DNA barcoding of the protected horned helmet, *Cassis cornuta* (Linnaeus 1758)†

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The horned helmet *Cassis cornuta* (Linnaeus 1758) is the largest of all helmet shells belonging to the family Cassidae. In India, *C. cornuta* is protected under the Wildlife Protection Act, 1972 (Schedule-I, Part IV-B) due to its conservation importance. Also, it is one of the most sought after tropical marine molluscs in illegal trading. In the present study, we have performed DNA barcoding for this species using the mitochondrial marker gene, cytochrome c oxidase subunit 1 (COI), and deposited the data at GenBank (accession no. MK878541). The evolutionary history was inferred adopting the maximum likelihood method and Kimura 2-parameter model by encompassing representative organisms belonging to the genus *Cassis*. As *C. cornuta* is in great demand among shell collectors and is illegally traded across countries, the DNA barcode data available in the public database would provide an additional checkpoint in reducing the chance of unlawful trade of this shell. Further, it strengthens conservation management practices, particularly at the port of entries where portable DNA barcoding facilities are in practice.

Keywords: *Cassis cornuta*, COI gene, DNA barcoding, protected species.

DNA barcoding is a molecular method that enables species identification by amplifying short DNA sequences from a specific target gene. It helps in species identification, species discovery, delineation of new species and resolving cryptic speciation. Being an important molecular technique, it critically helps wildlife trafficking control authorities (Wildlife Crime Control Bureau (WCCB) as in India) to identify the trade prohibited/protected species even when the specimen is not in its actual form. Wildlife exploitation followed by ecosystem damage is a serious concern worldwide. Abusing wildlife by market-
The FASTA sequence of the COI gene for *C. cornuta* was searched in the databases of NCBI (https://blast.ncbi.nlm.nih.gov) and Barcode of Life Data (BOLD) Systems (http://www.boldsystems.org/index.php). This exercise led us to recognize that there were no earlier records for *C. cornuta* in the databases. Based on the classical taxonomic details and COI gene sequences, the DNA barcode data were submitted to GenBank through BankIt submission.

To understand the phylogenetic relationship of *C. cornuta* with other organisms of this Cassidae family, the COI gene sequences for members of genus *Cassis*, *Cypraecassis*, *Dalium*, *Galeodea*, *Oocorys*, *Scosnia*, *Casmaria*, *Echinophoria*, *Phalium* and *Semicassia* were downloaded from NCBI to construct a phylogenetic tree. In total, 23 COI sequences representing 10 genera from the Cassidae family were aligned using MUSCLE prior to phylogenetic tree construction. The evolutionary relationship was inferred using the maximum likelihood method and Kimura 2-parameter model. The bootstrap consensus tree inferred from 500 replicates was chosen to represent the evolutionary history of the taxa analysed.

The evolutionary analysis was performed using MEGA X software.

**Taxonomic position:**

Phylum: Mollusca  
Class: Gastropoda  
Subclass: Caenogastropoda  
Order: Littorinimorpha  
Superfamily: Tonnoidea  
Family: Cassidae  
Genus: *Cassis* Scopoli, 1777  
Species: *Cassis cornuta* (Linnaeus, 1758)

**Distribution:** *Cassis cornuta* (Linnaeus 1758) is the largest and heaviest shell under the genus *Cassis* and has a wide distribution from East Africa to Polynesia, including...
Madagascar and the Red Sea to eastern Polynesia (Figure 1). In Indian waters, it has been recorded in the Gulf of Mannar and the Andamans\textsuperscript{15}.

Shell structure: The shells are normally characterized by an ovate shape; one or more varices; a large body whorl; well-developed parietal or columellar shield and a small bulimoid shell nucleus (Figure 2).

Diagnostic characters: Massive shell structure, up to 350 mm length, spherical, attains large size through a wide and flat apertural side. The body whorl with spiral rows of large tubercles. Large calloused shield connects columella with outer lip. Calloused ventral side is glossy and cream or orange in colour. Dorsal side and spire are grayish-white in colour. Large and prominent teeth, spire low, corona with prominent axial varices at approximately right angles to each other; sculpture with heavy knobs and spiral cords\textsuperscript{16,17}. The helmet shell occurs in colonies at depths ranging between 1 and 15 fathoms (~27 m), where the bottom is made of sand and broken coral rocks\textsuperscript{16}. It is active at night and buries itself partially under the sand during inactive period. \textit{C. cornuta} preys on \textit{Acanthaster planci} (crown of thorns), the thorny sea star feeding on corals and causing large-scale destruction to reefs.

The amplified COI PCR product of size ~710 bp from \textit{C. cornuta} mitochondrial DNA was qualitatively confirmed by agarose gel (Figure 3a). The electropherogram for the COI gene sequence was reconstructed from the

\textbf{Figure 3.} COI PCR amplification and gene sequencing. \textit{a}, Representative gel photograph with amplifications of COI PCR product (710 bp) from \textit{C. cornuta} DNA. The 100 bp DNA ladder is also shown. \textit{b}, Electropherogram of the COI gene displaying the quality of DNA sequencing. \textit{c}, The COI gene sequence of \textit{C. cornuta} in FASTA format.
raw sequence file (.abi) using chromatogram viewer, Chromas V 2.6.6 application (Figure 3b). The DNA sequence of COI gene in FASTA format has also been provided (Figure 3c). The final processed DNA barcode data were submitted to GenBank (accession no. MK878541).

Within the genus Cassis, excluding C. cornuta, the COI DNA barcode data are available only for three species. The sequence homology of Cassis flammea (MH581308.1), C. madagascriensis (MH581309.1), C. fimbiaria (MH581307.1) and C. cornuta (MK878541.1) was analysed using multiple sequence alignment (Figure 4). As the Cassidae Latreille, 1825 family includes medium-sized to very large sea snails commonly called helmet shells or bonnet shells, the C. cornuta relationship study was extended with other members (outgroup) of the Cassidae family. It is evident from the phylogenetic tree that there are three major clades based on the size of the shells. Clade 1 (Casmaria) includes medium-sized shells, clade 2 (Semicassis, Echinophoria, Phalium) contains medium sized to large shells, and clade 3 (Cassis, Oocorys, Eucorys, Galeodea, Scionia, Cypraecassis) has mostly large-sized shells (Figure 5). The tree with the highest log likelihood (~5986.89) is shown in Figure 5. Initial tree was obtained by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances assessed using the maximum composite likelihood approach, and then selecting the topology with superior log-likelihood value.

Mollusca is the second largest phylum in the animal kingdom. Despite their large numbers, molluscan populations are decreasing locally, regionally and globally due to varied reasons. The shells of molluscs, particularly marine, have always been objects of fascination for humans18,19. Owing to their size, diverse morphology, eye-catching colours and decoration, molluscan shells are treated as extremely popular souvenirs, and are either collected from the wild or purchased from traders18,20. In overseas trade, they are merchandized as pieces of decorative as well as utility items, and even as sentimental items19,21. The trade of marine ornamental molluscs and other marine life-forms such as corals, hard-structured/stuffed marine vertebrates and invertebrates as curios, is fetching a major source of revenue. The overexploitation of molluscs due to their high commercial demand is one of the formidable reasons driving them to an endangered
status. Many studies have documented large-scale shell trade across the globe, especially in the Philippines\textsuperscript{22}, Brazil\textsuperscript{19}, Java\textsuperscript{8}, South India\textsuperscript{1}, Zanzibar\textsuperscript{14}, Hainan Island\textsuperscript{24}, Bali\textsuperscript{20} and Singapore (freshwater mollusc)\textsuperscript{25}. Other than \textit{C. cornuta}, the protected species involved in trade are chambered nautilus (\textit{Nautilus pompilius}), giant clams (\textit{Tridacna spp.}), top shell (\textit{Trochus niloticus}) and marbled turban (\textit{Turbo marmoratus})\textsuperscript{7}. Besides their trade value, molluscs have a crucial role in the ecosystem well-being. They play a key role as feeders of decomposed organisms in the terrestrial ecosystem and their faecal matter forms organic detritus in estuaries. Therefore, focus on conservation strategies for these ecological engineers is of paramount importance to maintain a healthy ecosystem. DNA barcoding has been extensively used for chitons\textsuperscript{26}, gastropods\textsuperscript{27-31}, bivalves\textsuperscript{32,33} and cephalopods\textsuperscript{14}. Using molecular techniques, the conservation management strategies would be much more rapid, effective and beneficial to the community.

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 1973)\textsuperscript{15} is the main international statutory regulation to control the international trade in wild fauna and flora, to which India has been a signatory since 1976. Being a participant country in CITES and Convention on Biological Diversity (CBD), India has established several legal frameworks to protect biodiversity and use the natural resources in an ecologically sustainable manner. To the best of our knowledge, no DNA barcoding data have been previously deposited for \textit{C. cornuta}, a protected gastropod mollusc species in India.

In India, under the Wildlife Protection Act, 1972, various threatened species are protected by categorizing them in schedules from I to VI based on their significance. Under the Act 24 species of marine molluscs, including \textit{C. cornuta} are protected. In this study, we report DNA barcode data for \textit{C. cornuta} using COI gene. Although \textit{C. cornuta} is a most popular collection item in illegal trading, it is not yet listed under CITES; only four of the scheduled species (\textit{Hippopus hippocus}, \textit{N. pompilius}, \textit{Tridacna maxima} and \textit{Tridacna squamosa}) are included in the CITES appendix II (Checklist.cites.org). Access to the DNA barcode data of this species will aid in reducing its unlawful trading significantly.

Conflict of interest: The authors declare that they have no conflict of interest.

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Natural regeneration dynamics of tree species along the altitudinal gradient in a subtropical moist deciduous forest of northern India

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The present study was conducted to examine the regeneration pattern in 72 random plots of six tropical forest sites of Rajaji Tiger Reserve, Uttarakhand, India. The population structure of the forest was determined through density of seedlings, saplings and trees from the sampling quadrat. Based on individual density of seedling, sapling and adult, the regeneration potential of the species was determined. A total of 58 tree species under 50 genera belonging to 30 families were recorded in the forest. The overall density ranged from 1525 to 6600 ind/ha and the total basal area ranged from 0.577 to 46.81 m²/ha in case of trees and saplings, whereas for seedlings, the value ranged from 511.96 to 1221 cm²/ha. The good regeneration pattern of tree species varied from 12.12% to 31.57%, fair regeneration pattern from 12.12% to 31.57%, new regeneration from 5.26% to 39.13%, poor regeneration from 0% to 10.52%, and no regeneration of trees from 15.78% to 42.42%. Inadequate regeneration status and population structure of tree species like Shorea robusta, Careya arborea, Ficus auriculata were observed which could be due to looping, scraping, grazing and trampling. Anthropogenic disturbances have resulted in the population decline of tree species which may lead to many species becoming endangered, rare and threatened. Therefore, proper management and conservation initiatives with active involvement of the locals must be taken to protect the tropical forest sites of the Reserve.

Keywords: Population structure, natural regeneration, saplings, seedlings, Tiger reserve, trees, tropical forest.

THE tropical forest biomes cover about 6 million km² of the earth’s surface with the tropical rainforest, dry deciduous and savannah. About 50% of the total plant species grow in these tropical forests. The tropical moist deciduous forests are found throughout India, except in the northwestern and western regions of the country. These forests are more pronounced in regions which receive rainfall in the range 100–200 cm. They are found in the North East states of the country and in the foothills of the Himalaya, and are succeeded by wet temperate forests between altitudes 1000 and 2000 m. In NE India and the hilly areas of Uttarakhand and West Bengal, these forest mainly comprise of Tectona grandis, Shorea robusta, Poecilogonous species of sea slug (Opisthobranchia: Sacoglossa) Krug, P. J., Ellingson, R. A., Burton, R. and Valdes, A., A new species of planktonic gastropods (Pteropoda and Heteropoda) from six ocean regions based on DNA barcode analysis. Deep Sea Res. Part II, 2010, 57(24–26), 2199–2210; https://doi.org/10.1016/j.dsr2.2010.09.022.

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