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[1]. 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 marketing it, either whole or its parts, is one of the most lucrative activities across the globe[2,3]. Simultaneously, trade activities in wildlife are a key threat to biodiversity[3–5]. According to the International Union for Conservation of Nature (IUCN), 1141 species representing the phylum Mollusca are being traded, and 695 of them are categorized in the sport hunting/specimen collecting group of trade and use[6]. One of the important species belonging to the genus *Cassis*, and often involved in illegal trade is *Cassis cornuta* (Linnaeus 1758) (horned helmet). *C. cornuta* and other few molluscs are traded because of their value as ornaments and in medicine, or as a source of protein. Between 2008 and 2013, more than 32,000 shells of *C. cornuta* valued at USD 500,000 were confiscated in Indonesia[7].

In India, *C. cornuta* is protected under the Wildlife Protection Act, 1972 (Schedule-I, Part IV-B) due to its conservation significance[8]. Although this species is popular in the international market and protected by law, there is no molecular evidence or DNA barcode data validation for its identification. In the present study, we have performed DNA barcoding for this protected marine organism using the mitochondrial cytochrome c oxidase subunit I (COI) gene and also studied the evolutionary relationship with other species under the genus *Cassis*.

The sample was collected from fish by catch discards in the landing centre at Thirespuram village (8°49′34.88″N and 78°10′2.76″E), Thoothukudi, Tamil Nadu, India.

The total DNA was isolated using standard CTAB–chloroform protocol based on Doyle *et al.*[9] for animal tissue samples. Briefly, about 20–25 mg of tissue from the foot muscle was dissected, washed with sterile Milli-Q water and preserved in 90% ethanol to prevent the DNA from degradation. The sample was stored at –20°C for later handling or processed immediately. The tissue sample was cut into smaller pieces and 700 µl of CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM, Tris-Cl (pH 8), 20 mM EDTA, 2% β mercaptoethanol) and 20 µl of proteinase K (20 mg/ml) were added. Tissue samples were vortexed briefly prior to incubation at 60°C in a water bath for 2–3 h. For every 30 min, the sample was removed from the water bath and vortexed for 15 sec for complete homogenization. The aqueous phase was gathered after adding 500 µl chloroform followed by centrifugation for 15 min at 12,000 rpm at a set temperature of 4°C. The aqueous layer was carefully removed to another fresh microcentrifuge tube and 1 ml of ice cold ethanol was added to precipitate the DNA. The precipitated DNA was pelleted down using centrifugation for 15 min at 12,000 rpm and at 4°C. The DNA pellet was washed twice with 70% ethanol and finally, dissolved by adding 40–50 µl of sterile Milli-Q water. The DNA was diluted to a final concentration of 75–100 ng/µl.

The COI gene was amplified in a 25 µl reaction volume with 2.5 µl of 10× PCR buffer, 1.25 µl of dNTPs mix (1 mM), 1.5 µl of $MgCl_2$ (25 mM), 1 µl of each primer

†All data generated or analysed during this study are included in this communication. The DNA sequence of COI gene from *C. cornuta* from this study has been submitted to the public database, GenBank with accession number MK878541. It can be accessed at https://www.ncbi.nlm.nih.gov/nuccore/MK878541. The datasets generated and/or analysed during this study are available from the corresponding author on request.

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Figure 1. Distribution of *Cassis cornuta* from East Africa to Polynesia. The distribution map was adopted from Abbott[16] and redrawn using Google Maps.

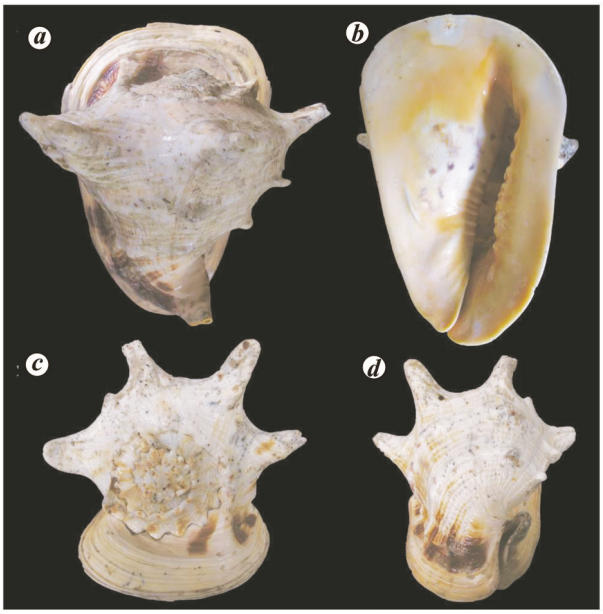

Figure 2. Shell of *C. cornuta*. ***a***, Dorsal view; ***b***, ventral view; ***c***, anterior apex view; ***d***, posterior tail view. (Photo credits: Dr R. Ravinesh, University of Kerala, Thiruvananthapuram, India.)

(20 µM), 1 U of *Taq* DNA polymerase (Sigma, USA) and 1 µl of diluted genomic DNA. The primer sequences LC01490: 5′GGTCAACAAATCATAAAGATATTGG-3′ and HC02198: 5′TAAACTTCAGGGTGACCAAAAAA-TCA-3′ were used to amplify the *COI* gene[10]. The PCR conditions were 5 min at 95°C as the initial denaturation followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C followed in turn by final extension of 10 min at 72°C. The PCR products were observed on 1.5% agarose gels, and the prominent products were selected for sequencing. The PCR products were sequenced at Eurofins Genomics, India using ABI 3730XL (Applied Biosystems, USA), following the manufacturer's instructions. The sequence data files were processed using Sequence Analysis V5.3 software.

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*, *Sconsia*, *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[11], prior to phylogenetic tree construction. The evolutionary relationship was inferred using the maximum likelihood method and Kimura 2-parameter model[12]. The bootstrap consensus tree inferred from 500 replicates was chosen to represent the evolutionary history of the taxa analysed[13]. The evolutionary analysis was performed using MEGA X software[14].

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

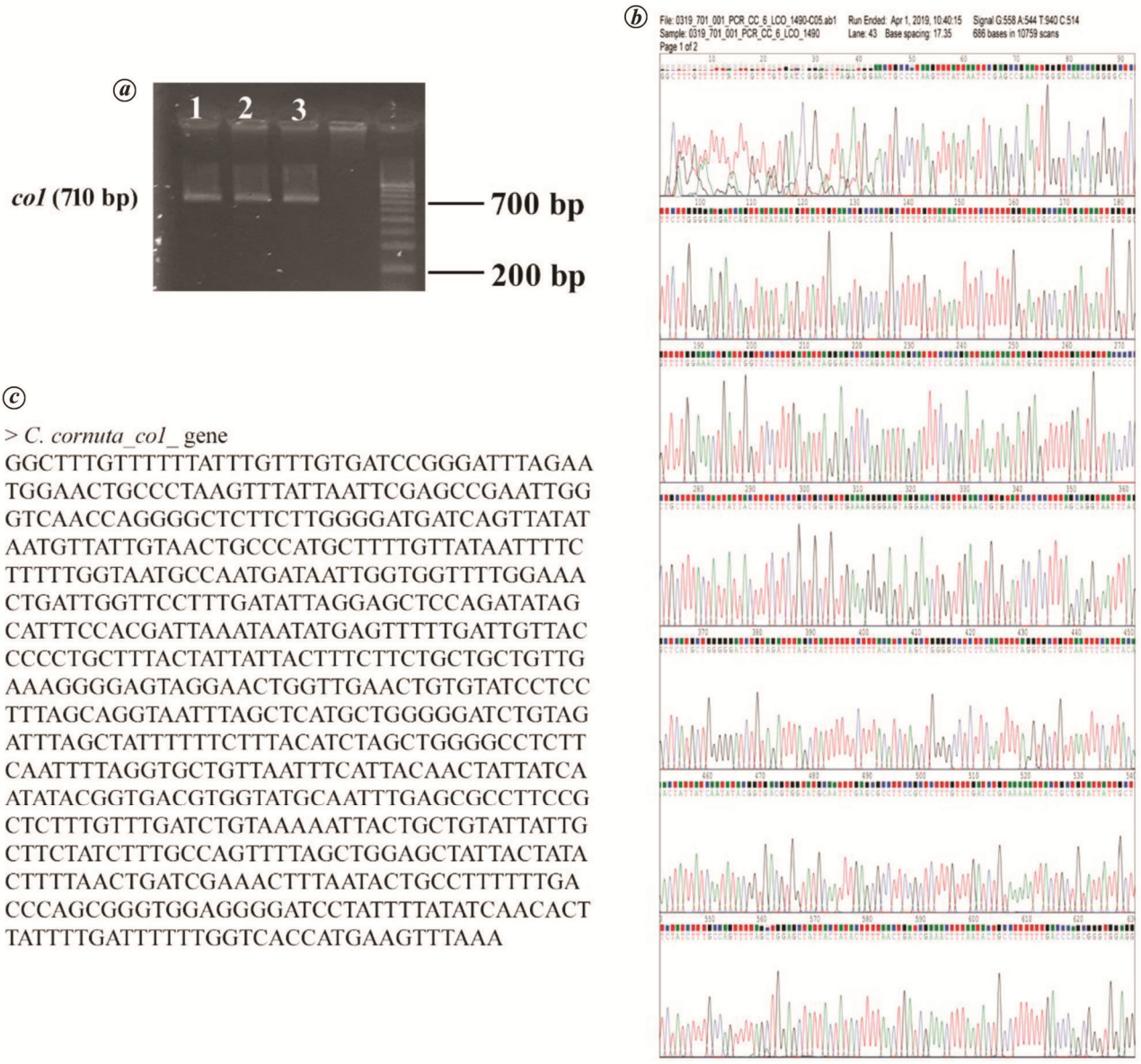

Figure 3. COI PCR amplification and gene sequencing. ***a***, Representative gel photograph with amplifications of COI PCR product (710 bp) from *C. cornuta* DNA. The 100 bp DNA ladder is also shown. ***b***, Electropherogram of the COI gene displaying the quality of DNA sequencing. ***c***, The COI gene sequence of *C. cornuta* in FASTA format.

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[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, coronate with prominent axial varices at approximately right angles to each other; sculpture with heavy knobs and spiral cords[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[16]. It is active at night and buries itself partially under the sand during inactive period. *C. cornuta* preys on *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 *C. cornuta* mitochondrial DNA was qualitatively confirmed by agarose gel (Figure 3 *a*). The electropherogram for the COI gene sequence was reconstructed from the

raw sequence file (.abi) using chromatogram viewer, Chromas V 2.6.6 application (Figure 3 *b*). The DNA sequence of COI gene in FASTA format has also been provided (Figure 3 *c*). 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. fimbriata* (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*, *Sconsia*, *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 humans[18,19]. 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 traders[18,20]. In overseas trade, they are merchandized as pieces of decorative as well as utility items, and even as sentimental items[19,21]. 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

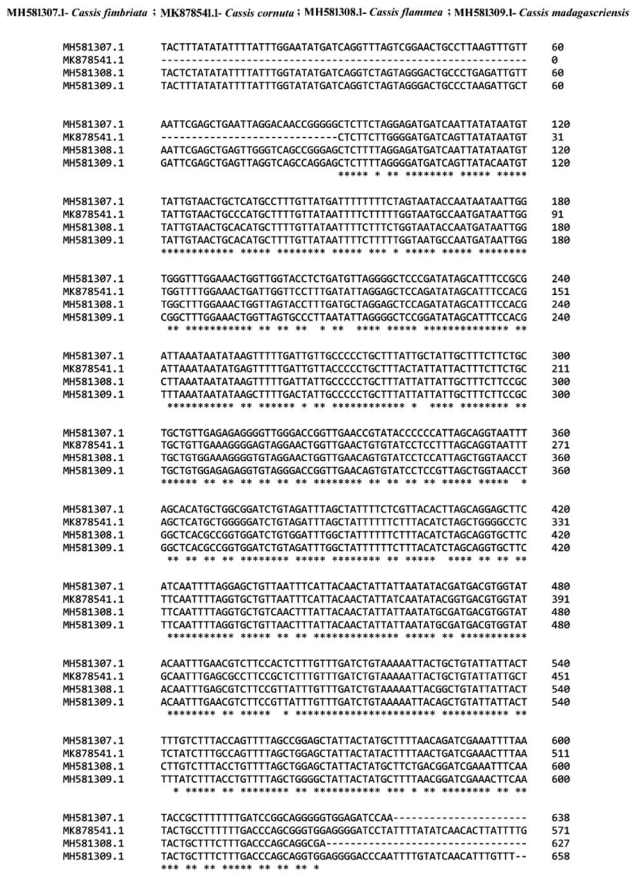

Figure 4. Multiple sequence alignment of COI gene sequences. The *COI* gene nucleotide sequences, *Cassis fimbriata*, *Cassis cornuta* (the present study), *Cassis flammea* and *Cassis madagascriensis* are aligned and the conserved nucleotide positions are marked with *.

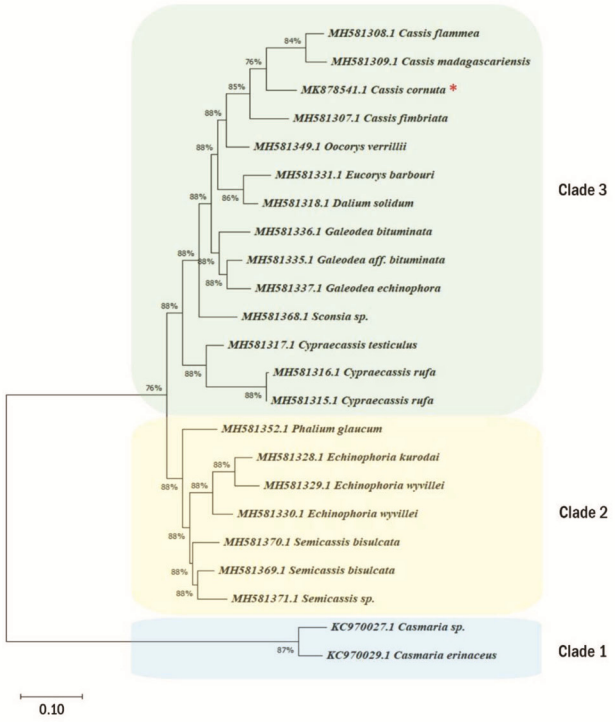

Figure 5. Evolutionary analysis using maximum likelihood method. Phylogenetic tree using COI sequences to understand the phylogenetic relationship of *C. cornuta* with other members of the genus *Cassis* and outgroup members in the Cassidae family, was constructed employing maximum likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is seen next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic tree has been constructed using MEGA X. *Represents the *C. cornuta* COI gene sequence generated in this study.

status. Many studies have documented large-scale shell trade across the globe, especially in the Philippines[22], Brazil[19], Java[7], South India[23], Zanzibar[18], Hainan Island[24], Bali[20] and Singapore (freshwater mollusc)[25]. Other than *C. cornuta*, the protected species involved in trade are chambered nautilus (*Nautilus pompilius*), giant clams (*Tridacna* spp.), top shell (*Trochus niloticus*) and marbled turban (*Turbo marmoratus*)[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[26], gastropods[27–31], bivalves[32,33] and cephalopods[34]. 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)[35] 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 *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 *C. cornuta* are protected. In this study, we report DNA barcode data for *C. cornuta* using COI gene. Although *C. cornuta* is a most popular collection item in illegal trading, it is not yet listed under CITES; only four of the scheduled species (*Hippopus hippopus*, *N. pompilius*, *Tridacna maxima* and *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.

1. Hebert, P. D. N., Cywinska, A., Ball, S. L. and deWaard, J. R., Biological identifications through DNA barcodes. *Proc. R. Soc. London*, *Ser. B*, 2003, **270**, 313–321; http://doi.org/10.1098/rspb.2002.2218
2. Robinson, J. E. and Sinovas, P., Challenges of analyzing the global trade in CITES-listed wildlife. *Conserv. Biol*., 2018, **32**(5), 1203–1206.
3. Scheffers, B., Brunno, O., Ieuan, L. and David, E., Global wildlife trade across the tree of life. *Science*, 2019, **366**, 71–76; 10.1126/science.aav5327.
4. Sutherland, W. J. *et al*., One hundred questions of importance to the conservation of global biological diversity. *Conserv. Biol*., 2009, **23**(3), 557–567; doi:10.1111/j.1523-1739.2009.01212.x.
5. Smith, K. F. *et al*., Reducing the risks of the wildlife trade. *Science*, 2019, **324**, 594–595.
6. IUCN, Red List version 2019-2: Table 1a, International Union for Conservation of Nature, United Kingdom, 2019.
7. Nijman, V., Spaan, D. and Nekaris, K. A. I., Large-scale trade in legally protected marine mollusc shells from Java and Bali, Indonesia. *PLoS ONE*, 2015, **10**(12), e0140593; https://doi.org/10.1371/journal.pone.0140593.
8. The Wild Life (Protection) Act 1972, The arrangement of sections; http://legislative.gov.in/sites/default/files/A1972-53_0.pdf
9. Doyle, J. J., Doyle, J. L., Doyle, J. A. and Doyle, F. J., A rapid DNA isolation procedure for small amounts of fresh leaf tissue. *Phytochem. Bull.*, 1987, **19**, 11–15.
10. Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R., DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol*., 1994, **3**(5), 294–299.
11. Edgar, R. C., MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*., 2004, **32**(5), 1792–1797.
12. Kimura, M., A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, 1980, **16**, 111–120.
13. Felsenstein, J., Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 1985, **39**, 783–791.
14. Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol*., 2018, **35**, 1547–1549.
15. Hornell, J., Indian molluscs. Bombay Natural History Society, Bombay, 1951.
16. Abbott, R. T., The helmet shells of the world (Cassidae). Part 1. *J. Indo-Pacific Mollusca*, 1968, **2**(9), 7–198.
17. Ramakrishna, A. D., *Manual on the Identification of Schedule Molluscs from India: 1-40*, Director, Zoological Survey of India, Kolkata, 2003, ISBN: 81-85874-97-2.
18. Gössling, S., Kunkel, T., Schumacher, K. and Zilger, M., Use of molluscs, fish, and other marine taxa by tourism in Zanzibar, Tanzania. *Biodivers. Conserv*., 2004, **13**, 2623–2639; https://doi.org/10.1007/s10531-004-2139-0.
19. Dias, T. L., Neto, N. A. L. and Alves, R. R., Molluscs in the marine curio and souvenir trade in NE Brazil: species composition and implications for their conservation and management. *Biodivers. Conserv*., 2011, **20**, 2393–2405; https://doi.org/10.1007/s10531-011-9991-5.
20. Nijman, V. and Lee, P., Trade in nautilus and other large marine molluscs as ornaments and decorations in Bali, Indonesia. *Raffles B. Zool.*, 2016, **64**, 368–373.
21. Wood, E. and Wells, S. M., The shell trade: a case for sustainable utilization, In *The Conservation Biology of Mollusk* (ed. Kay, E. A.), Occasional Paper of the IUCN Species Survival Commission No. 9, IUCN, Cambridge, 1995, pp. 41–52.
22. Dolorosa, R. G., Conales, S. F. and Bundal, N. A., Status of horned helmet *Cassis cornuta* in Tubbataha Reefs Natural Park, and its trade in Puerto Princesa City, Philippines. *Atoll Res. Bull*., 2013, 1–17; https://doi.org/10.5479/si.00775630.595.
23. John, S., Batu, M. P. K., Kuppusamy, S. and Choudhury, B. C., An assessment of legally protected marine fauna in curio trade – a market study from Tamil Nadu, India. *Int. J. Conserv. Sci.*, 2012, **3**, 217–230.
24. Larson, C., Shell trade pushes giant clams to the brink. *Science*, 2016, **351**, 323–324; https://doi.org/10.1126/science.351.6271.323.

25. Ng, T. H. *et al.*, Molluscs for sale: assessment of freshwater gastropods and bivalves in the ornamental pet trade. *PLoS ONE*, 2016, **11**(8), e0161130; doi:10.1371/journal.pone.0161130.
26. Kelly, R. P., Sarkar, I. N., Eernisse, D. J. and Desalle, R., DNA barcoding using chitons (genus *Mopalia*). *Mol. Ecol. Notes*, 2007, **7**, 177–183; doi:10.1111/j.1471-8286.2006.01641.x.
27. Hunt, B. *et al*., Poles apart: the 'bipolar' pteropod species *Limacina helicina* is genetically distinct between the Arctic and Antarctic oceans. *PLoS ONE*, 2010, **5**(3), e9835; doi:10.1371/journal.pone.0009835.
28. Jennings, R. M., Bucklin, A., Ossenbrügger, H. and Hopcroft, R. R., Species diversity 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.
29. Krug, P. J., Ellingson, R. A., Burton, R. and Valdes, A., A new Poecilogonous species of sea slug (Opisthobranchia: Sacoglossa) from California: comparison with the planktotrophic congener *Alderia modesta*. *J. Mollus. Stud*., 2007, **73**, 29–38.
30. Puillandre, N., Baylac, M., Boisselier, M. C., Cruaud, C. and Samadi, S., An integrative approach to species delimitation in *Benthomangelia* (Mollusca: Conoidea). *Biol. J. Linn. Soc.*, 2009, **96**, 696–708; https://doi.org/10.1111/j.1095-8312.2008.01143.x.
31. Puillandre, N., Samadi, S., Boisselier, M. C., Cruaud, C. and Bouchet, P., Molecular data provide new insights on the phylogeny of the Conoidea (Neogastropoda). *Nautilus*, 2009, **123**, 202–210.
32. Feng, Y., Li, Q., Kong, L. and Zheng, X., DNA barcoding and phylogenetic analysis of Pectinidae (Mollusca: Bivalvia) based on mitochondrial COI and 16S rRNA genes. *Mol. Biol. Rep*., 2011, **38**(1), 291–299; doi:10.1007/s11033-010-0107-1.
33. Mikkelsen, N. T., Schander, C. and Willassen, E., Local scale DNA barcoding of bivalves (Mollusca): a case study. *Zool. Scr*., 2007, **36**, 455–463; doi:10.1111/j.1463-6409.2006.00289.x.
34. Allcock, A. L. *et al.*, Cryptic speciation and the circumpolarity debate: a case study on endemic Southern Ocean octopuses using the COI barcode of life. *Deep Sea Res. Part II*, 2011, **58**(1–2), 242–249; https://doi.org/10.1016/j.dsr2.2010.05.016.
35. Convention on International Trade in Endangered Species of Wild Fauna and Flora, Text of the Convention, 1973; https://www.cites.org/eng/disc/ text.php

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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 m^2/ha in case of trees and saplings, whereas for seedlings, the value ranged from 511.96 to 1221 cm^2/ha. The good regeneration pattern of tree species varied from 12.12% to 31.575%, 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 km^2 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[1]. 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*,

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