СЕЛЕКЦІЯ, ГЕНЕТИКА ТА БІОТЕХНОЛОГІЇ

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MOLECULAR IDENTIFICATION AND BARCODING OF TWO SHRIMP SPECIES (*PARAPENAEOPSIS SCULPTILLIS* AND *PARAPENAEOPSIS HARDWIKII*) COLLECTED FROM GORAI CREEK OF MUMBAI, WEST COAST OF INDIA

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Purpose. *Molecular identification of two shrimp species (Parapenaeopsis sculptillis and Parapenaeopsis hardwikii) from Gorai creek, Mumbai, west coast of India.*

Methodology*. The specimens of Shrimps Parapenaeopsis sculptillis and Parapenaeopsis hardwikii were collected from Gorai creek. The samples were morphologically identified as per the FAO guidelines manual and by using taxonomic keys. Genomic DNA was extracted from muscle tissue using DNA isolation kit (Hi Media, India). Molecular identification was carried out by using cytochrome oxidase subunit I (COI), gene sequencing by using specific primers LCO1490 and HCO2198. Phylogenetic tree was constructed by neighbour-joining method using mega 6 software to determine the relationship of the samples with known sequences in database.*

Findings. *The P. sculptillis and P. hardwikii showed closest sequence similarities with P. cornuta (84%) and Thysanopoda obtusifrons (83%). A phylogenetic tree was constructed based on COI gene, which separates the populations into thirteen stable clades. The results on DNA barcoding and current distribution of P. culptillis and P. hardwikii and their haplotype P. cornuta and Thysanopoda obtusifrons showed phylogenetic relationship among them, providing insights into the adaptive evolution of DNA sequences. The phylogenetic divergence analyses of the selected specie showed worldwide distribution because the above said species and their haplotype species showed complex sequence diversities that are having functional relevance with energy metabolism and environmental adaptation.*

Originality. *First attempt to use molecular genetic techniques for the identification of Parapenaeopsis sculptillis and Parapenaeopsis hardwikii and to compare their DNA sequences with other haplotype species such as P. cornuta and Thysanopoda obtusifrons.*

Practical value. *The obtained data can be used for shrimp species identification and studies of their phylogenetics and population genetics.*

Keywords: *species identification, barcoding, shrimp, phylogenetic, Gorai Creek.*

PROBLEM STATEMENT AND ANALYSIS OF LAST ACHIEVEMENTS AND PUBLICATIONS

Shrimps and prawns have great economical value as they earn valuable foreign exchange. Generally, more than 10 million tons of crustaceans are produced annually for human consumption. Crustacean often referred to as "Insect of Sea" as the range of morphological characteristics for taxonomical identification exceeds than that of the insecta. There are approximately 50,000–67,000 crustaceans have been estimated worldwide. They show an enormous diversity and different range of sizes. Morphological identification of crustaceans is very critical because, this group has different larval stages, sexual dimorphism, plasticity trading etc. [26]. The unique colour system in crustacean often plays an important role in aquaculture because their colour affects the quality and market price CSIRO [8]. Prawns, like most other crustaceans are able to change colour depending upon growth, background coloration and time of day due to chromatophores Montgomery [23]. These problems can be overcome by molecular identification or DNA Barcoding.

HIGHLIGHT OF THE EARLIER UNRESOLVED PARTS OF THE GENERAL PROBLEM. AIM OF THE STUDY

The term 'DNA barcode' was recently used in the literature by Floyd, et al., and Hebert, et al. [9, 14]. It is generally use for rapid and accurate species identification [13]. The term 'DNA barcodes' was first used by Arnot, et al. [3], in their research paper but they have not received more attention from the scientific community. Even though the concept of species identification using molecular tools was older one and it was proposed by Kangethe, et al. [17]. However, the work carried out by Hebert, et al. [14] has given the golden age of DNA barcoding which embark on 2003, due to which many biological scientist have started their work on DNA barcoding. Therefore, DNA barcoding is currently used as a powerful and efficient tool for species level identification and also found useful for taxonomic and biodiversity research. Nowadays, a number of DNA barcode sequences provides a unique 'horizontal' genomics perspective with broad implications [22]. Computational technology found very useful for sequencing and it also provides a major source of new information for advancing our understanding about the evolutionary and genetic relationship with the help of DNA barcoding. The footprints of DNA sequence are now useful in almost all areas of biological sciences [29].

Classification and identification of Species by traditional method has become specialized domain of taxonomists. Nomenclature of species provides a backbone and key prerequisite for numerous biological studies. Nowadays, human society is facing numerous crucial biological issues including maintenance of biodiversity, wild life conservation, bio-security and many others to protect the species. To achieve such goals successive action plans are needed in a global basis to identify and describe the species and prevent them from extinction. Therefore to achieve this goal we have applied a tool of molecular identification of shrimp species and find its molecular phylogenetics and population genetics by DNA barcoding.

MATERIALS AND METHODS

a) Sample Collection and Identification:

The specimens of Shrimps *Parapenaeopsis sculptillis* and *Parapenaeopsis hardwikii* were collected from 200 meters away from Gorai creek of Mumbai from September, 2014 to, December 2014. The Shrimp samples, packed in propylene bags, were stored at – 20 °C in deep freeze in the Department of Zoology, S.S & L.S. Patkar College, Goregaon (West) Mumbai for further analysis. The samples were identified as per the FAO guidelines manual and by using taxonomic keys described by Kathirvel and Thirumilu [18],"Edible Penaeid Shrimps in India" in the Training Manual "GIS and Marine Biodiversity" edited by John Milton (2008).

b) Genomic DNA isolation and PCR amplification of cytochrome oxidase gene

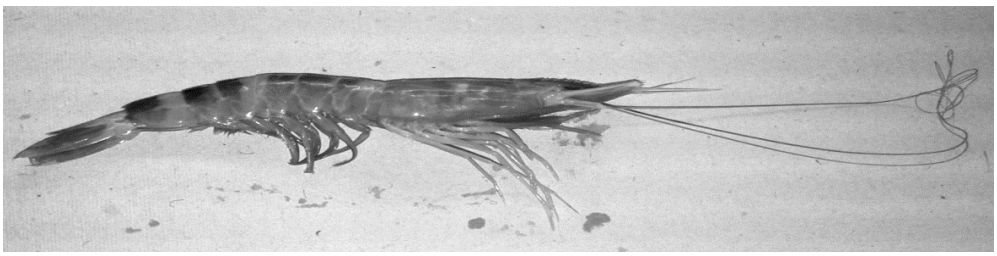
Genomic DNA was extracted from muscle tissue of sample MT1 and MT2 using DNA isolation kit (Hi Media, India) as per manufacturer's instructions. Integrity of the extracted DNA was assessed by 0.8% agarose horizontal gel electrophoresis in TAE buffer (40 mM Tris, 20 mM acetate, and 2 mM EDTA) visualized by Gel Red staining on a gel documentation unit (Protein simple). The concentration of the extracted DNA was analyzed by Nano Drop Lite spectrophotometer (Nano Drop Biotechnologies). PCR amplification of 700 base pair fragment of cytochrome oxidase subunit I (COI) gene was carried out using specific primers LCO1490(5′-GGTCAACAAATCATAAGATATTGG-3′) and HCO2198(5′TAAACTTCAGGGT GACCAAAAAATCA-3′) [10]. The PCR was carried out in 50 µL of reaction mixture containing 10 nM (each) primer (Eurofins), 200 µM (each) deoxynucleoside triphosphate (dNTP) (Genei), 1 U of Taq polymerase (Genei) in the appropriate reaction buffer, and 100 ng of DNA extract as a template. PCR conditions were 35 cycles of 60 s at 95 °C, 60 s at 58 °C, and 1 min 30 s at 72 °C. PCR amplified products were purified using Exo-rSAP (USB) and sequenced in ABI 3500xl genetic analyzer (Thermo fisher). The COI sequences of MT1 and MT2 have deposited under gen bank accession number (genbank accession MT1_ KT898913 and genbank accession MT2 _KT898914) respectively.

c) Phylogenetic analysis

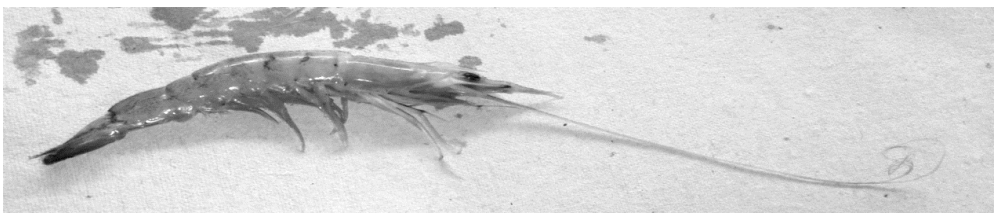
The edited mitochondrial COI gene sequences from shrimp samples collected from west cost of India were analyzed by BLASTN and BLASTX databases from NCBI [2]. High quality alignment of around 550 bp sequences was used to construct Phylogenetic tree. For both the samples MT1 and MT2, phylogenetic tree was constructed by neighbour-joining method using MEGA 6 software to determine the relationship of these samples with known sequences in database. One thousand bootstrap iterations were generated to derive a consensus tree (Tamura et al. 2013).

STUDY RESULTS AND THEIR DISCUSSION

In this study, a total of two species of Penaeid shrimps namely *Parapenaeopsis sculptillis* and *Parapenaeopsis hardwikii* were identified by morphologically. Among these, according to Kathirvel and Thirumilu [18], the Penaeid shrimps family comprises of 13 genera and 105 species. The photographs of *Parapenaeopsis sculptillis* and *Parapenaeopsis hardwikii* species and their morphological differences are presented in Figure 1.



Parapenaeopsis sculptillis



Parapenaeopsis hardwikii

Figure 1. **The Shrimp samples Parapenaeopsis sculptillis and Parapenaeopsis hardwikiiwere collected from Gorai creek of Mumbai**

Among the above species identified morphologically were further subjected to DNA barcoding. These species successfully amplified with the universal decapods COI specific primer used in this study. This may be due to several reasons right from the handling of tissue samples to the steps involved in PCR amplification, since the same primer has produced successful amplification. The isolated genomic DNA showed greater than 1Kb nucleotides (Figure 2).

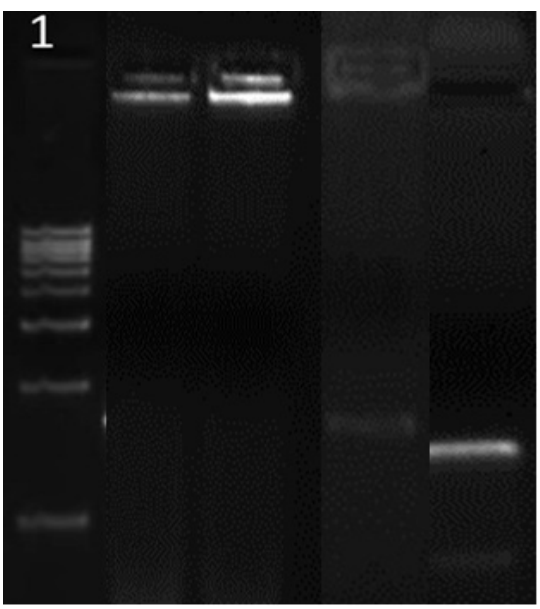


Figure 2. **Genomic DNA and PCR amplification of Cytochrome oxidase gene. Lane 1; 1Kb DNA ladder (Genei), Lane 2; Genomic DNA of MT1, Lane 3; Genomic DNA of MT2, Lane 4; PCR amplified product of MT1Lane 5. PCR amplified product of MT2.**

The amplified products showed approximately 700bp (Figure 2). Several studies have been reported that sequence diversity in a ~650 bp region near the 5' end of the MT-COI gene provides strong species-level resolution for varied animal groups, including birds [1, 12], fishes [7, 30], springtails [5, 24], spiders [5, 6] and moths [16, 20]. It has been reported that the primer pair, (LCO_MT1_NC101214 and LCO_MT2_NC021214) was not so ''universal'' as thought before, as it would still fail to amplify some taxa [21, 28]. While performing BLAST (using BLASTn and BLASTx), the sequences of the above said species showed varied degrees of similarity with existing data in the NCBI database (Table 1).

Table 1. **Sequence analysis of MT1 and MT2 Samples in the NCBI database**

Sr. No	Sample	Gene sequence	NCBI BLASTn	NCBI BLASTx
1	MT1_NC101214	COI	*KP072694* *Parapenaeopsis cornuta voucher NTOU-M0186* Identities:436/516(84%) Yang et. al.(Zool. Scr. 44 (3), 312-323 2015	AGV33575 *Parapenaeopsis stylifera* Identities: 140/159(88%) unpublished
2	MT2_NC021214	COI	GU183782 *Thysanopoda obtusifrons* voucher UCONN:Eu02.2.1 Identities:444/534(83%) Bucklin et. al. (Deep Sea Res. Part II Top. Stud. Oceanogr. 57 (24-26), 2234-2247 2010) ++ AY350987 *Munidapsamathe* voucher Fo89 Identities:434/522(83%) Machordom et. al. (Mol. Phylogenet. Evol. 33 (2), 259-279 2004)	AEX10285 *Munidarutllanti* Identities: 138/156(88%) Matzen et al PLoS ONE 6 (5), E19449 (2011)

For BLASTn (i.e. at nucleotide level) *Parapenaeopsis sculptillis* showed 84% similarity with the *Parapenaeopsis cornuta*) and the species *Parapenaeopsis hardwikii* showed 83% similarity with the *Thysanopoda obtusifrons* voucher UCONN:Eu02.2.1 cytochrome oxidase subunit I (COI) gene genbank accession MT2 _KT898914 voucher NTOU-M01863 cytochrome oxidase subunit I (COI) gene (GenBank accession MT1_ KT898913) (GenBank accession MT2 _KT898914). For BLASTx (i.e. at amino acid level) *P. sculptillis* showed 88% similarity with *P. stylifera* (Genbank accession AGV33575) and *P. hardwikii* showed 88% similarity with *Munidarutllanti* (GenBank accessionAEX10285).

Divergence estimation:

DNA barcoding has become a promising tool for rapid and accurate identification of various taxa and it has been used to reveal unrecognized species in several animal groups. The COI gene was selected as the standard barcode to establish phylogenetic relationships among species *Parapenaeopsiss culptillis and Parapenaeopsis hardwikii*

from major habitats, in West coat of India including Gorai Creek west coast of Mumbai. This study has revealed in identifying the shrimp species *Parapenaeopsiss culptillis* and *Parapenaeopsis hardwikii* with the help of potentiality of DNA barcodes. Phylogenetic tree of MT1 and MT2 samples based on COI gene, and determined using MEGA tool, wherein 1000 bootstrap replicates. For maximum likelihood phylogenetic tree showed polyphyletic clades (Figure 3).

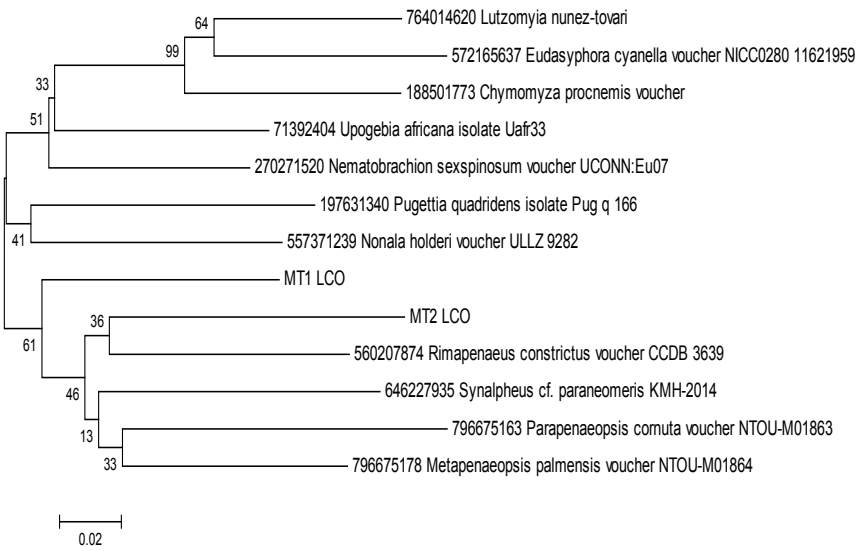


Figure 3. **Phylogenetic tree of MT1 and MT2 samples based on COI gene, and determined using MEGA tool, wherein 1000 bootstrap replicates were used.**

The non-linear tree exhibited two major clades among the subjected and retrieved species. This may be due to the differences in primers used. Similar opinion has been postulated by [25]. In the present study, the nucleotide divergence for the selected shrimp species was calculated as between 0.00 -0.207% with the average interspecies nucleotide divergent value of 0.103% which is less than the significant 3% threshold value as per the 10X rule of [11]. Our results are also found similar with the reports drawn by Avise [4] who found genetic divergence in majority of the recorded species are less than 1% in the case of same species, whereas the divergence exhibited greater than 2% for mitochondrial DNA in few cases. Ratnasingham and Herbert [27] also drawn similar conclusion by using similar criteria for identification of species through matching of query sequence with the reference barcode records. Ward et al. [30] reported a divergence level of 0.39% and 9.93% for individuals within species and species within genera for Australian fishes respectively, while Lakra et al. [19] observed the average distances within species and genera as 0.30% and 6.60% respectively in Indian marine fishes. Our results are also in agreements with the above said demonstrations. Therefore, both the species studied are closely related to each other, and also with the retrieved species. The phylogram constructed in this study showed that the *Parapenaeopsis cornuta* and *Thysanopoda obtusifrons* are having a separate branch which indicates the reliability of phylogenetic tree. The pair-wise analysis suggests that the mean pair-wise distance between LCO_MT1 and LCO_MT2 was relatively low when compared with out-group. The nucleotide sequence diversity

was found to be low within the different clades of LCO_MT1 and LCO_MT2. BOLD analysis has precisely identified both the sequence as *Parapenaeopsis cornuta* and *Thysanopoda obtusifrons*. Figure 3 depicts the details of distinct haplotypes available in the database for the selected shrimp species. While searching for species haplotypes only one sequence was retrieved from the GenBank databases *Parapenaeopsis sculptillis* showed its haplotype *Parapenaeopsis cornuta* where as we were unable to find the sequence in GenBank databases of *Parapenaeopsis hardwikii* its haplotype *Thysanopoda obtusifrons.*

Table 2: The distance matrix based on COI gene (similarity index) between MT1 and MT2 sample. The standard sequences from genbank were used for comparison.

[1] #MT1_LCO
[2] #MT2_LCO
[3] #796675163_Parapenaeopsis_cornuta_voucher_NTOU-M01863
[**4]** #560207874_Rimapenaeus_constrictus_voucher_CCDB_3639
[5] #197631340_Pugettia_quadridens_isolate_Pug_q_166
[6] #796675178_Metapenaeopsis_palmensis_voucher_NTOU-M01864
[7] #764014620_Lutzomyia_nunez-tovari
[8] #646227935_Synalpheus_cf._paraneomeris_KMH-2014
[9] #71392404_Upogebia_africana_isolate_Uafr33
[10] #572165637_Eudasyphora_cyanella_voucher_NICC0280_11621959
[**11]** #270271520_Nematobrachion_sexspinosum_voucher_UCONN:Eu07
[**12]** #188501773_Chymomyza_procnemis_voucher
[13] #557371239_Nonala_holderi_voucher_ULLZ_9282
[1 2 3 4 5 6 7 8 9 10 11 12 13]

[1]
[2] 0.207
[3] 0.188 0.253
[4] 0.183 0.173 0.193
[5] 0.188 0.217 0.238 0.193
[6] 0.183 0.173 0.178 0.164 0.212
[7] 0.207 0.270 0.238 0.227 0.222 0.259
[8] 0.173 0.197 0.202 0.183 0.212 0.173 0.253
[9] 0.183 0.207 0.217 0.197 0.193 0.188 0.178 0.207
[10] 0.212 0.264 0.297 0.253 0.227 0.270 0.136 0.286 0.197
[11] 0.173 0.183 0.238 0.197 0.183 0.183 0.193 0.217 0.136 0.202
[12] 0.212 0.264 0.280 0.212 0.227 0.248 0.141 0.270 0.183 0.154 0.169
[13] 0.178 0.243 0.238 0.233 0.173 0.217 0.248 0.202 0.164 0.233 0.159 0.227

Geographical distribution:

We have also found the general distribution pattern of *Parapenaeopsis sculptillis* and its haplotype *Parapenaeopsis cornuta* and it was found that the general distribution of *Parapenaeopsis sculptillis* from west coast of India through Malaysian waters and Indonesia to Hong Kong in the north and tropical Australia and New Guinea in the south. Along the Indian coasts this is mostly represented in the northern region of the west coast and the east coast. In these places the species contribute to the fishery to a certain extent, where as the distribution pattern of its haplotype *Parapenaeopsis cornuta* was found in two different geographical regions, an equatorial spread from the west coast of India and Ceylon through Malaysia to the Philippines and New Guinea. In Indian waters although not contributing to a fishery it has been recorded from Bombay and Kerala on the west coast and off Madras on the east coast. The general distribution pattern of *Parapenaeopsis hardwickii* and its haplotype *Thysanopoda obtusifrons* was found, in Indian coasts including Gorai Creek west coast of Mumbai. It was found that the *Parapenaeopsis hardwickii* reported in Indian waters the species is distributed in the North West coast in Bombay waters and on the east mainly off river Godavari estuary. In these two places it contributes to a fairly good fishery, especially in Bombay waters. With respect to the global distribution it was reported in Malaysia to southern China waters where as its haplotype *Thysanopoda obtusifrons* the general distribution pattern was found in the Indian Ocean from the equator to about 30°S. With respect to the global distribution the known range is approximately 40°N-30°S in the Atlantic, including the Gulf of Mexico and off northwest Africa but not the Gulf of Guinea. In the Pacific it is from 35°N to 35°S, but is lacking in the Eastern Tropical Pacific, eastern boundary currents, and much of the western tropical Pacific.

CONCLUSION AND PERSPECTIVES OF FURTHER DEVELOPMENT

In this study, the universal decapods primer, LCO_MT1_NC101214 and LCO_MT2_NC021214 has worked well with both the species *Parapenaeopsis sculptillis* and *Parapenaeopsis hardwikii.* Further studies with other species are necessary to conclude that whether the primer used is species specific. The subjected species and literature data showed average nucleotide divergence of *Parapenaeopsis is* 1.43% [26] from its closest relative. Consequently, our results on DNA barcoding and comparative analysis reveal the current distribution of *Parapenaeopsiss culptillis and Parapenaeopsis hardwikii* and their haplotype *Parapenaeopsis cornuta* and *Thysanopoda obtusifrons* showed phylogenetic relationship among them, providing insights into the adaptive evolution of DNA sequences. Based on their phylogenetic and divergence analyses, the selected species showed worldwide distribution because these species and their haplotype species reveal complex sequence diversities that are expected to have functional relevance such as energy metabolism and environmental adaptation. Therefore the species could not be discriminated.

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МОЛЕКУЛЯРНА ІДЕНТИФІКАЦІЯ І ДНК-ШТРИХКОДУВАННЯ ДВОХ ВИДІВ КРЕВЕТОК (*PARAPENAEOPSIS SCULPTILLIS* І *PARAPENAEOPSIS HARDWIKII*) З ГОРАЙ-КРІК, МУМБАЇ, ЗАХІДНЕ УЗБЕРЕЖЖЯ ІНДІЇ

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Мета. *Провести молекулярну ідентифікацію двох видів креветок (Parapenaeopsis sculptillis та Parapenaeopsis hardwikii) з Горай-Крік, Мумбаї, західне узбережжя Індії.*

Методика. *Креветки Parapenaeopsis sculptillis і Parapenaeopsis hardwikii були виловлені в Горай-Крік і ідентифіковані за морфологічними ознаками з використанням таксономічних ключів визначника ФАО. Геномна ДНК була виділена з м'язової тканини із застосуванням набору для виділення ДНК (Hi Media, Індія). Молекулярна ідентифікація зразків здійснювалася з використанням цитохромоксидазної субодиниці I (COI), секвенування генів проводилося з використанням специфічних праймерів LCO1490 and HCO2198. Філогенетичне дерево було побудоване методом «приєднання сусідів» за допомогою програми Mega 6, яка визначає зв'язок досліджуваних зразків з відомими послідовностями, що знаходяться в базі даних.*

Результати. *Показано, що ДНК-послідовності креветок P. sculptillis і P. hardwikii дуже схожі з ДНК-послідовностями P. cornuta (84%) та Thysanopoda obtusifrons (83%). Було побудовано філогенетичне дерево, яке розділяє популяції на тринадцять стабільних клад. Результати ДНК-штрихкодування і сучасне поширення креветок P. sculptillis і P. hardwikii і їх гаплотипів P. cornuta і Thysanopoda obtusifrons показали близькі зв'язки філогенезу між ними, що дозволяє зрозуміти адаптивну еволюцію їх ДНК-послідовностей. Аналіз філогенетичної дивергенції досліджуваних видів показав їх широке поширення, що пов'язано з тим, що ці види, а також їх родинні види, характеризуються значною різноманітністю ДНК-послідовностей, що відіграє активну роль в енергетичному метаболізмі і адаптації до різних умов навколишнього середовища.*

Наукова новизна. *Була виконана перша спроба проведення молекулярної ідентифікації креветок Parapenaeopsis sculptillis і Parapenaeopsis hardwikii та порівняння їх ДНК-послідовності з іншими спорідненими видами, такими як P. cornuta і Thysanopoda obtusifrons.*

Практична значимість. *Отримані дані можуть бути використані для ідентифікації видів креветок і дослідження їх філогенетики і популяційної генетики.*

Ключові слова: *ідентифікація видів, ДНК-штрихкодування, креветки, філогенетика, Горай-Крік.*

МОЛЕКУЛЯРНАЯ ИДЕНТИФИКАЦИЯ И ДНК-ШТРИХКОДИРОВАНИЕ ДВУХ ВИДОВ КРЕВЕТОК (*PARAPENAEOPSIS SCULPTILLIS* И *PARAPENAEOPSIS HARDWIKII*) ИЗ ГОРАЙ-КРИК, МУМБАИ, ЗАПАДНОЕ ПОБЕРЕЖЬЕ ИНДИИ

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Цель. *Провести молекулярную идентификацию двух видов креветок (Parapenaeopsis sculptillis и Parapenaeopsis hardwikii) из Горай-Крик, Мумбаи, западное побережье Индии.*

Методика*. Креветки Parapenaeopsis sculptillis и Parapenaeopsis hardwikii были выловлены в Горай-Крик и идентифицированы по морфологическим признакам с помощью таксономических ключей определителя ФАО. Геномная ДНК была выделена из мышечной ткани при помощи набора для выделения ДНК (Hi Media, Индия). Молекулярная идентификация образцов осуществлялась с использованием цитохромоксидазной субъединицы I (COI), секвенирование генов проводилось с использованием специфических праймеров LCO1490 и HCO2198. Филогенетическое дерево было построено методом «присоединения соседей» с помощью программы Mega 6, которая определяет связи исследуемых образцов с известными последовательностями, находящимися в базе данных.*

Результаты. *Показано, что ДНК-последовательности креветок P. sculptillis и P. hardwikii очень схожи с ДНК-последовательностями P. cornuta (84%) и Thysanopoda obtusifrons (83%). Было построено филогенетическое дерево, которое разделяет популяции на тринадцать стабильных клад. Результаты ДНК-штрихкодирования и современное распространение креветок P. sculptillis и P. Hardwikii, а также их гаплотипов P. cornuta и Thysanopoda obtusifrons показали близкие филогенетические связи между ними, что позволяет понять адаптивную эволюцию их ДНК-последовательностей. Анализ филогенетической дивергенции исследованных видов показал их широкое распространение, что связано с тем, что эти виды, а также их родственные виды, характеризуются значительным разнообразием ДНК-последовательностей, что имеет функциональную значимость в энергетическом метаболизме и адаптации к разным условиям окружающей среды.*

Научная новизна*. Была выполнена первая попытка проведения молекулярной идентификации креветок Parapenaeopsis sculptillis и Parapenaeopsis hardwikii и сравнения их ДНК-последовательности с другими родственными видами, такими как P. cornuta и Thysanopoda obtusifrons.*

Практическая значимость. *Полученные данные могут быть использованы для идентификации видов креветок и исследования их филогенетики и популяционной генетики.*

Ключевые слова: *идентификация видов, ДНК-штрихкодирование, креветки, филогенетика, Горай-Крик.*