Current Trends in Biotechnology and Pharmacy
Vol. 12 (4) 371-376, October 2018, ISSN 0973-8916 (Print), 2230-7303 (Online)

Molecular Phylogenetic Analysis of Indian Apple Snail

Silpi Sarkar and S. Krupanidhi
Department of Biotechnology, VFSTR
Vadlamudi, 522213 A P, India
Corresponding author : krupanidhi.srirama@gmail.com

Abstract

Apple snails (Ampullariidae) belong to a diversified freshwater family which occurs in pan-tropical habitats. The Indian apple snail, *Pila globosa* and other operculate snails are grouped in Architaenioglossa which also includes Viviparidae and Cyclophoridae. In our study, a concise relationship of Ampullariidae with Viviparidae and Cyclophoridae has been elucidated by using partial nucleotide sequence of cytochrome b gene. *Pila globosa* was collected from freshwater habitats of Berhampore (West Bengal) and its morphometric analysis is determined as an addendum to its phylogeny. The multiple sequence alignment is performed in MEGA v5.2 to reconstruct molecular affinities within the family, Ampullariidae. The phylogenetic tree is constructed with 1000 bootstrap replications in RAxML software. The outgroup species considered for the analysis in our study is chosen from Heterobranchia as it is a sister group to the clade Caenogastropoda. It is observed that family Ampullariidae formed a nested cluster and showed a polyphyletic affinity with Viviparidae and Cyclophoridae.

Keywords: - *Pila globosa*, Morphometry, Cytochrome b gene, RAxML , MEGA-v5.2

Introduction

The Phylum Mollusca is an extensive group of unsegmented and soft-bodied coelomates which have a prominent ventral foot and dorsal visceral mass (1). Phylum Mollusca consists of seven classes, among which class Gastropoda belongs to large assemblage of soft bodied invertebrates such as slugs and hard shelled snails which are adapted to varied habitats in comparison to rest of the representatives of molluscs (2). The genera of class Gastropoda, represented in all realms as their habitat varies namely benthic, epifaunal, burrowers, pelagic drifters, active swimmers, detritus, sedentary and suspension feeders (3). Gastropods are commercially exploited due to ornamental, edible and medicinal value and thus there is a risk for their sustainability (4). The clade Caenogastropoda as mentioned in taxonomic classification of Bouchet and Rocroi 2005 is the largest and most diverse among the living snails which consist of 136 extant, 65 extinct and 41 superfamilies. Caenogastropoda includes a) Architaenioglossa (Cyclophoridae - a major group of operculate land snails) and b) freshwater families – Ampullariidae and Viviparidae (5). The taxonomic history, affinities and systematic positions within the families namely Ampullariidae, Viviparidae and Cyclophoridae were studied to reconstruct the phylogenetic affinities (6). Thus, the morphology and phylogeny related study would give an insight into the deeper aspects of these families. The evolutionary hypothesis for major groups within the phylum is controversial due to varied habitats adaptability and extreme diversity of the phylum. A recent insight into the correlation and taxonomic identity of the studied genera with the existing findings on caenogastropods by Ponder and Linderberg (1997), Simone (2001, 2004, 2005) and Strong (2002) had been deduced

within this article (7). Ampullariids are freshwater amphibious snails of tropics and sub-tropics of Africa, America and Asian continents, where they are found as major native freshwater molluscan fauna. Our study is based on the characterisation of morphological features which includes morphometric analysis of *Pila globosa* (Ampullariidae) and construction of its phylogenetic affinities using partial nucleotide sequence of cytochrome b, which is a highly conserved representative of mitochondrial enzymes. The MT-Cyb gene provides the necessary instructions to make a protein called cytochrome b. There are 11 components forming group of proteins called complex III among which cytochrome b is one among the 11 components. Complex III performs a step of a process known as oxidative phosphorylation in mitochondria where oxygen and sugars are used in synthesis of adenosine triphosphate (ATP), cells main energy source. During oxidative phosphorylation, the protein complexes which include complex III, carries the production of ATP through a step-by-step transfer of negatively charged particles called electrons. Cytochrome b is mainly involved in the transfer of these negatively charged particles through complex III. In complex III cytochrome b is the only component which is produced from a gene found in the mitochondrial DNA (8).

Materials and methodology

A) Collection of Samples : *Pila globosa,* also known as apple snails, are freshwater dwelling animals habituated in ponds, streams, lakes, rice fields and in rivers. They are collected by skimming from the riverine areas of Berhampore, West Bengal. A total size of ten samples is procured in the months of the monsoon from June to August 2016 because of their abundance in freshwater habitats. The coordinates for the study area are Lat. 24.098 N and Long 88.267 E for specific taxonomical identifications.

B) Maintenance of the samples prior to study **:** The procured snails after collection from the river site are taken to the laboratory, washed properly to remove the dirt and greenish algae attached to its shell, kept in tubs with water and fed them with lettuce and green leaves available from the market (9).The snails are kept for observation for a week to check for their mortality rates. To maintain the health of snails it is appropriate to change the water in the tubs daily.

C) Morphometric Study : The cleaned snails are kept aside over a bed of filter papers separately for 24 hours to remove the excess water. The colouration of *P.globosa* shell is generally pale to dark brownish with a globose shape in appearance. The collected *Pila* specimens are medium, large adults in size, body whorls are 5-6 in number. Morphologically the specimens' shells are transverse with oblique suture, sculpture coarser and less regular. The size and weight of snails are recorded on the live samples and it varied externally (10). The height, width of the *P.globosa* is measured using Vernier callipers to 0.1 mm precision as shown in Fig.1. The shell height (H), shell breadth (B), length of the operculum (LO) and breadth of operculum (BO) of *Pila globosa* are measured. The measurement of length and breadth of operculum provided information on the shell shape and hence the calculated values are determined from the ratio given in Table 1 whether any variation in shape existed within the species of snails which is not possible to visualize by apparent vision. The operculum of *Pila* is hard, concentric and calcareous. Also, the morphometric analysis might give an insight to the characters to be explored for further studies with large sample size. These studies may give the possibility to identify the cryptic species, which are morphologically identical can be hidden in a same species without proper taxonomical classification but literally belonging to different species and cannot interbreed among themselves within a particular geographical region (11).

D) DNA extraction by phenol- chloroform : The snails before dissected are kept aside separately and the snails are narcotized to isolate 0.5 g foot muscle tissue for genomic DNA extraction. Genomic DNA of good quality is vital as it is significant in deciphering the information which can be required to study evolutionary

interrelationships among taxa. There has been a recent study that developed a new methodology to generate high quality genomic DNA from terrestrial gastropod *Achatina fulica* (12). In our study, we followed the total genomic DNA extraction by the well known phenol-chloroform method (13). The purity of the extracted genomic DNA is checked and it is found to be 1.68 ng/ml respectively. The samples are loaded to 0.8% agarose gel prepared in 0.5 X TBE (Tris-Borate-EDTA) buffer which contained 0.5 mg/ml ethidium bromide. The gels are visualized in a UV transilluminator (Genei) and the image is captured under UV light using Gel documentation system (Bio-Rad). For PCR amplification, the 100 ng template DNA is taken (14).

E) PCR amplification : PCR is performed in a 20 µl reaction volume which contained 100 ng template DNA, 5 pmol of each specific primers, 0.2 mM of each dNTPs, 1.0 U *Taq* DNA polymerase and 1X PCR buffer contained 1.5 mM of $MgCl_2$. The PCR is driven for 40 cycles in a Thermal Cycler (Effendorf, Germany). The partial cyt b gene is amplified using the template DNA with reported universal primers (15).The PCR products are run on 1.2% agarose gel prepared in 0.5X TBE buffer containing 0.5 mg /ml ethidium bromide against a DNA ladder. The gel is visualised in UV transilluminator (Genei) and the image is captured under UV light using the gel documentation system (Bio-Rad). The size of amplicon of cytochrome b is found to be 490 bp (Fig.2).

F) Phylogenetic Analysis : The PCR product of cytb (Fig.2) is eluted. The sequencing of the eluted gene is carried by Big Dye Terminator 3.1 v sequencing kit to acquire partial nucleotide sequences of cytb in *P.globosa.* The sequences obtained are annotated and submitted to NCBI. The sequence is given the accession number viz., KR297240. The BLAST analysis is performed to check the similarity and match identity of the species, after which the alignment is done with default parameters in Clustal W algorithm using MEGA v5.2 (16). Regions of excess gaps and lengthy inserts are exempted during the alignment analysis in MEGA. The FASTA format generated is uploaded in ALTER (Alignment Transformation Environment), to be used in RAxML software. The software package RAxML GUI V 1.3 is used in the construction of phylogenetic tree for maximum likelihood analysis. This method is based on a firm statistical principles and is most powerful in recovering correct tree topologies by computer simulation studies. The unpartitioned nucleotide sequences are subjected to ML phylogenetic analysis through RAxML GUI v1.3 software package supported with 1000 bootstrap replications (17). The bipartition file obtained from RAxML is opened in Fig Tree v4 software to obtain the final cladogram.

Results and Discussion

The morphological parameters of *P.globosa* such as weight, colour and shape of shell are

Fig.1 Measurement of height of shell of Pila globosa (H) using a Vernier Callipers.

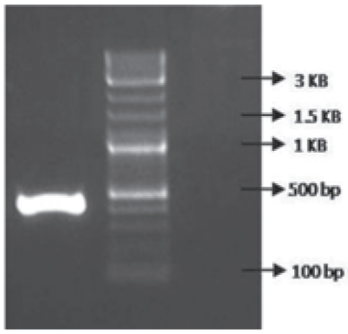

Fig.2. PCR amplification of partial Cyt b gene with an amplicon stretch of 490 bp in 1.2% agarose gel electrophoresis.

given in Table 1. The shell of the *P.globosa* is globose shaped like an apple and hence commonly known as 'Indian Apple snail'. Analyses using morphological and molecular approaches invariably highlight the phylogenetic affinities among the genera of Ampullariidae, Viviparidae and Cyclophoridae (18). The morphometric analysis of snails collected is studied to check whether any unusual variation exists among the population from the Berhampore region. In the present study, 11 species of Architaenioglossa are retrieved based on the available closest sequences of species obtained from GenBank. The representative species of families namely Ampullariidae, Cyclophoridae and Viviparidae are selected to derive phylogenetic affinities. Maximum likelihood (ML) phylogenetic analysis of 12 genera including *P.globosa* belonging to Architaenioglossa is shown in Fig.3 and the derived tree using RAxML tool is rooted on the outgroup taxon namely *Anguispira* (19).The tree does not show nodal support to the genera, *Pomacea* and *Pila* though both of them belong to Ampullariidae and the same might be in compliance with their divergent habitats due to their geographical location. This result also indicated that the ampullariids are polyphyletic within Architaenioglossa as they are clustered with the species of Cyclophoridae and Viviparidae. Our focal taxa, *P. globosa* formed a tertiary cluster with the genera of Viviparidae. Thus, the Maximum Likelihood tree reconstructed with the species of families, Ampullariidae, Viviparidae and Cyclophoridae within the Architaenioglossa is in synchronicity with Ponder and Lindberg's (1997) based topology which is also strongly supported by taxonomical classifications of Bouchet and Rocroi 2005. The bootstrap values of the cladogram supported the clustering of the chosen genera of families of Ampullariidae and Cyclophoridae despite their divergent habitats (20). It is further reported by Healy (1988) that Ampullariidae and Cyclophoridae shared a few specialized features namely eusperm and parasperm (21).

Conclusion

Understanding, the freshwater fauna of gastropods is essential as India has a peculiar diverse biota of invertebrate species which encompasses greater than 99 % animal biodiversity (22). The freshwater gastropods species are representative of Caenogastropoda

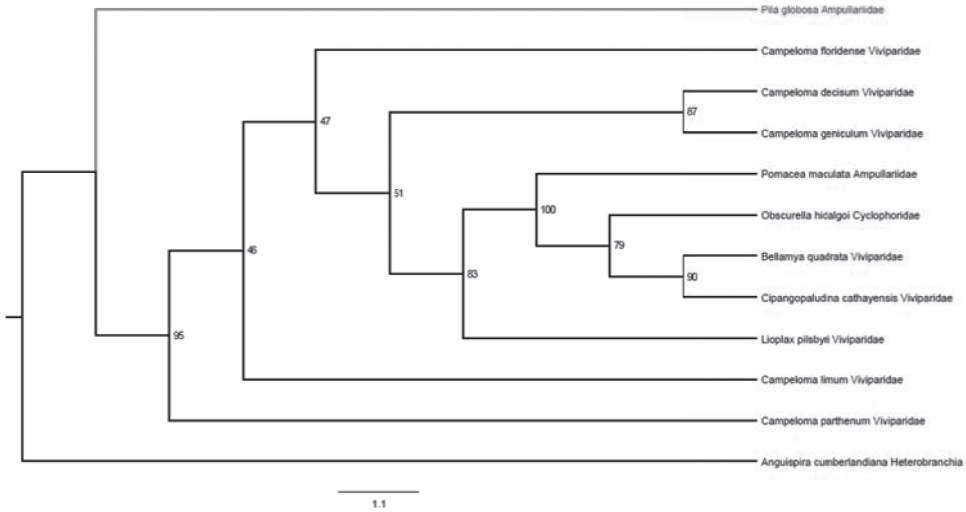

Fig.3. Maximum Likelihood cladogram based on RaXML analysis of the full concatenated dataset on the nucleotide sequence of partial gene, cytochrome b of the chosen gastropod taxa with 1000 bootstrap replications.

Table 1 The morphometric analysis of *Pila globosa* sampled from Berhampore (WB), India.

No. of samples	Snails weight g.	Vol. ml	Height of shell in mm (H)	Height up to 2nd whorl (H2)	Breadth of shell in mm (B)	Length of operculum in mm (LO)	Breadth of Operculumn in mm (BO)	Operculum shape LO/BO	Shell shape H2/B
5	28.82 ±12.56	42.4 ±7.83	46.24 ±4.08	45.60 ±0.38	40.04 ±6.18	35.53 ±2.70	24.44 ±1.40	1.453 ± 1.92	1.13 ±0.061

and pulmonate heterobranchs. In India, phylogeny studies of *Pila globosa* (Ampullariidae) along with other sister taxa families of Cyclophoridae and Viviparidae have not been done previously. In this study, it was found that Ampullariidae is found to be polyphyletic and showed a sister taxon relationship both with Cyclophoridae and Viviparidae although there is no apparent variation among the morphometric study done using the snails from Berhampore West Bengal. Nevertheless, an increase in number of *Pila globosa* from diverse areas is required which needs to be considered to deduce a definitive conclusion. To find out the diversity of Ampullariidae species, which can add to the evolutionary relationships in the reconstruction of phylogeny tree for Indian Apple snails, it is necessary to identify and locate these species through a scaling up process which explores the conservation stragies of freshwater gastropod species in a holistic way.

Acknowledgements:

Prof. S Krupanidhi thanks DST MRP (SB/SO/AS-138/2012), New Delhi India for providing financial support to carry out the work presented in this article. Authors acknowledge DST FIST (2015-20) support to the Department of Biotechnology, VFSTR during the time of which the present work was done.

References

1. Jena, C., Sarkar, S., Jalaja, N. and Krupanidhi, S. (2017).Molecular Phylogenetic relations of *Achatina fulica* based on partial sequence of COI gene. *National Academy of Science Letters.* 40(2):101-103.
2. Sarkar, S. and Sreerama, K. (2018). Phylogenetic Affinities Of Indian Apple Snails- An Insight into the Tibetan Tectonic Terranes. *Proceedings of Zoological Society* 71(2):194-201; https://doi.org/10.1007/s12595-017-0257-4.
3. Rao, S.N.V. (1989). Handbook Freshwater Molluscs of India. Zoological Survey of India, Kolkata, pp 411.
4. Baby, R.L., Hasan, I., Kabir, K.A. and Naser, M.N. (2010).Nutrient analysis of some commercially important molluscs of Bangladesh. *Journal of Scientific Research* 2(2): 390-396.
5. Bouchet, P. and Rocroi J.P. (2005). Classification and nomenclator of gastropod families. *Malacologia*., 47(1–2) :1– 397.
6. Colgan, D.J., Ponder,W.F., Beacham, E. and Macaranas, J. (2007). Molecular phylogenetics of Caenogastropoda (Gastropoda: Mollusca). *Molecular Phylogenetics and Evolution*. 42: 717–737.
7. Hayes, K.A., Burks, R.L., Vazquez, C.A., Darby, P.C., Heras H., Martin, R.P., Qui, W.J.,Thiengo, C.S., Vega,A.I., Wada,T., Yusa,Y., Burela,S., Cadierno, P.M., Cueto, A.J., Dellagnola, A.F., Dreon,S.M., Frassa, V.M., Billoud,G.M., Godoy, S.M., Ituarte, S., Koch, E., Matsukura, K., Pasquevich, Y.M., Rodriguez, C., Saveanu, L., Seuffert, E.M., Strong E.E., Sun, J., Tamburi, E.N., Tiecher,J.M., Turner,L.R., Darby- Valentine L.P. and Cowie, H.R.(2015). Insights from

an Integrated View of the Biology of Apple snails (Caenogastropoda: Ampullariidae). *Malacologia* .58(1-2): 245-302.

8. Esposti, D.M., Vries, De. S., Crimi, M., Ghelli, A., Patamello, T. and Meyer, A.(1993) Mitochondrial cytochrome b: evolution and structure of the protein. *Biochimica et Biophysica Acta.,*1143(3):243-271.

9. Ramakrishna and Dey,A. (2007). Handbook on Indian freshwater molluscs. Zoological Survey of India. Kolkata. pp1- 399.

10. Strong, E.E. (2003). Refining molluscan characters: morphology, character coding and a phylogeny of Caenogastropoda. *Zoological Journal of Linnean Society*.137(4): 447- 554.

11. Ponder, W.F. and Lindberg, D.R. (1997). Towards a phylogeny of gastropod molluscs: an analysis using morphological characters. *Zoological Journal of Linnean Society*. 119: 83–265.

12. Ayyagiri, V.S., Jalaja,N. and Krupanidhi,S.(2017).Optimization of the Isolation procedure of genomic DNA from a mucus laden pulmonate gastropod, *Achatina fulica.*40(2):109-112.

13. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

14. Brown, T.A. (1991). Essential molecular biology, A practical approach. (Volume 2) Oxford University Press, New York pp 296.

15. Meritt, T.J.S. and Shi, L.(1998). Universal cytochrome b primers facilitate intraspecific studies in molluscan taxa. *Molecular Marine Biology and Biotechnology.* 7(1):7–11.

16. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*., 28: 2731- 2739.

17. Stamatakis, A., Hoover, P. and Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML web servers. *Systemic Biology*.57: 758–771.

18. Thompson, J.D., Plewniak, F. and Poch, O. (1999). A comprehensive comparison of multiple sequence alignment programs. *Nucleic Acids Research*. 27: 2682–2690.

19. Raven, J.G.M. (1990). A revision of Obscurella Clessin, 1889 (Gastropoda Prosobranchia: Cyclophoridae) *Basteria*. 54:17-62.

20. Harasewych,M.G.,Adamkewicz,S.L. and Gillevet, P.M. (1998). Phylogenetic relationships of the lower Caenogastropoda (Mollusca, Gastropoda, Architaenioglossa, Campaniloidea, Cerithioidea) as determined by partial 18s rDNA sequences. *Zoologica Scripta*. 27(4) : 361–372.

21. Healy, J.M. (1988). Sperm Morphology and its systematic importance in the Gastropoda *Malacological Review Supplement*. 4: 251-266.

22. Sarkar, S. and Krupanidhi, S. (2018) (in press). A review to necessitate conservation of Indian terrestrial and freshwater gastropods. *Journal of Threatened Taxa.*