

Phylogenetic Analysis of Indian Freshwater Pond Mussels *Lamellidens corrianus* and *L. phenchooganjensis* (Bivalvia: Unionidae) from the Upper Brahmaputra Basin of Assam, India

Jyotish Sonowal, Pankaj Chetia and Devid Kardong

Department of Life Sciences, Dibrugarh University, Dibrugarh, Assam, India.

<http://dx.doi.org/10.13005/bbra/2907>

(Received: 14 December 2020; accepted: 19 March 2021)

To update the present knowledge of freshwater molluscs, a phylogenetic analysis of two species of Indian pond mussels was carried out using amplified sequence of 18S rRNA gene. From the phylogenetic study of 18S rRNA gene sequencing, it was found that the *Lamellidens corrianus* and *L. phenchooganjensis* are phylogenetically closely related to 18S rRNA gene sequences of other freshwater bivalve mussels belonging to the family Unionidae of order Unionida. Like other members of the Unionidae family, the two *Lamellidens* spp. showed monophyletic evolutionary lineage and shared a common ancestor. The result obtained from the phylogenetic analysis of *Lamellidens* spp. was significant as the 18S rRNA gene sequence of *L. phenchooganjensis* was submitted for the first time in the global nucleotide database (Genbank, NCBI). Similarly, the 18S rRNA gene sequence of *L. corrianus* was also submitted to the database for the first time from this region having unique ecological niche. Therefore, the present study on phylogenetic analysis is a contribution to the global database of 18S rRNA gene sequences of freshwater mollusc, particularly from this part of the region.

Keywords: Unionidae, monophyletic, common ancestor, database, Data Deficient.

The members of class Bivalvia shows unique evolutionary radiation as it displays parental care through brooding of eggs and larva¹ and they (Glocidial larva) depend on the species-specific fish host for completion of their life cycle². Taxonomically, the freshwater mussels are placed in the order Unionida which is the largest group among the five orders of class Bivalvia represented by 6 families, 181 genera and about 840 species. On the other hand, the family Unionidae shows the largest radiation among the 6 families of class Bivalvia that comprises of 142

genera and 620 species worldwide. Though the families of Unionida are ubiquitously distributed throughout the globe, except Antarctica, this is considered as one of the most threatened groups of freshwater animals alive today³⁻⁵. Being an ecosystem engineer, these freshwater bivalves of order Unionida and family Unionidae play many significant roles to maintain the balance of aquatic ecosystem health^{3,6-14} and need utmost conservation priorities. However, due to the lack of detailed taxonomic and ecological study, application of management and conservation planning is still

*Corresponding author E-mail: kardongdevid@dibru.ac.in



found to be very difficult. This highlights the urgent need for detailed study in the areas of phylogenetic and evolutionary relationships within the order, family or at the genus level¹⁵. Due to their interesting biological characters, for example, reproductive dependence on a host-specific fish for completion of the life cycle, double uniparental inheritance (a particular form of mitochondrial inheritance)¹⁶⁻¹⁹ and ecological as well as economic importance, there is a development on phylogenetic studies on freshwater bivalve in recent years^{20, 21}. However, conservation efforts focused on species-based and habitat-based unionids are impeded by uncertainties like taxonomic, morphological, ecological, phylogenetic^{22, 23} and most of the species around the globe have been assigned as Data Deficient (DD) or Least Concerned (LC) species of IUCN Redlist, especially those outside of North America and Western Europe^{2, 14, 15, 24} where the majority of phylogenetic study on freshwater unionids have been going on^{25, 26}. Recently, several systematic and phylogenetic studies have been carried out on comparatively diverse tropical Asian lineages that have improved our understanding of the classification, morphological evolution, and geographic distribution of many tropical freshwater bivalves^{15, 26-28}. But still, the south-east Asian and Indo-tropical freshwater unionids have received less attention from the systematic perspective and most of the freshwater unionids taxa remain poorly understood phylogenetically.

India harbours 263 freshwater mollusc species found in two mega biodiversity hotspots, *viz.* The Western Ghats and Eastern Himalayas of which 95 species are belonging to class Bivalvia^{14, 29}. Out of these total species richness, most of the freshwater bivalves are under DD or LC category of IUCN Redlist. These high numbers of species under DD and LC category are mainly due to unknown species distribution pattern and population trends, lack of information on potential threats and phylogenetic lineages. On the other hand, most of these freshwater mollusc species known only from morphological descriptions of the late 19th and early 20th century. Since then no vast amount of knowledge has been added on the freshwater mollusc species and many morphologically described species seems to be doubtful^{14, 24, 30, 31}. The study on systematics and evolutionary lineages of freshwater unionids in the mainland

of India is very limited with the aid of molecular and modern bioinformatics tools^{32, 33}. However, with a single exception of the report by Jadhav and Jamkhedkar 2009³⁴ studied the phylogeny of freshwater bivalve, *L. corrianus* collected from Maharashtra, India, there is absolutely no other report available on DNA sequencing of freshwater mussels based on 18S rRNA sequences from the Assam as well as north-east India. The 18S rRNA is a structural component of the small eukaryotic ribosomal subunit and popularly used in biodiversity research and phylogenetic studies³⁵⁻³⁸. Compared to conserved cytochrome oxidase subunit I (COI) gene, the 18S rRNA gene is more conserved and its evolutionary progress is much slower making it a suitable molecular marker for phylogenetic studies and for differentiating between taxon at each taxonomic levels³⁹. Recently, a good number of new species, new information on their biology, bionomics and distribution have been added. A good number of taxonomic account was given by different authors, but at the same time, much confusion was created by the addition of several isolated and inadequate description of species⁴⁰. So these work needs revision and up to date the present knowledge of freshwater molluscs based phylogenetic studies using sophisticated molecular markers and modern bioinformatics tools. Considering this fact, an attempt was made to a phylogenetic study of two *Lamellidens* spp. using the amplified sequence of the 18S rRNA gene and to study evolutionary lineage of different *Lamellidens* spp. concerning available 18S rRNA sequences of other unionids in the global database.

MATERIALS AND METHODS

Collection and morphological identification *Lamellidens* specimens

The specimens of the *Lamellidens* spp. were collected from the upper Brahmaputra basin of Assam covering a total geographical area of approximately 3900 km² between latitude (27°16'20"–27°47'77") N and longitude (94°35'30"–95°22'42.16") E (Figure 1). The large specimens were handpicked and the smaller ones were collected from the bottom substrata by using a metal sieve of mesh size 2mm². Specimens were then washed, sorted into morpho-species and representatives were brought to the laboratory for

future reference. Identification of the recorded specimens was done according to Subba Rao (1989)⁴¹, Ramakrishnan and Dey (2007)⁴⁰ and by tallying with authentic voucher specimens deposited at Zoological Survey of India (ZSI), Kolkata.

DNA extraction and sequencing of the 18S rRNA gene

The DNA was extracted from mantle using the Qiagen Blood and Tissue Extraction Kit (Cat No./ID: 69504). The quality of the extracted DNA from gills and mantle tissue was evaluated in 1% agarose gel. A single band of high molecular weight DNA was observed. Its quantity was determined by measuring the optical density at λ 260/230 and λ 260/280. After that, the fragment of the 18S rDNA region was amplified by the polymerized chain reaction (PCR) using forward NS1 and reverse NS4 universal primers. A single discrete PCR amplicon band of ~1300 base pairs (bp) was observed when resolved on the agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with the NS1 and NS4 universal primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Phylogenetic analysis of the sequenced *Lamellidens* spp.

The consensus sequence was generated from the forward and the reverse sequences, and it was subjected to BLAST-n against non-redundant nucleotide collection. Twenty similar sequences were taken from BLAST result, and the phylogenetic tree was constructed using MEGA 7 software and based on BLAST similarity and phylogenetic affinity of the sequence, the species were identified as *L. corrianus* and *L. phenchooganjensis* (Figure 2). The sequences were then deposited to NCBI Genbank and accession numbers MT11611 and MT112199 were obtained for *L. corrianus* and *L. phenchooganjensis* respectively.

Comparison of *Lamellidens* sequence with other available freshwater unionid bivalves

The partial sequence of the 18S rRNA gene was analysed by Clustal W software to find out the pairwise distance of 18S rRNA gene between *Lamellidens* spp. and other unionid bivalve sequences present in the nucleotide database of NCBI. A distantly related 18S rRNA

sequence was selected as an outgroup to find out the phylogenetic lineages of *Lamellidens* spp. All the sequences including the outgroup sequence were loaded as FASTA format and multiple sequence alignment (MSA) was performed following Kumar *et al.* 2016⁴² using MEGA 7 software. After trimming off the flanking ends of the aligned sequences, a phylogenetic tree was constructed using the maximum likelihood method with a bootstrap replication value of 1000.

RESULT AND DISCUSSION

After blast analysis and construction of the phylogenetic tree, it was observed that *L. corrianus* generated a sequence of about 903 bases and the first 10 hits comprised of partial sequences of 18S rRNA gene of freshwater mussel *Lamprotula leai* (Accession No. MF072524), *Potomida littoralis* (Accession No. KU763287), *Lampsilis cardium* (Accession No. KX713305), *Elliptio complanata* (Accession No. JF899209), *Aculamprotula tientsinensis* (Accession No. MF072525), *Thyasira* sp. (Accession No. LC187037), *Unio pictorum* (Accession No. KC429349), *Anodonta cygnea* (Accession No. AM774476), *Psilunio littoralis* (Accession No. AF120536), *Elliptio complanata* (Accession No. AF117738) and *Anodonta* sp. (Accession No. AY579090). The phylogenetic tree generated showed four major clusters (Figure 3a). The *L. corrianus* (Accession No. MT11611) forms a cluster with *Lamprotula leai* and *Potomida littoralis* at the bootstrap confidence level of 77. The three *Neotrigonia* spp. (Accession No.s AF 120538, AM774478, and AF411690) forms the second cluster at the bootstrap confidence level of 100. *Triplodon corrugatus* (Accession No. KX713352), *Hyridella australis* (Accession No. KX713301) and *Velesunio ambiguous* (Accession No. KC429346) formed the third cluster at the bootstrap confidence level of 99. The fourth major cluster was formed by the sequences of freshwater mussels *Psilunio littoralis*, *Elliptio complanata*, *Aculamprotula tientsinensis*, *Unio pictorum*, *Anodonta cygnea* and *Lampsilis cardium*.

The *L. phenchooganjensis* generated a sequence of about 458 bases and after blast analysis and, it was observed that the first 10 hits comprised of partial of 18S rRNA gene of

freshwater mussel *Aculamprotula tientsinensis*, *Lamprotula leai*, *Lampsilis siliquoidea* (Accession No. KY978476), *Lampsilis cardium*, *Fusconaia flava* (Accession No. KX342024), *Unio pictorum*, *Anodonta cygnea*, *Elliptio complanata*, *Psilunio littoralis* etc. The phylogenetic tree generated showed three major clusters (Figure 3b). The three *Neotrigonia* spp. forms the first cluster at a bootstrap confidence level of 96. The second cluster comprises *Triplodon corrugatus*, *Vesunio ambiguous* and *Hyridella australis* at a bootstrap confidence level of 87. The partial 18S rRNA gene sequence of *L. phenchooganjensis* forms cluster comprises *Aculamprotula tientsinensis*, *Lamprotula leai*, *Lampsilis cardium*, *Unio pictorum*, *Anodonta cygnea*, *Psilunio littoralis* and *Elliptio complanata* (Figure 3b).

Identification of species is the basis of studies like biological, ecological, genetic, conservation as well as different culture operations.

Almost all freshwater mollusc species of Indo-tropical region were identified by the pioneer researchers based on external morphological characters of shells, glochidial larva and internal morphological characters such as the structure of marsupial gills^{40, 41} and still, morphological identification has taken as the major tool for the identification of freshwater mussels in India. Although, morphological, anatomical and reproductive keys are found to be important features of basic classification. But, in many instances due to overlapping characters, it is very difficult to make species distinction. Simpson (1900)⁴³, Modell (1942)⁴⁴, and Starobogatov (1970)⁴⁵ had largely used on external shell characters for the establishment of the non-phylogenetic classifications of various new taxa at genus and family level. Since numerous classification and reclassification has been made for unionid bivalve, different and inconsistent classification schemes create many taxonomic

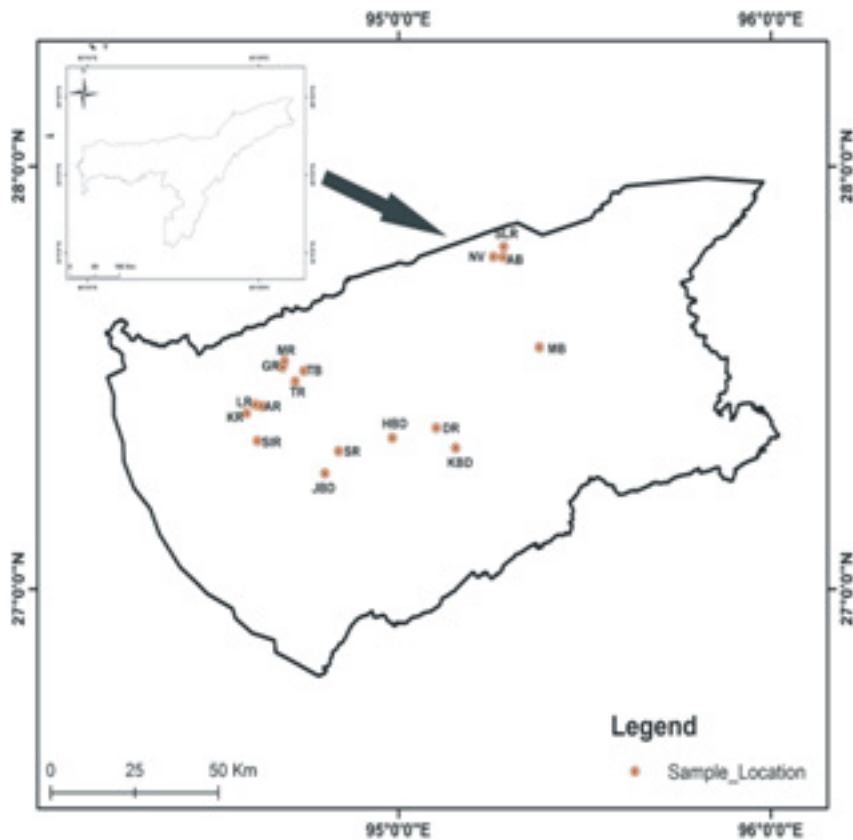


Fig. 1. Map of the study area between latitude 27°16'20"–27°47'77" N and longitude 94°35'30"–95°22'42.16" E. Coloured dots are different sampling stations of the study area

confusions and uncertainties in the classification of these freshwater bivalves⁴⁶.

Some members of genus *Lamellidens* is more or less widespread in India and some are confined to a limited geographic region within the

Indian subcontinent. Due to allopatric distribution pattern and parapatric speciation, the genus display wide range of shell structural variability and colour pattern which remains a challenging task to identify accurately based on morphological, anatomical

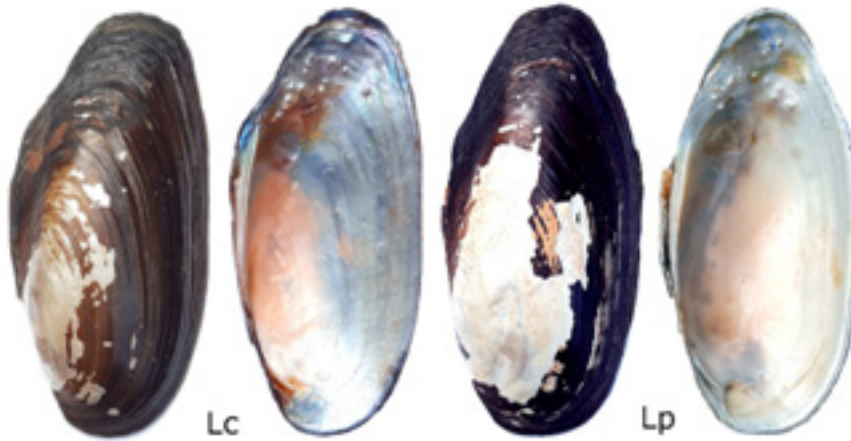


Fig. 2. Photographs of *Lamellidens* spp. Lc – *Lamellidens corrianus*(Lea, 1834), Lp – *Lamellidens phenchooganjensis* Preston, 1912

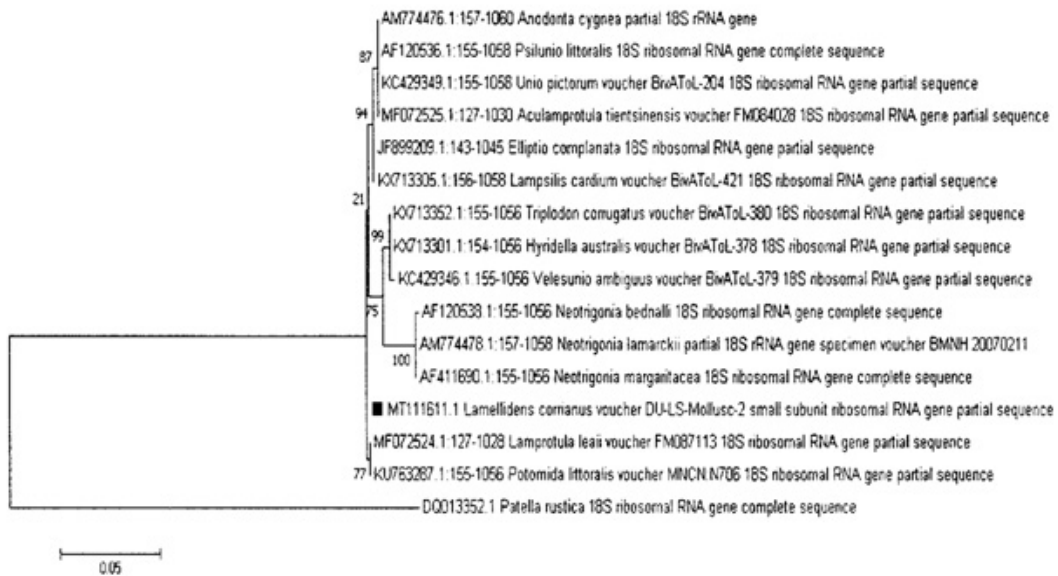


Fig. 3a. The evolutionary history of *L. corrianus* was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-4755.98) is shown.

The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences and one outgroup sequence of *Patella rustica*. There were a total of 896 positions in the final dataset

and reproductive taxonomic keys. Taxonomic keys are still a major problem in establishing a correct database for freshwater mollusc especially the freshwater mussel of India^{14, 24, 31}. With the lack of correct database and inconsistency classification therefore, it is difficult to implement conservation strategies and management measures that result from commercial, municipal and residential development.

From the phylogenetic study of 18S rRNA gene sequencing, it was found that the *L. corrianus* is closely related to 18S rRNA gene sequences of *Lamprotula leai* and *Potomida littoralis* at an indent 99.89% and *Lampsilis cardium*, *Elliptio complanata*, *Aculamprotula tientsinensis*, *Unio pictorum* and *Anodonta cygnea* at an indent of around 99.23%. Like *L. corrianus*, all these species are freshwater mussels belonging to the family Unionidae of order Unionida. After generation of the phylogenetic tree using maximum likelihood method, it was observed that the *L. corrianus* is phylogenetically neighbour/ very close to *Lamprotula leai* and *Potomida littoralis* at 77% bootstrap confidence of trees in which the associated taxa are clustered together (Figure 3a). It was also observed that the *L. corrianus* showed a high degree of association with the other freshwater mussel from the family Unionidae as branch lengths measured in the number of substitutions per site was very minimum. Therefore, the present study indicates that the *L. corrianus* showed a single evolutionary lineage with the other considered freshwater mussel species as they showed a common ancestor which was separate from that marine gastropod lineage. The species *Patella rustica* commonly called a true limpet, a marine gastropod belonging to the family Patellidae was taken as an outgroup and from the analysis, it was observed that the freshwater mussels and marine gastropods were evolved separately in due course of time from different ancestors as this marine gastropod species group from the rest in the phylogenetic tree (Figure 3a). Similarly, after sequencing analysis of 18S rRNA gene, it was observed that *L. phenchooganjensis* was also closely related to 18S rRNA gene sequences of *Lamprotula leai*, *Aculamprotula tientsinensis*, *Lampsilis cardium*, *Unio pictorum*, *Potomida littoralis* and *Anodonta cygnea* with an indent of 99.78% and *Elliptio complanata* and *Triplodon*

corrugates with 99.56% indent. From the analysis of the phylogenetic tree, it was observed that the *L. phenchooganjensis* was grouped with the above mentioned freshwater mussel species at a bootstrap confidence level of 87% except for *Triplodon corrugatus*. The *Triplodon corrugates* formed a separate group, although the branch length and substitution per site were very less than 0.02 (Figure 3b). Therefore, like other freshwater mussel species, *L. phenchooganjensis* was also evolved from a common ancestor which was separate from that of marine lineage as the considered outgroup *Patella rustica* formed a separate lineage in the phylogenetic tree (Figure 3b). Though *L. corrianus* and *L. phenchooganjensis* were morphologically distinct species^{40, 41}, their evolutionary relationship was found to be similar and showed monophyletic origin. Like other freshwater mussels species, the members of *Lamellidens* spp. shared a common ancestor and showed linear evolutionary lineage (Figure 4).

The result obtained from the phylogenetic analysis of *Lamellidens* spp. based on 18S rRNA sequencing was found to be significant, because there is only one report of 18S rRNA gene sequence of *L. corrianus* by Jadhav and Jamkhedkar, 2009³⁴ from Maharashtra, India. After that, there is no other report about 18S rRNA sequences available/ submitted to the global database except for the present report. On the other hand, 18S rRNA gene sequences of *L. phenchooganjensis* was submitted for the first time in the Global nucleotide Database (Genbank, NCBI). No previous record on molecular data for this species is available.

According to IUCN, there are 10 globally recognized species under the genus *Lamellidens* and found to be endemic to Indo-tropical ecoregion. But recent assessment reports suggested that there are only seven species of *Lamellidens* that are found in mainland of India and some species are still doubtful^{40, 47, 48, 49}. For instance, *Lamellidens narainporensis* is known only from a single location KamdaiNadi of Nepal and thought to be possibly a synonym of *L. corrianus*. Detailed taxonomic study at the molecular level is required to confirm the status of the species⁴⁷. Similarly, the status as a separate species for *Lamellidens unioides* is still doubtful and more taxonomic review is required to confirm it⁵⁰. The *Lamellidens lamellatus* is known only from early 20th century

records by Preston (1915)⁵¹ and detailed survey and taxonomic study are required to confirm its status as a species, geographical distribution and IUCN Redlist status⁴⁸. The information about the *Lamellidens scutum* is only based on museum specimens collected only from some localities of Myanmar and India⁴⁹. On the other hand, information on some *Lamellidens* spp. in global mollusc databases like Molluscabase, Malacolog, UnitasMalacologica is not available or whatever the information available is seem to outdated (Worldwide mollusc species database, Catalogue of Life).

CONCLUSION

Due to the difficulties in developing a database on population trend, distribution pattern, current geographic range, species coverage and lack of information on potential threats, a comprehensive phylogenetic study on *Lamellidens* spp. had not been attempted in the past. Therefore the outcome of the present phylogenetic study will help to establish an accurate database and add new information to the existing database especially from those regions where information on freshwater mollusc is considerably lacking.

ACKNOWLEDGEMENTS

Authors are thankful to Eurofins Scientific, Bengaluru and ZSI, Kolkata for their technical support; DST, Govt. of India for financial support and DST-FIST, Department of Life Sciences for providing necessary facilities for carrying out this work.

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