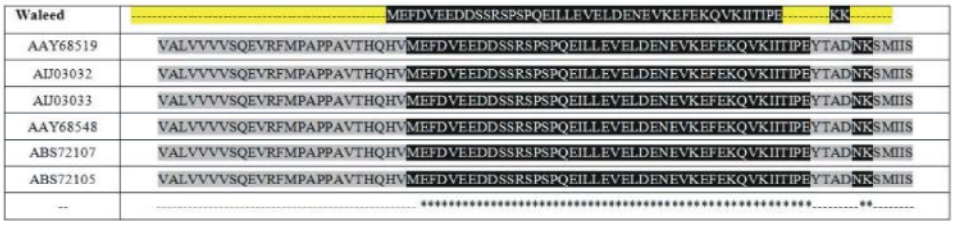

Fig. 3: Sequence alignment of TMO-4C amino acids of *P. areolatus* sample compared with other *Plectropomus* protein sequence in NCBI GenBank.

Table 3: BLASTP search of translated polypeptide amino acids of TMO-4C4 gene sequence identity of *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia compared with other *Plectropomus* TMO-4C4 protein sequence.

Accession	TMO-4C4 protein	Identity %
AAY68519	*Plectropomus leopardus*	100
AIJ03032	*Plectropomus laevis*	100
AIJ03033	*Plectropomus leopardus*	100
AAY68548	*Plectropomus areolatus*	100
ABS72107	*Plectropomus laevis*	100
ABS72105	*Plectropomus leopardus*	100

The phylogenetic dendrogram divided all named GenBank *Plectropomus* species into one main discrete cluster *P. areolatus* with accession AAY68548. It is interesting to note that, *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia was occupied the first distinctive cluster separated from all and originated from *P. areolatus* (AAY68548). The other five *Plectropomus* species were constructed the second cluster with two sub-clusters. The first sub-cluster combined two closely linked *P. laevis* species and the second sub-cluster comprises three *Plectropomus leopardus* as shown in Fig. 4.

Much research remains to be addressed in order to optimize management plans for conservation goals or gain a more complete molecular understanding of *Plectropomus* species, especially *P. areolatus*. The following proposals would augment the findings of this thesis, providing critical information for fisheries management and phylogeographic study [16]. In addition, the research topics have the potential to build a rapport

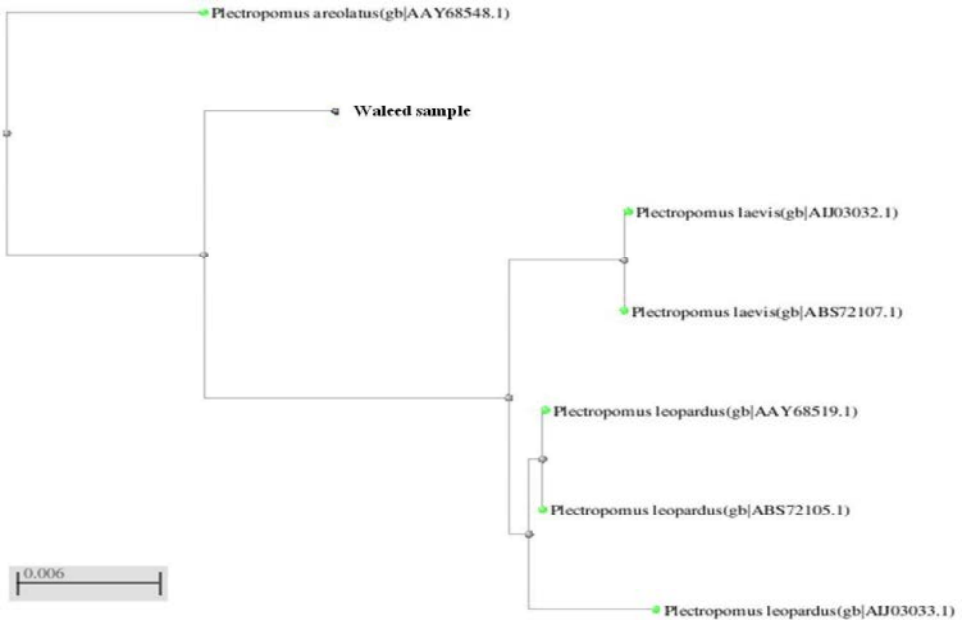

Fig. 4: Phylogenetic relationships between TMO-4C amino acids of *P. areolatus* sample compared with other *Plectropomus* protein sequence in NCBI GenBank.

with marine stakeholders in Saudi Arabia. The latter point is valuable in two ways. Considering the current lack of any efficient marine management regime, the first benefit of good stakeholder relationships relates to the degree of success which will be achieved by nascent fisheries management. Management success in any country depends on compliance by fishers, which is affected by their understanding of the situation and the degree to which their objectives are considered in the process [17]. Plectropomid population genetics is yet another field that is actively studied to the benefit of fisheries management elsewhere [18]. Microsatellite mapping of individuals can describe the relatedness of individuals on numerous scales, from the level of reefs to basins. The samples collected for this study are being used in a phylogeographic study to explore the relationship between populations of Red Sea plectropomids with other populations outside the Red Sea. Preliminary results indicated that the Red Sea population of *P. pessuliferus* is more closely related to Pacific populations of *P. laevis* than Pacific populations of *P. pessuliferus* [19]. An analysis of potential differences between the Red Sea and the Indian Ocean samples could indicate the history of the species expansion into the relatively young Red Sea. While the benefits of this research are primarily for evolutionary biology, further analysis of gene flow within or among populations in the Red Sea may illustrate the presence and distribution of meaningful and effective management units. Studies on differential resource use could be conducted to resolve the regional demographic differences observed in *Plectropomus* species of Red Sea plectropomids.

No information is yet available on the levels of environmental contaminants in Red Sea fishes. Despite minimal terrestrial runoff, it is unknown to what extent chemicals are released into the Red Sea. For a region that depends so heavily on the petrochemical industry, it is possible that there is a high level of mercury and/or PCBs in the environment. A small gram sample of muscle tissue should be preserved from every fish in further molecular studies. This allows the potential for a regional toxicity study which may be more useful in identifying sources of contamination, if there are any appreciable levels. In a study of 34 published datasets, Reed and Frankham [20] found that population fitness was significantly positively correlated with heterozygosity, population size and quantitative genetic variation. Such processes may impact on a population's short-term survival and the long-term adaptive potential under changing environmental conditions. The nature and extent of genetic changes observed in TMO-4C4 gene in *P. areolatus* sample collected from Yanbu coast on the Red Sea in Saudi Arabia suggested that fluctuations in nucleotide sequences underlain by significant changes in genetic composition and population integrity.

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