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Application of Molecular Phylogeny and Bioinformatics for Identification of Cryptic Species in a Community

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ABSTRACT

Molecular markers like *mt*DNACOI have proved their identification efficiency in discriminating closely related species which may possibly have a vital effect on biodiversity estimation.Traditional methods based on morphology often fail to identify closely related species causing an over or under estimation of biodiversity. Studies have demonstrated that marine fauna exhibits high degree of cryptism. Thus, a lot of species either remain unidentified or have been identified erroneously. Present study involves the morphological identification of a group of limpet species found in the intertidal zones of Saurashtra coastline Gujarat followed by their COI gene sequence analysis. They were identified as *Cellana karachiensis*.Interestingly the three sample types: X, Y and Z, sharing the same habitat not only exhibit clear variations in their shell banding pattern but also show minor variations in their COI gene sequence. A total of six COI gene haplotypes were found using DnaSP version 5. A striking association of COI haplotypes to specific morphotypes was seen. Construction of phylogenetic tree using Mr. Bayes 3.2 clarifies the phylogenetic relationships of these samples indicating a split of lineages. This intriguing observation has put forth many possibilities. Is this plasticity due to polymorphism? Could they be a complex of cryptic species undergoing sympatric or peripatric speciation? It is also possible that they had been geographically isolated earlier and after genetic divergence occurred, came back to same location. This study thus points towards the need to reinvent an accurate baseline database of marine speciesand highlights the potential of molecular taxonomy in distinguishing between closely related species.

Key Words: Biodiversity Estimation; Molecular Taxonomy; *mt*dna Coi; Speciation; Limpet; Kathiawar Peninsula

INTRODUCTION

Research all over the world suggests that marine fauna exhibit a high degree of cryptic, sibling or closely related species (Vrijenhoek 2009). A database created without considering this feature of the marine ecosystem leads to an over- or underestimation of the marine biodiversity. A thorough study of these closely related species would give us a deeper understanding of the evolutionary aspects of these species. It has been seen that pressures like food, mating, etc leads to a pressure for natural selection and the organisms start to diverge or show variance. If this variation is beneficial to the organisms and is successfully inherited by the offspring this new variant becomes a part of the population. However, during the initial stages of divergence, the difference is so negligible that it can go unnoticed. If the species show no considerable genotypic variation while exhibiting morphological plasticity or vice versa it becomes difficult to discriminate these species, especially when they have to be identified and classified. Traditional morphological identification often fails to differentiate these closely related species (Herbert et al. 2003). Again, lack of expertise in taxonomy can lead to incorrect identification. Modern approach of molecular taxonomy can be accepted for biodiversity estimation with faster and accurate identification (Neusser et al. 2011). This is a vital matter to consider for conservation of marine

fauna as the extinction may outpace species identification (Bucklin et al. 2011).The present study was therefore undertaken to show the possible effect of cryptism in biodiversity estimation and to demonstrate the limitations of traditional identification methods in discriminating between closely related species. The hypotheses to be answered were: (1) can cryptism have a significant effect on biodiversity estimation? (2) Whether there are any challenges faced by traditional methods for species identification. If yes, whether molecular taxonomy using novel methods like DNA barcode (*mt*DNA COI gene analysis) is efficient in discriminating between closely related species. We studied a group of intertidal marine limpets (phylum Mollusca) collected from the Saurashtra coastline, off Veraval coast, Gujarat. Of the data collected for population estimation, few exhibited morphological plasticity. Molecular taxonomy of these species using the DNA barcode region viz. COI sequence of the mitochondrial DNA, showed minor dissimilarities in their sequences which raise concerns regarding identification and biodiversity estimation so far.

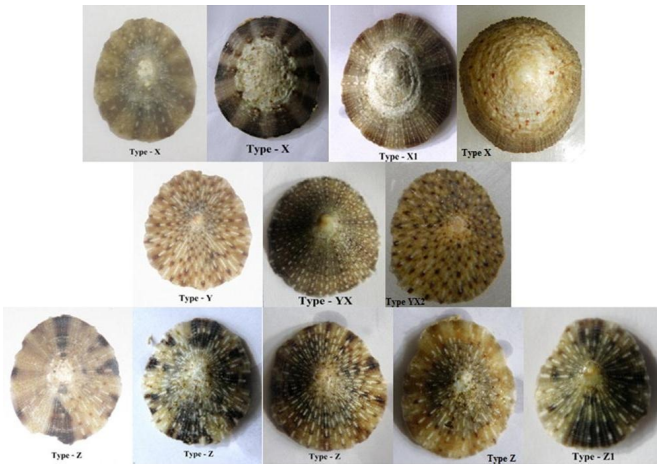

Figure 1. *Cellana karachiensis* with different shell banding patterns (Sneha Joseph et al. 2016 b)

METHODS

The Patellogastropod mollusc samples were collected from the intertidal zones of Veraval ($20^0$54' N, $70^0$21' E) coast, Gujarat, by using belt transects. The samples were identified morphologically using available keys (Prasad et al. 1984).This data was very useful in calculating the population density of the species on this coastline (Faladu et al. 2014, Vakani et al. 2014). The samples that were difficult to identify by morphology alone were sent for COI sequencing of mtDNA. Three such samples belonging to the genus *Cellana* showed slight variation in their shell banding pattern categorized as type X, type Y and type Z (Figure 1).

To check the intraspecific variation, twelve such samples were collected from different sites: Dwarka (22^0 13' N, 68^0 58' E), Okha (22° 28′ N, 69° 4′ E), Veraval (20^0 54'N, 70^0 21'E), Sarkeshwar (20^0 50' N, 71^0 19' E) and Mahuva (21° 4' N, 71° 48' E) along the Gujarat coastline, off the northern Arabian Sea.

COI Gene Analysis

The sequencing of the COI gene of mitochondrial DNA which is typically taken as the DNA barcode region for the metazoans, was done at the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India. The sequences were amplified using the universal primers LCO: 5'-GGTCAACAAATCATAAAGATATTGG-3' (forward) and HCO: 5'-TAAACTTCAGGG TGACCAAAAAATCA-3' (reverse) (Folmeret al. 1994), and analysed using BLAST algorithm (NCBI, BOLD) and submitted to Genbank and BOLD (Table 1). Total number of COI gene haplotypes of the *C. karachiensis*-variants observed along the coastline was determined using DnaSP version 5 (Rozas 2009)A Median joining network was generated to establish the relationship between the haplotypes X, X_1, X_2, Y, Z and Z_1 setting 'parsimony informative sites' as the statistical analysis and mutations indicated with hatch marks using raxmlGUI version 1.5 (Silvestro and Michalak 2012**)**. To understand the phylogenetic relationships between the COI gene sequences of the morphotypes, the COI sequences were aligned using ClustalW (MEGA version 6.06). A Bayesian phylo-genetic tree was constructed using Mr. Bayes 3.2 (Ronquist et al. 2012). For this analysis the evolutionary model was set to GTR (generalized time reversible model) with gamma-distributed rate variation across sites and a proportion of invariable sites. X haplotype was set as outgroup based on the result of the median joining network. The number of generations was set to 20,000 and sampling frequency was set to tenth generation. Since the standard deviation of split frequencies obtained was below 0.01, summaries of the parameter values were obtained with 95% credibility interval of each parameter. The potential scale reduction factor was close to one, hence a cladogram was generated and phylogenetic tree constructed using

TreeView version 1.6.6 (Page 1996). The amino acids coded by the codons of the variant nucleotide were analyzed.

Table 1. Accession number of sequences submitted to GenBank

Sample Id	GenBank Accession No.	Specimen morphotype	COI gene haplotype
ZMGDNC5.3	KF840075	Z	Z
ZMGDNC5.1	KF840077	X	X
ZMGDNC5.2	KF840076	Y	Y
ZMGDNC5.6	KP698009	Z	Z
ZMGDNC5.5	KP698010	Z	Z
ZMGDNC5.4	KP698011	X	X
ZMGDNC1.9	KP698012	Z	Z_1
ZMGDNC13.8	KP698013	Z	Z
ZMGDNC13.7	KP698014	Y	X
ZMGDNC13.6	KP698015	X	X_1
ZMGDNC9.14	-	X	X
ZMGDNC10.15	-	Y	X_2

RESULTS

The sampled organisms were morphologically identified as *Cellana karachiensis*. The BLAST result of COI gene sequence of the specimens showed approximately 99% similarity with *C. karachiensis*, which is a new record from the Indian subcontinent (Faladu et al. 2014, Sneha et al. 2016a, 2016b). Six COI gene haplotypes were obtained X, X_1, X_2, Y, Z and Z_1 (DnaSP version 5). A median joining tree was constructed which indicated X as the parent haplotype (Figure 2). Bayesian phylogenetic analysis using Mr. Bayes 3.2 showed similar results with three diverging lineages, wherein the haplotypes X-Y, X_1-X_2 and Z-Z_1 formed different lineages (Figure 3). All the X morphotypes showed the presence of either X or X_1 haplotypes, Y morphotypes showed the presence of Y, X or X_2 haplotypes while the Z morphotypes showed the presence of either Z or Z_1 haplotypes. The amino acids coding demonstrated that in all the three cases if the variant nucleotide was in the first or second position of the codon they would code for different amino acids. However, if this variant nucleotide was at the third position of the codon all the three COI sequences would code for the same amino acids.

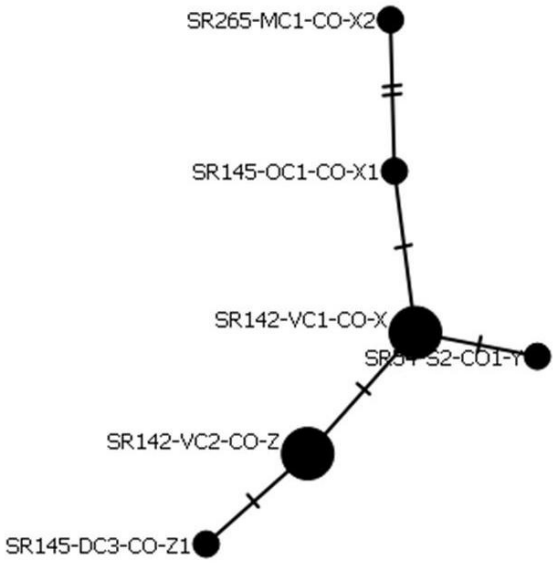

Figure 2. A Median joining network showing relationship between the haplotypes X, X_1, X_2, Y, Z and Z_1 (Hatch marks indicates mutation).

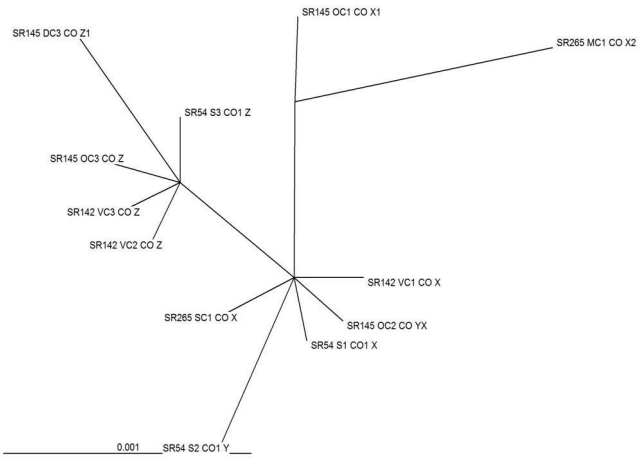

Figure 3. Two diverging lineages: X –Y, X_1-X_2 and Z-Z1 shown by Bayesian phylogenetic analysis using Mr. Bayes 3.2

DISCUSSION

C.karachiensis has been observed from the Gulf of Oman, Pakistan (Zafar and Ayub 2013) and Gulf of Kuchchh, with rocky muddy coastline different from the rest of the Indian coastline (Sneha et al. 2014, Sneha et al. 2016a). The variation in the species found in both the ecologically supporting habitats could possibly be due to allopatric speciation. The three variants of *C. karachi-*

ensis observed showed apparent morphological plasticity and minor variation in their COI sequences which is a highly conserved sequence. Moreover, the different haplotypes were associated with specific morphotypes. Many explanations are possible for these mysterious observations which raise critical question regarding the identification and speciation of species. For example, is it simply a case of polymorphism? If so, it is expected to find many such shell banding patterns among this species complex without any genetic variation. The present population estimation data however suggests the presence of only three such banding patterns (Faladu et al. 2014). The second possibility is that they are a complex of cryptic species undergoing speciation. The three X, Y and Z types of *C. karachiensis*, share same habitat between themselves, possibly exhibiting sympatric speciation, with X and Y morphotypes exhibiting either partial or unidirectional mating, while Z morphotype shows lack of genetic exchange. It is also possible they had been geographically isolated earlier and after genetic divergence occurred, merged or came back to same locations (Schön et al. 2012). It may be an example of peripatric speciation (Payne et al. 2011) where small founding population from the same source as in Oman population, entered into isolated niche between Gulf of Kutch and Gulf of Cambey.

CONCLUSIONS

The present investigation demonstrates a case study where morphological plasticity in *C. karachiensis* is supported by genetic divergence in the COI gene, a highly conserved region often considered as a reliable molecular clock in the animal system to distinguish closely related species. Can we rely on molecular phylogeny? If so, what about the variants YX with phenotypic characteristics of Y type and X COI gene haplotype? Whether there is a partial or complete reproductive barrier in these variants is a hypothesis yet to be tested. If so, this could possibly be the beginning of a speciation process which often goes unnoticed in nature until the variation becomes large enough to be visible to us.

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REFERENCES

Bickford, D.; Lohman, D.J.; Sohdi, N.S.; Ng, P.K.L.; Meier, R.; Winker, K.; Ingram,K.K. andDas,I. 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22:148–155.

Bucklin, A.; Steinke,D. andBlanco-Bercial,L. 2011. DNA barcoding of marine metazoan. Annual Review Marine Science 3: 471-508.

Faladu, J.; Vakani, B.; Poriya, P. and Kundu, R. 2014. Habitat preference and population ecology of Limpets *Cellana karachiensis* (Winckworth) and *Siphonaria siphonaria* (Sowerby) at Veraval Coast of Kathiawar Peninsula, India. Journal of Ecosystems. Article ID 874013, p 1-6. [Online] doi.org/10.1155/2014/874013.

Folmer, O.; Black, M.; Hoeh, W.; Lutz, R. and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Herbert, P. D. N.;Cywinska, A.; Ball,S. L. anddeWaard,J. R. 2003. Biological identification through DNA Barcodes. Proceedings of the Royal SocietyB. 270(1512): 313-21.

Neusser, T.P.; Jörger, K.M. and Schrödl, M. 2011. Cryptic species in tropic sands – interactive 3d anatomy, molecular phylogeny and evolution of meiofaunal Pseudunelidae (Gastropoda, Acochlidia). PLoS ONE 6(8): e23313. [online] doi:10.1371/journal.pone.0023313.

Payne, J.L.; Mazzucco, R. and Dieckmann, U. 2011. The evolution of conditional dispersal and reproductive isolation along environmental gradients. Journal of Theoretical Biology 273: 147–155.

Prasad, M. N.;Malli, P.C. and Mansur, A.P. 1984. The color banding pattern and frequencies in a tropical limpet *Cellana radiata* (Born) on the Veraval coast of western India. Journal of the Marine Biological Association of India 24: 67-68.

Ronquist, F.; Teslenko, M.; Van Der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A. and Huelsenbeck, J.P. 2012. Mr Bayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3): 539-42.

Rozas, J. 2009. DNA sequence polymorphism analysis using DnaSp. Pages 337-350, In: Posada, D. (Editor) Bioinformatics for DNA Sequence Analysis: Methods in Molecular Biology Series 537. Humana Press, New Jersey.

Silvestro, D. and Michalak, I. 2012. raxmlGUI: a graphical front-end for RAxML. Organisms Diversity & Evolution 12: 335–337.

Schön, I.; Pinto, R. L.; Halse, S.; Smith, A.J.; Martens,K. and Birky, C.W. 2012. Cryptic species in putative ancient asexual Darwinulids (Crustacea, Ostracoda). PLoS ONE 7: e39844. [online] doi:10.1371/journal.pone.0039844.

Sneha Joseph; Poriya, P. and Kundu, R. 2014. Probing the phylogenetic relationships of a few newly recorded intertidal zoanthid of Gujarat coast (India) with mtDNA COI sequences. Mitochondrial DNA [online] doi/abs/10.3109/19401736.2014.971239.

Sneha Joseph; Poriya, P.; Vakani, B.; Singh, S. P. and Kundu, R. 2016a. Identification of a group of cryptic marine limpet species, *Cellana karachiensis* (Mollusca: Patellogastropoda) off Veraval coast, India, using *mt*DNA COI sequencing. Mitochondrial DNA Part A 27(2): 1328–1331.

Sneha Joseph; Vakani, B. and Kundu, R. 2016b. Molecular phylogenetic study on few morphotypes of a Patellogastropod *Cellana karachiensis* from northern Arabian Sea reveals unexpected genetic diversity. Mitochondrial DNA Part A. (In press).

Vakani, B.; Poriya, P. and Kundu, R. 2013. Spatio-temporal variations in the population ecology of two limpets in a rocky intertidal shore of south Saurashtra coast (Gujarat: India). The Ecoscan 8 (1 & 2): 71-75.

Vrijenhoek, R. C. 2009. Cryptic species, phenotypic plasticity, and complex life histories: Assessing deep-sea faunal diversity with molecular markers. Deep-Sea Research II 56:1713–1723.

Zafar, F.S.H. and Ayub, Z. 2013. Allometric variations and condition factor in *Cellana karachiensis* (Winckworth, 1930) found at two adjacent rocky of Karachi, Pakistan. Indian Journal of Geo-marine Sciences 42: 794-799.

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