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Enlightening a hidden diversity through DNA barcoding of gastropods for conservation and ecological studies: A critical review

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ABSTRACT

The class Gastropoda (Cuvier, 1795) are commonly known as slugs and snails belong to phylum Mollusca. Due to the rapid evolution of this group, it has become essential to utilize genetic markers to identify some species, as their physical characteristics are not always reliable. Therefore, the purpose of this review intended to assess the identification of gastropods using cytochrome-c oxidase-I(cox1) gene sequences of mitochondrial DNA. Partial cox1 gene sequencing from specimens can be used for DNA barcoding and consequently identification of gastropods with the aid of numerous online software programs, sequences can be further analyzed via phylogenetic reconstruction. Reviews on various research papers on extraction of genomic DNA, it may summarize that, the CTAB method is good for extraction of genomic DNA as gastropods have proteinaceous animals. Many research studies depicted that the species identification can be accomplished with success when using cox1 gene reconstruction of the phylogeny of gastropods, which may offer an alternative method of comprehending the evolution and dispersal of species.

Keywords: Gastropods, *cox1* gene, Sanger's DNA sequencing, DNA barcoding, phylogenetic reconstruction, NCBI, BOLD system

INTRODUCTION

Animals have been used as a food source and therapeutic remedies since the time of human civilization. They are rich sources of proteins, carbohydrates, lipids and fats. Modern systemic efforts established several therapeutically active animal derived compounds having anti-inflammatory, immunosuppressant, anti-tumor, anticancer activities. In India and other south Asian countries animal derived medicines are very popular, particularly in chronic disorders. Molluscs are soft bodied invertebrates and second largest phylum after Arthropods. Among them, gastropods such as snails and slugs, represent the most abundant class.

Snails in particular are important from an evolutionary point of view, as they have survived extreme environmental conditions for more than 600 million years, due to their capacity to adapt to different environments and to reach dry land. This shows that they have an innate immune system due to which they can survive in an environment full of bacteria and viruses. They are an important part of the ecosystem, helping in the foundation of aquatic environments by providing habitat, protection and food to wide varieties of living organisms. They also recycle animal and plant waste, and are rich in minerals and nutrients which makes them commercially important for aquaculture and aquaponics (Panda *et al.,*2021).

Freshwater snails form the major part of molluscs and they are highly proteinaceous, having great ecobiological significance. They are a large reservoir for pharmacologically active compounds that show antibiotic, antifungal, anti-inflammatory, analgesic, anticancer and anti-arthritic properties (Saallah *et al.,*2020). Due to prevalent use of antibiotics, problems related to antibiotic resistance have increased. This has increased the need for new antibiotics obtained from natural sources. Many studies have revealed that snails contain bioactive compounds which can be used for manufacture of antibiotics. As snails are found in abundance, even as pests (Cowie, 2002), due to which they can be utilized for the need of human welfare. Because of tasty proteinous flesh the snails are sold in market in huge quantity, therefore they are categorized as conservation status as shown in table-1 (Jadhav *et al.,* 2023).

Table 1. List of freshwater molluscs sold in market of NE India (Jadhav *et al.,* 2023)

Family	Species Name	IUCN Red List	Carrier for disease	Uses
Ampullariidae	*Pila olea*	DD	No	Food, medicine, poultry
	Pila globosa	LC	No	Food, medicine, poultry
Viviparidae	*Filopaludina bengalensis*	LC	Yes	Food, medicine, poultry
	Angyulyagra oxychaetaphora	LC	May be	Food, medicine
	Cipangopaludina lecythis	LC	May be	Food, medicine
Pachychilidae	*Brotia costula*	LC	No	Food, medicine
	Brotia assamensis	NE	No	Food, medicine
	Brotia insolita	NE	No	Food, medicine
	Brotia sp. 1	NA	No	Food, medicine
	Brotia sp. 2	NA	No	Food, medicine
Paludomidae	*Paludonus* sp. 1	NA	No	Food, medicine
	Paludonus sp. 2	NA	No	Food, medicine
	Paludonus sp. 3	NA	No	Food, medicine
	Paludonus sp. 4	NA	No	Food, medicine
	Paludonus sp. 5	NA	No	Food, medicine
	Paludonus sp. 6	NA	No	Food, medicine
Unionidae	*Parreysia* sp. 1	NA	No	Food, medicine
	Lamellidens marginalis	LC	no	Food, medicine

Table 2. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Kane *et al.,* 2008)

Denaturation	Amplification (×45 cycles)			Elongation
	Denaturation	Annealing	Elongation	
94°C	94°C	40°C	72°C	72°C
5 min.	15 sec	30 sec	45 sec	7 min

Table 3. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Castillo *et al.,* 2021)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
94°C	94°C	50°C	72°C	72°C
3 min.	20 sec	30 sec	1 min	5 min

Table 4. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Park *et al.,* 2023)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
94°C	94°C	42°C	72°C	72°C
4 min.	30 sec	30 sec	1 min	10 min

Table 5. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Borges *et al.,* 2016)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
94°C	94°C	51°C	72°C	72°C
1 min.	30 sec	90 sec	60 sec	5 min

Table 6. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Menabit *et al.,* 2022)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
95°C	94°C	50°C	72°C	72°C
2 min.	30 sec	1.5 min	1 min	5 min

Table 7. PCR program cycles for amplification of cox1 gene of mollusks for DNA barcoding (Bravo *et al.,* 2021)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
94°C	94°C	47-49°C	72°C	72°C
5 min	45 sec	45 sec	45 sec	10 min

Table 8. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Raphalo *et al.,*2021)

Denaturation	Amplification(×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
95°C	94°C	42°C	72°C	72°C
4 min.	30 sec	45 sec	45 sec	10 min

Table 9. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Ran *et al.,* 2020)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
95°C	94°C	42-52°C	72°C	72°C
3 min.	30 sec	1 min	1 min	10 min

Table 10. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Galan *et al.,* 2018)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
95°C	94°C	45°C	65°C	72°C
2 min.	30 sec	30 sec	5 min	5 min

Table 11. PCR program cycles for amplification of *cox1* gene of mollusks for DNA barcoding (Thangaraj*et al.,* 2020)

Denaturation	Amplification (×25 cycles)			Elongation
	Denaturation	Annealing	Elongation	
94°C	94°C	45°C	72°C	72°C
30 sec	30 sec	30 sec	1 min	1 min

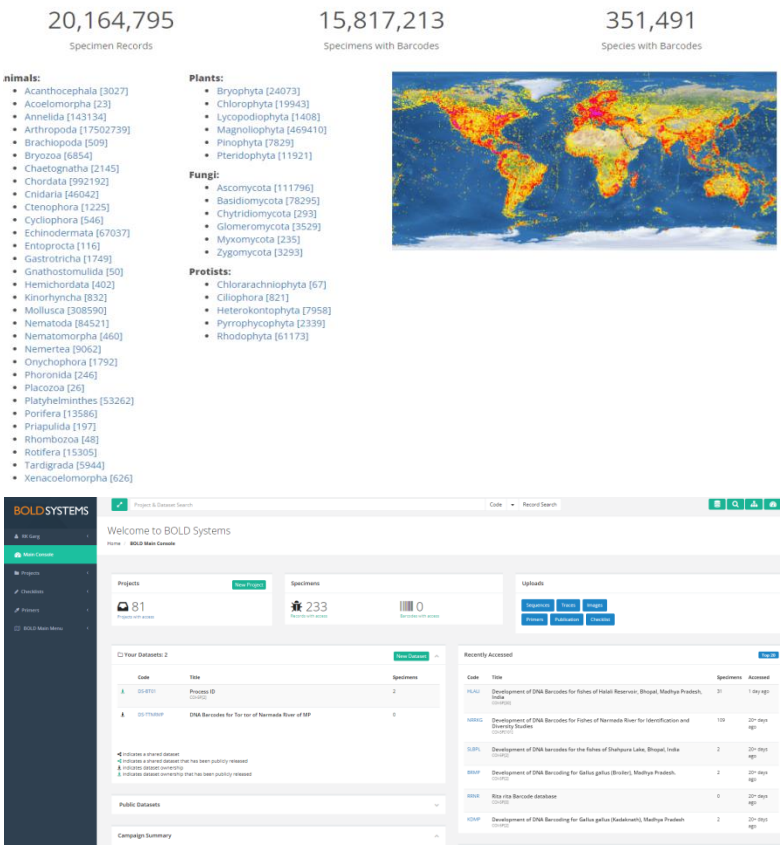

Fig. 1 BOLD systems (www.boldsystems.org) tool for development of DNA barcodes

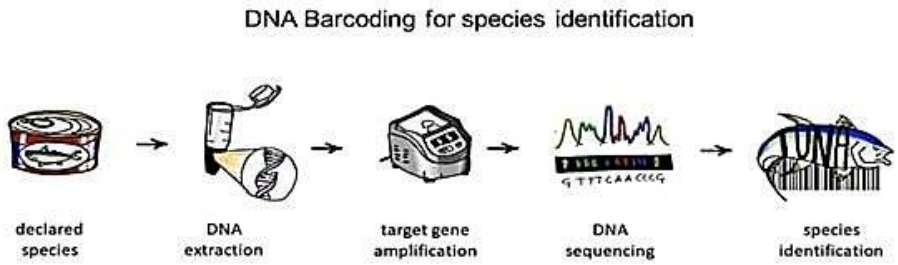

Fig. 2. Source: Flow for development of DNA barcodes animals

Prof. Paul Herbert (2003) suggested the use of mitochondrial cytochrome-c oxidase subunit I (COI) gene as the basis for animal DNA barcoding.A taxonomical technique called DNA barcoding is used to molecularly identify any species. We employ a distinct, brief genomic sequence as a marker in this. The most often used barcode area for mammals is the mt COI gene, which has 500–600 base pairs. On the other hand, the maturaseK gene (*matK*) / *rbcL* and the Internal Transcribed Spacer (*ITS*) are used for plants and fungi, respectively (Sarvananda, 2018).

In *cox1* and six additional genes, there were several introns that could potentially impede the amplification of polymerase chain reaction. The genes encoding for ATPase subunit 6, cytochrome oxidase subunit 3, and NADH dehydrogenase subunit 6 have the most promising properties for DNA barcoding among the mitochondrial genes studied, taking into account these results and the minimal length of 600 bp that is optimal for a fungal barcode. Nevertheless, no single mitochondrial gene provided a superior taxonomic resolution than the ITS, the region already extensively utilized in fungal taxonomy, according to biological validation on two fungal data sets (Vialle *et al.,* 2009).

Many apicomplexan parasites carry the mitochondrial cytochrome-c oxidase subunit-I (*cox1*) locus, which

has been widely used for sequence-based genotyping and species identification (also known as "DNA barcoding") of various organisms that use oxidative phosphorylation as a source of energy (Hebert et al. 2003). One benefit of the mitochondrial genome replication process is that it follows strict maternal inheritance, which reduces the likelihood of recombination occurrences. These factors have led to the widespread usage of genes encoded in the mitochondrial genome in molecular phylogenetics of numerous organisms (Hafeez *et al.,* 2015).

Observations on methodologies for development of DNA barcodes for identification of gastropods

In the past time, the morphometric parameters were considered for identification of large animals. But now a days, are many things required to do identification by molecular techniques, one of them is using mitochondrial *cox1* gene sequencingbased identification by development DNA barcodes. This DNA barcoding needs good amount and quality wise DNA, so, that could amplify targeted gene. DNA extraction from Snail is difficult due to the presence of high protein content which acts as a barrier for DNA extraction. To solve this problem, various methods can be used that claim to obtain a good quantity of DNA with less protein and RNA contamination. There are many scientists who worked on techniques of the isolation of genomic DNA, few are discussed below:

Genomic DNA extraction from mucus of snail

Kawai *et al.* (2004) extracted genomic DNA using chloroform-isoamyl alcohol (24:1) method where collected snails were putted on one end of a glass microscope slide and given time to go to the other side. A cotton swab that had been previously soaked in a 0.9% NaCl solution containing 1mMEDTAwas used to wipe the mucus secreted on the glass after the snail had crawled. After that, the cotton swab was moistened and turned inside a plastic tube containing 1 ml of the same solution in order to extract the mucus. There were at least five iterations of this process. After that, 9 ml of 95% ethanol were added to the tube, making the solution's ultimate volume 10 ml. For ten minutes, the fluid was centrifuged at 2500 rpm and the supernatant was discarded. Using a pipette, a portion of the precipitate was stained with May-Grünwald-Giemsa stain, and the entire mixture was inspected under a microscope. 500 µl of STE buffer (0.1 M NaCl, 10 mMTris, and 1mM EDTA, pH 8.0) was added to the residual precipitate. Using a pipette, the solution was moved into a fresh 1.5 ml tube, and it was centrifuged for 10 minutes at 2500 rpm. Following the removal of the supernatant, 250 µl of STE buffer, 25 µl of 10% sodium dodecyl sulphate, and 25 µl of proteinase-K (5 mg/ml) were added to the precipitate and allowed to sit at 55°C for 2 hours. 200 µl of phenol saturated with TE-CIA (chloroform-isoamyl alcohol 24:1) and 30 µl of 5 M NaCl were used to extract the hydrolysates, and the DNA was precipitated with a DNA precipitating kit (Ethachin Mate, Nippon Gene). 20 µl of TE (10 mMTris and 1.0 mM EDTA, pH 8.0) were used to dissolve the precipitates. The UV/VIS Spectrophotometer was used to measure the amount of DNA that was extracted from the mucus. Every snail's foot was gave good amount of genomic DNA using this technique for genomic DNA extraction.

Conventional salting out protocol

Morinha *et al.* (2014) used the conventional salting out protocol for extraction of genomic DNA, in which 300 µl of lysis buffer,10 mMTris (pH 7.5), 400 mMNaCl, 2 mM EDTA (pH 8.0) (pH 7.3-7.5), 15 µl of 20% SDS and 20 µl(20 mg/ml proteinase-K) were added and samples were incubated at 55°C for 2 hrs on a heat shaker. After removing the swabs using sterile tweezers, 50 µl of a saturated solution of 6 M NaCl was added to the extraction mixture. The samples were well mixed by vortexing for 10 seconds, and then centrifugation at 8000 g for 10 minutes was performed to precipitate any remaining cellular debris. After transferring the supernatant to a sterile Eppendorf tube, 500 µl of 100% ethanol was added to every sample, after vigorously mixing for 10 seconds with a vortex, the DNA was pelleted by centrifuging at 8000 g for 5 minutes. After washing the DNA pellets with 250 µl of 70% ethanol, they were centrifuged for five minutes at 8000 g. After fully drying by air, the pellets were reconstituted in 100 µl of sterile, nuclease-free water which gave also gave good amount of genomic DNA.

Modified CTAB DNA extraction method

Chakraborty *et al.* (2020) explained that molecular taxonomy is significantly better with a number of freshwater and terrestrial gastropod taxa. This could be explained by the fact that the main distinction between modified CTAB DNA extraction methods for animals and normal procedures is the elimination of polyphenolic chemicals using polyvinyl pyrrolidone during their studies. Because of its ability to impede amplification, too much slime (mucopolysaccharides)

is harmful to gastropod molluscs and bivalves. During PCR, it co-precipitates with DNA and inhibits the enzyme's activity. First, all of the specimens were properly cleaned in 100% ethanol and left submerged in it. Since the animal's discharge of slime caused the ethanol to become murky, it was changed every two days for the next week. Furthermore, ethanol (which was very small) was submerged in twice as much pure ethanol for an additional two days in order to get rid of any remaining slime on the tissue. Using a pestle, the alcohol-soaked tissue was slightly crushed and then placed in 400 µL of TE buffer to soften before the excess alcohol was removed. The samples were incubated for one hour at 25 to 30 °C at room temperature with light shaking at 400 rpm. 400 µl of CTAB buffer that had been heated to 60 °C was added to the softened tissue. Next, the tissue was mechanically disrupted with a bead beater after 20 µl of Qiagen Proteinase-K was added. 400 µL of chloroform:isoamyl alcohol (24:1) was used three times to extract the suspension at 12,000 rpm for five minutes at 4 °C. To guarantee that the white layer remained intact, the supernatant was carefully separated and precipitated using 800 µl of pure ethanol and 40 µl of 3 M sodium acetate at 12,000 rpm for 10 min at 4 °C. For long-term storage, the pellet was air dried, re-suspended in TE Buffer, and cleaned once more with 200 µl 70% ethanol. With the exception of the lysis buffer, which had CTAB added after autoclaving, all reagents were autoclaved after preparation. Chakraborty *et al.,* (2020) stated that the extraction of genomic DNA by using CTAB method gave more and more good quality and quantity of genomic DNA. The reviewing various research works on extraction of genomic DNA; it may conclude that, the CTAB method is good for extraction of genomic DNA as gastropods have proteinaceous animals.

Dilution of extracted genomic DNA for PCR amplifications

While using manual methods scientists often get a high quantity of DNA which is not required for PCR amplification. For amplification we only need a small concentration of DNA, which is between 40 to 60 ng/µl. Concentration and 260/280 ratio of DNA can be checked using Nano-Drop Spectrophotometer (Seipp *et al.,*2010) 260/280 ratio gives information about the quality of DNA, if ratio is <1.6 it indicates protein contamination, if ratio is nearly 1.8 it is generally accepted for pure DNA and if ratio is >2 then it indicates RNA contamination in the extracted DNA (Lucena-Aguilar *et al.,*2016).

Amplification of cox1 gene and agarose gel electrophoresis for development of DNA barcodes

Specific part of the extracted DNA needs to be amplified with certain primers which are specific for a particular phylum or kingdom. For snails, the commonly used universal primers are LCO1490 (5' GGTCAACAAATCATAAAGATATTGG3') and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA3') which were developed by Folmer *et al.* (1994). Different scientists have been used different cycles for PCR amplification as per their primers conditions as shown in table-2 to table-10. However, the more suited PCR conditions for universal primer LCO1479 (F) and HCO2198 (R) is as shown in table-6 by Bravo *et al.* (2021).

It is prepared by agarose and TAE buffer. The PCR products are generally visualized in 1% agarose gel (Mahjabin *et al.,* 2023). Ethidium bromide (EtBr) is used as fluorescent (0.003%) dye for clear visualization of DNA bands under UV rays with UV Transilluminator. The clear DNA bands are then purified with a purification kit for further sequencing (Sultana *et al.,* 2021).

DNA Sangers Sequencing

Sequencing is generally done by the Sanger sequencing method on an automatic sequencer after which the sequences are assembled using forward and reverse sequences (Sengupta *et al.,* 2009). The sequences are needs to edit using various software's like MegaX, DNA Sequencing Analysis 5.2 ver. Software, BLAST, using ORF finding online tool.

Bioinformatic analysis

The BOLD System (the Barcode of Life Data) can be used to analyze obtained sequences (Fig. 1). However, complete flow for development of DNA barcodes in animals has been depicted in Fig. 2. GenBank-NCBI can use CLUSTAL-X to align sequences, and MEGA software can be used for phylogenetic analysis using the Neighbor-Joining tree (NJ) and Maximum Likelihood (ML) method. The Kimura-2 Parameter (K2P) model can be used to calculate genetic distance (Djoemharsjah *et al.,* 2023).

Bravo *et al.,* (2021) worked on cladograms studies on molluscs by using K2P distances. Galan *et al.* (2018) studied evolutionary history inferred by using the

Maximum Likelihood (ML) method based on the General Time Reversible model and analyzed 25 nucleotide sequences using Mega software and depicted evolutionary analyses. However, the neighbor-joining tree of gastropod species based on *cox1* gene sequences studied by Thangaraj *et al.* (2020).

Earlier many scientists have worked on ecology, habitat, biochemical, antimicrobial, antioxidant and molecular taxonomy of molluscs at the international, national and local level. Some of the quantitative works have been disused below to under the current scenarios of research. Many researchers have worked on estimation of habitat by analyzing of the water quality. Few of the researchers are discussed as below on habitat and ecology of the reservoir.

Panda *et al*. (2022) analyzed the biochemical and molecular responses of *Pila* towards environmental pollutants and found that heavy metals such as Ni and Hg induce stress factors in *Pila* and degrade the protein content. This can also affect the antimicrobial and antioxidant potential of *Pila* in a negative way. Khan *et al*. (2015) studied on seasonal variations of physic-chemical parameters such as temperature, transparency, TDS, conductivity, pH, dissolved oxygen, chloride, total alkalinity, total hardness, calcium hardness, magnesium, free CO_2, nitrate and orthophosphate and found better water quality in winter season as compared to summer season due to optimum conditions during winter season. Paul *et al*. (2022) concluded that the waste shell of freshwater snails can be used as a biosorbent of phosphate ions from polluted water and phosphate loaded snails can be further utilized as fertilizer to improve the soil quality. Snails are widely used for human consumption and as a feed in aquaculture, in this way snails can play a more ideal role in the ecosystem. Talwar *et al*. (2014) revealed that the concentration of heavy metals, specifically increases in the post monsoon season because that is the period after idol immersion, also the discharge from industries and sewer lines easily merge with water bodies and results in the increase in pollutant and heavy metals concentration in water and this needs to be controlled for good health of water bodies. Chainy *et al*. (2016) concluded that animals cannot modify environmental factors like photoperiod, temperature, humidity, salinity, etc. That is why they have evolved their mechanisms to fit themselves with changing environment for survival, one such biochemical mechanism is antioxidant defense system. If temperature increases above the tolerance point, then the antioxidant defense acts against the organism because they impair cellular functions due to oxidation of biomolecules.

Basavaraju and Krupanidhi (2013) reported about the phenotypic, physiological and biochemical changes in snails during aestivation period such as closure of operculum, mucus secretion, withdrawal into shells, aperture closure, decrease in weight, shell thinning, mineral salts transport from shell to calcic cells and intestinal fluid to survive hot season and scarcity of food. This shows the potential of snails to cope against the extreme climate conditions.

Before starting of research work it is necessary to identify model animals in correct way, therefore may techniques have been developed by scientist for identification of the animals. Currently molecular taxonomy is well known and authenticated techniques of which many scientist worked, some of researches are listed below.

Araujo *et al*. (2023) concluded that the region of COI gene can be used to understand the evolution and dispersion of the *Biomphalaria* genus, which is a gastropod. Kane *et al*. (2008) concluded that DNA barcoding targeting COX I gene may offer the best method for identification of species. Jadhav *et al*. (2023) focused on the importance of molluscs as food and medicine as they studied about 18 molluscs species. They analysed DNA barcodes using COI gene as marker and also suggested snail farming for conservation. Menabit *et al*. (2022) successfully identified 16 gastropods and 12 crustaceans using DNA barcoding based on cytochrome-c oxidase I gene sequences. Merly and Saleky (2021) analyzed DNA barcodes of a gastropod species using LCO1490 and HCO2198 primers and successfully identified it as *Terebralia* species. They concluded that molecular identification is an easier, faster and more accurate method. Borges *et al*. (2016) used cytochrome oxidase I gene sequence from 108 specimens of gastropods for their molecular diversity investigation out of which 102 specimens were successfully identified on molecular level by using universal primer- LCO1490 and HCO2198. Mahjabin *et al*. (2023) used mitochondrial COI (primer - LCO1490 and HCO2198) and 16S rRNA (primer- 16 Sar and 16 SBR) gene to identify 8 species of gastropods and concluded that

DNA barcoding is a highly effective tool for molecular identification. Araujo *et al*. (2023) used cytochrome c oxidase I gene for gastropod identification and then analysed the obtained sequences by phylogenetic reconstruction and algorithm. They concluded that DNA barcoding methodology is successful for mollusc's identification.

CONCLUSION

The main dangers to freshwater environments are pollution, changing land uses, and dam construction. Numerous freshwater mollusks are employed in the lime and pearl industries, as well as in food and medicine. The majority of freshwater mollusks that are eaten are either classified as DD or LC or have the wrong taxonomic status. With the risks, extinctions, and underlying causes of freshwater biodiversity and their ecosystems in mind, it is critical to record, research, and create effective conservation measures. Reviews on various research papers on extraction of genomic DNA, it may summarize that, the CTAB method is good for extraction of genomic DNA as gastropods have proteinous animals. Conventional morphological identification techniques necessitate extensive training and expertise, and the phenotypic plasticity of taxa may result in incorrect organism identification. Because the mitochondrial *cox1* gene is largely conserved within species and represents a typical pattern of genetic variability between various species, it is typically utilized as a species barcode. It is concluded that the cytochrome oxidase I gene may distinguish between two distinct species that are not morphologically distinguishable, which is helpful in identifying gastropods.

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