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Comparison of Evolutionary Selection Acting on the Mitochondrial Protein-Coding Genes Between Intertidal and Deep-Sea Gastropods

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ABSTRACT

Marine molluscs are ecologically and economically important group of organisms that survive in the challenging environments of different oceanic zones. Of all the classes of the phylum Mollusca, gastropods have radiated into marine, freshwater and terrestrial habitats, successfully adapting themselves to thrive in changing environmental conditions. Hence, marine gastropods can be considered as an ideal system to study stress adaptation. In order to withstand the constant fluctuations in temperature, salinity and shifts in oxygen concentration of the intertidal zone, the gastropods inhabiting here rely on a modified and adaptive energy metabolism. The same is applicable for gastropods living in the deep sea environment, which is characterized by high hydrostatic pressure, low oxygen concentrations and abundance of heavy metals. Therefore, survival of these organisms may be correlated to their adaptive mitochondrial genome which serves as the principal site for energy metabolism and production in the cell. Here, we estimated selection pressure acting on the mitochondrial protein-coding genes of 13 intertidal and 2 deep sea gastropods based on site and branch-site specific models. The results exhibited higher number of sites under diversifying selection for the mitochondrial protein-coding genes of intertidal gastropods compared to deep sea species. Overall, this study focusses on the adaptive mitogenome evolution of marine gastropods for survival in the dynamic environments of the intertidal zone as well as deep sea.

Keywords- Deep sea, Gastropods, Intertidal, Mitochondria, Selection pressure.

I. INTRODUCTION

Mitochondrial DNA of animals usually consists of about 15 - 20 kilobase pairs [1]. Nevertheless, larger mitochondrial genomes exist due to the presence of large amounts of duplicated genes or non-coding nucleotide sequences [2]. Mitochondrial DNA of bilaterians usually consists of 13 protein-coding genes (PCGs), 2 rRNAs of the mitochondrial ribosome and 22 tRNAs essential for the translation of oxidative phosphorylation (OXPHOS) proteins. Cytochrome *c* oxidase subunit 1 - 3 (COX1 - COX3), cytochrome *b* (CYTB), NADH dehydrogenase subunit 1 - 6 (ND1 - ND6 & ND4L) and ATPase subunits (ATP6 & ATP8) are the subunits of the respiratory chain complexes, namely Complex I, III, IV and V, respectively, responsible for producing up to 95% of the energy of eukaryotic cells [3].

Oxidative phosphorylation as well as innate immune response are regulated by mitochondrial PCGs [4]. Therefore, cellular homeostasis and survival relies largely on the maintenance of mitochondrial integrity and signaling [5]. Changes in the constituent residues of the mitochondrial proteins might have an influence on their functional domains, thereby allowing organisms to adapt to challenging environments [6]. Thus, determination of selection pressures acting on mitochondrial PCGs could shed light into the adaptive evolution of the mitochondrial genome. Majority of the research on adaptive evolution of mitochondrial genomes in vertebrates involve fish and mammals [7] while knowledge about the molecular basis of the same in invertebrates, especially marine invertebrates, remain limited [8,9].

Mollusca is the second largest animal phylum after Arthropoda and constitutes classes – Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, Polyplacophora, Scaphopoda, Caudofoveata and Solenogastres [10]. In addition to serving as a global food source, this phylum is an ubiquitous and ecologically important group of invertebrates, many of which function as ecosystem engineers [11]. Although ecosystem deterioration results in the elimination of many species, certain molluscs exhibit an unusual resilience towards environmental fluctuations [12]. Gastropods represent the largest class of this phylum, with number of species ranging from 80,000 to 150,000 [13,14]. Apart from being the only terrestrial molluscs, gastropods live in every conceivable place on Earth with marine ones occupying habitats ranging from the deepest ocean basins to the intertidal zone, as well as freshwater and other inland water habitats.

The intertidal zone is the region between the highest and lowest tides, depicting the transition from ocean to terrestrial conditions. Organisms inhabiting this zone are able to maintain mitochondrial homeostasis amidst regular oscillations of immersion and emersion, along with tremendous variations of abiotic factors such as light, temperature, salinity and oxygen concentrations [15]. Consequently, gastropods living in this area need to withstand the dynamic conditions of the intertidal zone. On the other hand, gastropods are also an important component of the fauna in hydrothermal vents with

regard to abundance and biomass [16]. Deep sea hydrothermal vents are characterized by extreme physicochemical conditions, such as high hydrostatic pressure, fluctuating temperatures, low levels of oxygen and high concentrations of heavy metals and other toxic chemicals [17]. Since acclimation to extreme habitat conditions are often correlated to the evolution of mitochondrial PCGs, detailed studies of gastropod mitogenome may uncover interesting aspects of molecular adaptations to living in intertidal and deep sea environment. In the present study, we used fifteen gastropod mitochondrial genomes to detect signs of natural selection and ascertain the adaptive evolution of the mitochondrial PCGs. We performed site and branch-site specific likelihood analyses to estimate the probability of positive selection on each site and branch of individual mitochondrial protein-coding genes across these species.

II. MATERIALS AND METHODS

The nucleotide sequences of 13 mitochondrial PCGs of 15 gastropods were downloaded from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nucleotide). Among the 15 gastropods, 13 species inhabiting the intertidal zone and 2 deep sea species were considered. The details of the organisms are provided in **Table 1**.

Table 1: Details of the gastropods considered for this study

Serial No.	Accession no.	Species	Family	Habitat
1	AY588938.1	*Haliotis rubra*	Haliotidae	Intertidal (0 – 50m)
2	JN790612.1	*Fissurella volcano*	Fissurellidae	Intertidal
3	DQ862058.1	*Conus textile*	Conidae	Intertidal (0 – 10m)
4	NC_030536.1	*Conus striatus*	Conidae	Intertidal (1 – 50m)
5	NC_032377.1	*Californiconus californicus*	Conidae	Intertidal (0 – 45m)
6	KF728890.1	*Nerita versicolor*	Neritidae	Intertidal
7	NC_010090.1	*Reishia clavigera*	Muricidae	Intertidal
8	NC_017886.1	*Concholepas concholepas*	Muricidae	Intertidal (0 – 40m)
9	NC_012383.1	*Siphonaria pectinate*	Siphonariidae	Intertidal
10	NC_012376.1	*Onchidella celtica*	Onchidiidae	Intertidal
11	NC_016181.1	*Peronia peronii*	Onchidiidae	Intertidal
12	NC_031861.1	*Stomatella planulata*	Trochidae	Intertidal (0 – 30m)
13	NC_030595.1	*Littorina saxatilis*	Littorinidae	Intertidal (0 – 46m)
14	LC107880.1	*Lepetodrilus nux*	Lepetodrilidae	Deep sea (640 – 1580m)
15	MK404176.1	*Pseudorimula sp.*	Lepetodrilidae	Deep sea (750 – 4200m)

The datasets of the 13 mitochondrial PCGs (*cox1* - *cox3*, *cytb*, *atp6*, *atp8*, *nd1* - *nd6* and *nd4l*) were constructed with the mitogenomes obtained from the 15 gastropod species. Codon-based alignment was performed using MUSCLE [18] integrated in the MEGA-X software [19] selecting the invertebrate mitochondrial genetic code. The best-fitting substitution models for 13 mitochondrial PCGs were estimated using the option 'Find Best DNA/Protein Models' in the MEGA-X software (**Table 2**).

Table 2: The best-fitting models selected for the 13 PCGs using MEGA-X

PCGs	Model selected
atp6	GTR + G
atp8	T92 + G
cox1	GTR + G
cox2	T92 + G
cox3	HKY + G
cytb	GTR + G + I
nd1	HKY + G + I
nd2	GTR + G
nd3	T92 + G
nd4	GTR + G + I
nd4l	T92 + G
nd5	GTR + G + I
nd6	GTR + G

The estimation of ω, *i.e.*, the ratio of non-synonymous substitutions (dN) to synonymous substitutions (dS) has been extensively used as an indicator of selection pressure on protein-coding genes. For protein-coding nucleotide sequences, the ratio of non-synonymous to synonymous substitutions (ω) differentiates neutrally evolving sequences (ω = 1) from

those subjected to negative (ω < 1) or positive (ω > 1) selection. The ω values were measured in EasyCodeML v1.21 that uses the codon-based maximum likelihood (CODEML) algorithm [20,21]. We performed both site and branch-site model analyses to identify the contrasting selection pressure acting on individual mitochondrial PCGs. Seven codon substitution models namely M0 (one-ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (beta), M8 (beta and ω > 1) and M8a (beta and ω = 1) were considered and four likelihood ratio tests (M0 *vs.* M3, M1a *vs.* M2a, M7 *vs.* M8 and M8a *vs.* M8) were implemented to estimate the likelihood of positive selection on each site for individual PCGs [22–24]. On the other hand, branch-site models enable ω to differ among sites and across branches of the phylogenetic tree [25]. Therefore, we evaluated selection on each site considering a single foreground branch (branch of interest) at a time. Posterior probabilities were computed using Bayes Empirical Bayes (BEB) analysis in order to recognize sites under positive selection on the selected branches if the Likelihood Ratio Test (LRT) *p*-values are significant (BEB > 95%). The requirement of running EasyCodeML is an alignment file and a phylogenetic tree. For each of the 13 PCGs, 13 Maximum Likelihood trees were generated using MEGA-X software. In this study, only the leaf nodes were taken into consideration. HyPhy package v2.5 (available from www.hyphy.org) was used to assess codons under selection pressure [26]. We performed MEME (Mixed Effects Model of Evolution) and FEL (Fixed Effects Likelihood) analyses to detect individual sites subjected to episodic diversifying selection and purifying selection, respectively [27,28]. Both analyses were done using a *p*-value ≤ 0.05 significance level.

III. RESULTS & DISCUSSION

Episodic positive selection acts on the mitochondrial PCGs that allow the organisms to adapt to extreme environmental changes [29,30]. The intertidal zone is one of the most stressful environments with extreme alterations in temperature, pH, salinity, and oxygen concentrations. The gastropods inhabiting this zone have successfully adapted to this dynamic environment. On the other hand, deep sea gastropods also face high hydrostatic pressure, variable temperatures and pH, and high levels of hydrogen sulphide, methane, and heavy metals. Considering the challenging conditions of both the zones, we investigated positive selection in the mitochondrial genes of intertidal and deep sea gastropods using EasyCodeML.

According to the results from the site-specific models of CODEML, negative selection dominated the evolution of all mitogenes. In other words, the comparison of the LRTs based on site models (M0 *vs.* M3, M1a *vs.* M2a, M7 *vs.* M8 and M8a *vs.* M8) revealed that the evolutionary rates of the PCGs are under negative constraints. Additionally, purifying selection was prevalent on all mitochondrial PCGs as revealed from the FEL analyses, with few sites evolving under neutrality (*p*-value < 0.05). From Fig. 1, it is evident that the PCGs (*cox1* - *cox3*) encoding complex IV subunits had the highest percentage of codons under negative selection (Fig. 1). PCG-encoding complex III (*cytb*) also displayed high levels of purifying selection, whereas genes encoding complex I and V showed relatively more relaxed selection. In contrast, sixteen positively selected sites were identified in eight genes (*atp8*, *cytb*, *cox1*, *cox3*, *nd2*, *nd4*, *nd5* and *nd6*) using MEME and FEL (**Table 3**).

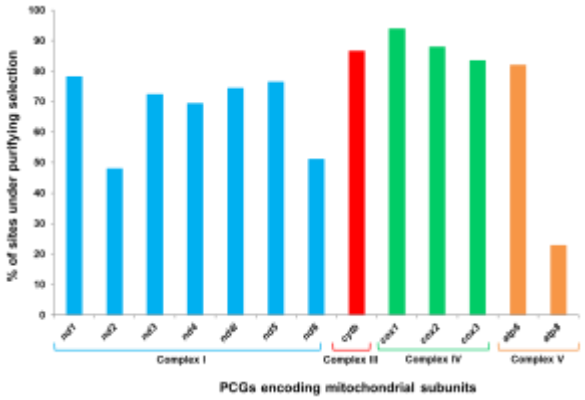

Figure 1: Percentage of sites under purifying selection in PCGs encoding mitochondrial subunits of gastropods, detected by FEL test. The subunits are colored according to their corresponding complexes: complex I (blue), complex III (red), complex IV (green) and complex V (orange).

Table 3: Sites of different mitochondrial PCGs under positive selection, as detected by MEME & FEL in the HyPhy package

Genes	***Sites under positive selection***	
	MEME	**FEL**
atp8	46	-
cytb	8	-
cox1	17, 254	-
cox3	50	-
nd2	133	-
nd4	46, 198, 400	188
nd5	188, 455, 551	581
nd6	92, 173	173

As diversifying selection occurs at only few sites in a certain lineage for most protein-coding genes, identification of natural selection becomes restricted based on-site models only. Hence, we applied branch-site model, which allows ω value to differ between branches and sites at the same time, to measure varying

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selection pressure on the specified foreground branch against the remaining background lineages. We detected potential positively selected sites in 12 PCGs with posterior probabilities ≥ 95% using the Bayes Empirical Bayes method (Fig. 2). Comparison of the mitochondrial PCGs of intertidal and deep sea gastropods revealed that the former lineage has experienced most diversifying selection. Only few sites located on the *nd2*, *nd3*, *nd4*, *nd5*, *cytb* and *atp8* gene were found to be positively selected along the branches of deep sea gastropods. Moreover, it is apparent from the results that for both intertidal and deep sea gastropods, PCGs encoding complex I subunits exhibited higher number of positively selected sites than PCGs encoding other complexes.

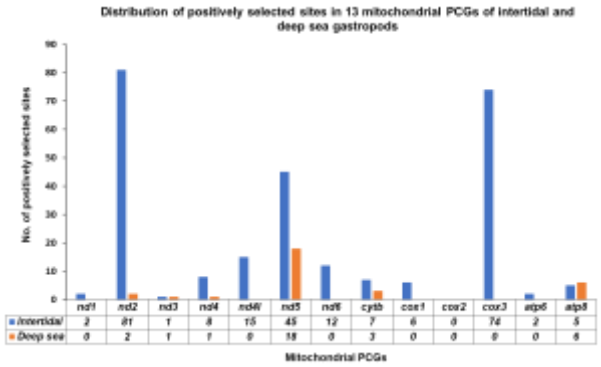

Figure 2: Distribution of positively selected sites, obtained from the branch-site models of CodeML, in 13 mitochondrial PCGs of intertidal and deep sea gastropods. The blue and orange bars represent the number of positively selected sites in intertidal and deep sea gastropods, respectively.

As a proton pump, the NADH dehydrogenase (Complex I) is the largest and most complicated enzyme complex of the respiratory chain. The efficacy of the proton pumping process can be influenced by mutation of this complex forming subunits, a feature crucial for adaptive evolution [31,32]. ND2 and ND5 are considered to be the central members for maintenance of the proton pump due to their sequence similarity with a class of Na^+/H^+ antiporters [33]. The effectiveness of the proton pump may either increase or decrease upon non-synonymous substitutions in these subunits. High number of positively selected sites are exhibited by these two ND protein-coding genes for both intertidal and deep sea gastropods that can be attributed to their high mutation rate [6]. CYTB of complex III plays a primary role in production of energy by catalyzing reversible electron transfer from ubiquinol to cytochrome *c* coupled to proton translocation [34]. We observed evidence of positive selection in *cytb*, which may be correlated with the adaptation of gastropods to their environment. In line with previous studies [35,36], signatures of positive selection were observed in *cox1* and *cox3* along the branches of intertidal gastropods, which probably allows intertidal organisms to adapt to intermittent hypoxia. Additionally, few sites that were identified under diversifying selection in members of complex V (*atp6* & *atp8*), may be involved with the regulation of the electrochemical gradient across the inner mitochondrial membrane to drive ATP production [37]. Therefore, the non-synonymous substitutions in ATPase genes provide possible implications on adaptive evolution of the stress-tolerant intertidal gastropods and also may help deep sea gastropods to thrive under harsh deep sea conditions.

Unlike two deep sea hydrothermal vent limpets (*Lepetodrilus nux* and *Pseudorimula sp.*), we found maximal instances of diversifying selection acting differentially on all the members of mitochondrial complexes in the 13 intertidal gastropods considered here. This may be attributed to the fact that though deep sea vent systems are driven by several challenging abiotic conditions, *i.e.*, no sunlight and highly acidic and toxic vent fluids emerging at extreme temperatures, most vent species are restricted to their particular biogeographic territory [38]. On the other hand, intertidal gastropods have to persist in the extremely variable environment of the intertidal zone caused by periodic changes of temperature, salinity, pH, and hydrodynamic forces [15]. Hence, survival of intertidal organisms depends on an extensively modified and adaptive energy metabolism. Although the mechanisms of physiological adaptation in intertidal gastropods remain elusive, our results clearly reflect the dynamic evolution of their mitochondrial genomes. Overall, this study provides insight into the mitogenomic adaptations of intertidal and deep sea gastropods.

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