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**Research Article**

# MOLECULAR GENETIC CHARACTERIZATION OF FRESHWATER MUSSEL *LAMELLIDENS MARGINALIS* USING MITOCHONDRIAL COI GENE

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**A R T I C L E I N F O**

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**A B S T R A C T**

Samples representing three populations of freshwater pearl producing bivalve *Lamellidens marginalis* were collected from different parts of India. Phylogenetic tree constructed based on COI gene indicated these populations are the subspecies of *L. marginalis*. The COI gene sequence analysis confirmed the traditional classification of *L. marginalis* at molecular level proving that it belongs to Unionidae family. The phylogenetic relationship among three population and taxonomic status of *L. marginalis* was validated using mt DNA (COI) gene sequences.



## INTRODUCTION

Mitochondrial DNA (mt DNA) is one of the most widely used genes in systematic, species characterization, population structure and phylogenetic studies. In general, animal mt DNA is a small, circular molecule with a high evolutionary rate and a very conserved gene order and content (Gray, 1989). The evolutionary rate as well as the genetic differentiation of mt DNA among populations is thought to be approximately 5-10 times higher than that exhibited by nuclear genes (Birky *et al.,* 1983, 1989; Wilson *et al*., 1985).

DNA Sequence analysis has been used for 30 years to assist species identification (Tautz *et al.,* 2002, 2003). Different sequences have been used for different taxonomic groups, a single gene sequence would be sufficient to differentiate all or atleast the vast majority of animal species. Hebert *et al.,* 2003 used mt DNA gene Cytochrome Oxidase Subunit- I (COI) as a global bio-identification system for animals.

*L. marginalis* is widely distributed in India, Bangladesh and Sri Lanka. It is a typical pond species and widely distributed in ponds and large bodies of perennial waters of Indian subcontinent (Nagabhushan and Lohgaonkar, 1978) as well as in number of reservoir and tanks of Maharashtra (SubbaRao, 1989). The present studies was aimed to characterize and establish phylogenetic relationship among three distinct population of *L. marginalis* using mitochondrial COI gene sequences.

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### *Sample Collection*

40 samples each of *L. marginalis* were collected from three different parts (waterbodies) of India viz. Patalganga river, Khopoli, district Raigad, Mumbai (Maharashtra) (18°N, 73°E), Ganga river, Balawali, district Bijnor (Uttar Pradesh) (29°N, 78°E) and Daya river, Dhauli, district Bhubaneswar (Odisha) (20°N, 85°E) between March and May 2007.

### *DNA Isolation*

The DNA was isolated following the modified phenol chloroform method of Ruzzante *et al*., 1996 with minor modifications. The concentration of isolated DNA was estimated using a UV spectrometer. The DNA was diluted to get a final concentration 50 ng/µl.

### *Amplification and Sequencing*

The mt DNA COI gene was amplified in a final concentration, 50µl volume reactions with concentration of 5µl of 1 x assay buffer (100 mM Tris-Cl, 500 mMv KCl, 0.1% gelatin, pH 9.0) with 2µl of 2mM $MgCl_2$ (Genei, Bangalore, India), 0.5µl of 5p moles of each primer, 1µl of 200mM dNTPs (Genei, Bangalore, India), and 0.4µl of 1.5 U Taq DNA polymerase, 38.6µl de-ionized water and 2µl of 50ng template DNA. An internal fragment of the COI gene was amplified using a pair of metazoan invertebrate COI primer *viz*.

LCO:5'-GGTCAACAAATCATAAAGATATTGG-3'and
HCO:5'TAAACTTCAGGGTGACCAAAAAATCA-3'
(Folmer *et al.,*1994).

The thermal regime consisted of an initial denaturation step 3min at 94°C followed by 35 cycles of 30s at 94°C, 45s at

$47^0$C and 60s at $72^0$C followed in turn by a final extension for 10min at $72^0$C. The PCR products were visualized on 2% agarose gel and the most intense products were selected for sequencing. All PCR products were purified using the QIA-quick PCR purification kit (Qiagen) and directly sequenced using an ABI 3730 (Applied Biosystems Inc.) sequencer following the manufacturer's instructions.

***Sequence Analysis***

Eight COI gene sequences of *L. marginalis* species of different locations were deposited to obtain GenBank Accession Number. The accession number given by GenBank is as follows GQ149468, GQ149469, GQ149470, GQ149471, GQ149472, GQ149473, GQ149474 and GQ149475. A total of eight sequences of mitochondrial COI gene of *L. marginalis* were analysed. Read lengths of all sequences were about 622 base pair long. Sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) with the default settings. Nucleotide sequence divergences were calculated using the Kimura 2-parameter (K2P) distance model (Kimura, 1980). DNA sequences were checked by FinchTV software Version 1.4.0 (Geospiza Inc.). Sequences were edited manually using EditSeq and aligned using MegAlign (The EditSeq and MegAlign both are DNA sequence editing and aligning tools from DNASTAR software).

## RESULTS AND DISCUSSION

Mitochondrial COI genes of all the three populations were investigated to authenticate taxonomic classification of *L. marginalis.* The COI gene sequences confirmed the traditional classification of *L. marginalis* at molecular level proving that it belongs to Unionidae family.

**Table 1** BLAST results of Cytochrome Oxidase Subunit-I (CO-I) gene for three populations

| Population | Library | Sequence Name | % Match | No. of Bases Searched |
|---|---|---|---|---|
| Maharashtra (LMK14) | NCBI | *Pleurobema plenum* | 88 | 622 |
| | | *Elliptio crassidens* | 87 | 622 |
| | | *Glebula rotundata* | 87 | 622 |
| | | *Fusconaia cor* | 88 | 622 |
| | | *Strophitus undulatus* | 86 | 622 |
| | | *Lexingtonia dolabelloides* | 87 | 622 |
| | | *Anodonta nuttalliana* | 86 | 622 |
| Uttar Pradesh (LMN7) | NCBI | *Pleurobema plenum* | 88 | 622 |
| | | *Elliptio crassidens* | 87 | 622 |
| | | *Glebula rotundata* | 87 | 622 |
| | | *Fusconaia cor* | 88 | 622 |
| | | *Strophitus undulatus* | 86 | 622 |
| | | *Lexingtonia dolabelloides* | 87 | 622 |
| | | *Anodonta nuttalliana* | 85 | 622 |
| Orissa (LMO42) | NCBI | *Pleurobema plenum* | 88 | 622 |
| | | *Elliptio crassidens* | 86 | 622 |
| | | *Glebula rotundata* | 87 | 622 |
| | | *Fusconaia cor* | 88 | 622 |
| | | *Strophitus undulatus* | 87 | 622 |
| | | *Lexingtonia dolabelloides* | 87 | 622 |
| | | *Anodonta nuttalliana* | 85 | 622 |

The sequence analysis revealed that three sequences (one from each population) matched 88% with *Pleurobema plenum,* 87% with *Glebula rotundata,* 88% with *Fusconaia cor* and 87% with *Lexingtonia dolabelloides* in all the three populations, while *Elliptio crassidens* matched 87% with Maharashtra and Uttar Pradesh and 86% with Odisha population. *Strophitus undulates* matches 86% with Maharashtra and Uttar Pradesh and 87% with Odisha population. *Anodonta nuttalliana* matches 86% with Maharashtra and 85% with Uttar Pradesh and Orissa population (Table. 1). The distance matrix of the three populations is shown in Table 2. Maharashtra, Uttar Pradesh and Odisha population sample generated a sequence of 622 base pair.

**Table 2** Distance matrix of the three population samples

| Population | Marker | Maharashtra | Uttar Pradesh | Orissa |
|---|---|---|---|---|
| Maharashtra | CO-I | 0.000 | 0.002 | 0.006 |
| Uttar Pradesh | CO-I | 0.002 | 0.000 | 0.008 |
| Orissa | CO-I | 0.006 | 0.008 | 0.000 |

Sequence data are useful for evaluating phylogenetic relationships and evolutionary status of populations (Zhang *et al*., 2003). In our study it was apparent from sequence analysis which revealed distinct phylogenetic relationships among three populations.

The phylogenetic tree was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.46533333 is shown in (Fig. 1, 2, 3).

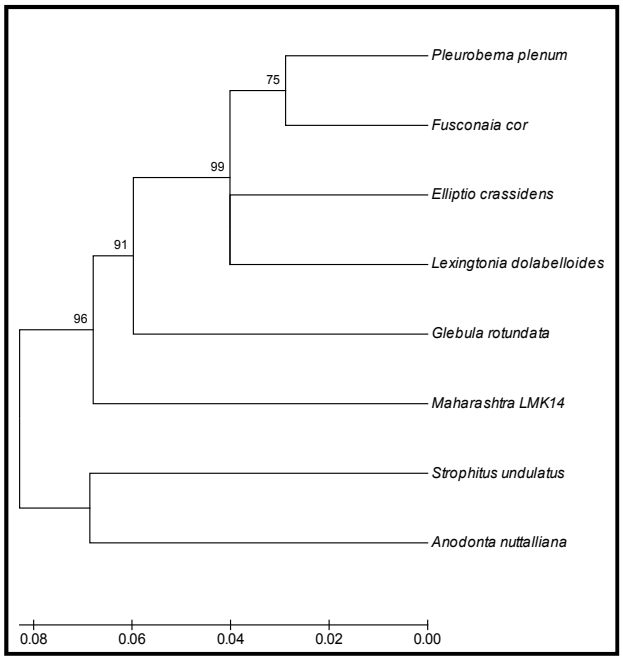


**Fig 1** Phylogenetic tree of Maharashtra sample with other freshwater mussels

Unit - no. of base substitution per site.

The percentage of replicate trees in which the associated taxa clustered together with bootstrap test (1000 replicates) are shown next to the branches. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Table. 3) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding.

**Table 3** Distance Score table of *L. marginalis* using Kimur 2-parameter model

| | LMK14 | LMK25 | LMK15 | LMN5 | LMN7 | LMN34 | LMO26 | LMO42 |
|---|---|---|---|---|---|---|---|---|
| LMK14 | | 0.0000 | 0.0016 | 0.0097 | 0.0081 | 0.0016 | 0.0179 | 0.0113 |
| LMK25 | 0.0000 | | 0.0016 | 0.0097 | 0.0081 | 0.0016 | 0.0179 | 0.0113 |
| LMK15 | 0.0016 | 0.0016 | | 0.0114 | 0.0097 | 0.0032 | 0.0196 | 0.0130 |
| LMN5 | 0.0097 | 0.0097 | 0.0114 | | 0.0016 | 0.0114 | 0.0146 | 0.0081 |
| LMN7 | 0.0081 | 0.0081 | 0.0097 | 0.0016 | | 0.0097 | 0.0130 | 0.0065 |
| LMN34 | 0.0016 | 0.0016 | 0.0032 | 0.0114 | 0.0097 | | 0.0196 | 0.0130 |
| LMO26 | 0.0179 | 0.0179 | 0.0196 | 0.0146 | 0.0130 | 0.0196 | | 0.0065 |
| LMO42 | 0.0113 | 0.0113 | 0.0130 | 0.0081 | 0.0065 | 0.0130 | 0.0065 | |

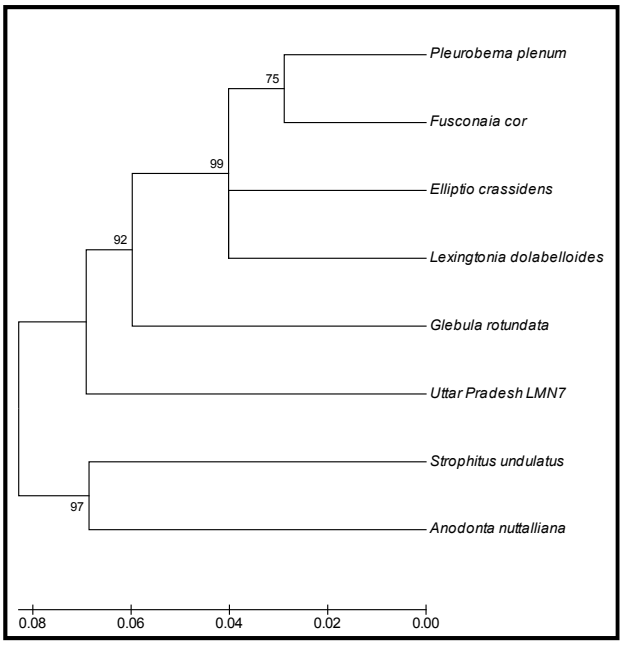


**Fig 2** Phylogenetic tree of Uttar Pradesh sample with other freshwater mussels

Unit - no. of base substitution per site.

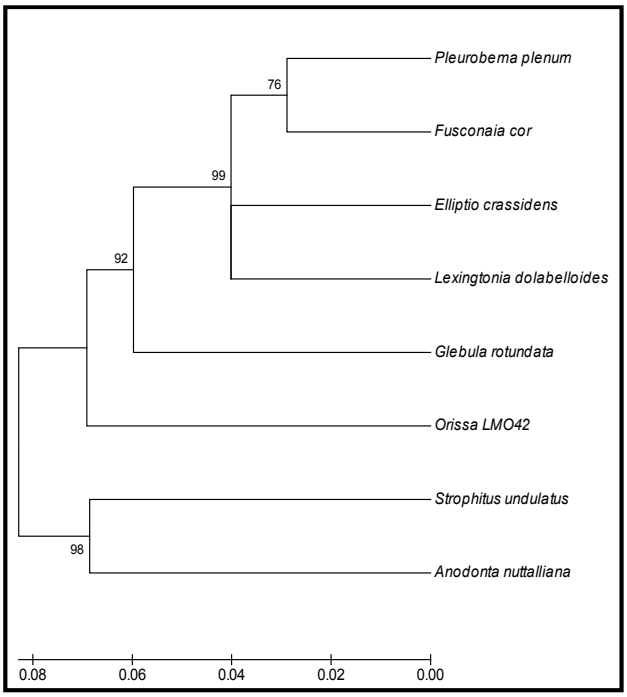


**Fig 3** Phylogenetic tree of Orissa sample with other freshwater mussels

Unit - no. of base substitution per site.

A Neighbor-joining tree (NJ tree) using Kimura 2-parameter method was constructed in (Fig. 4).

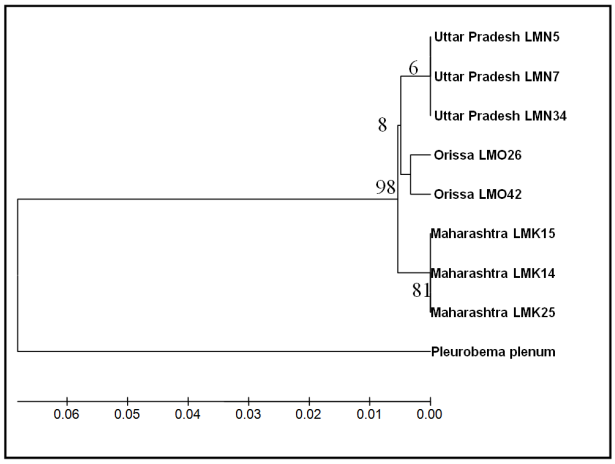


**Fig 4** Estimates of evolutionary pairwise divergence between sequences withoutgroup (Neighbor-Joining tree constructed using Kimura 2-parameter method)

Unit - no. of base substitution per site.

The NJ tree revealed a distinct population structure of this species from the Indian water bodies. The NJ tree depicted three clusters, in which *Pleurobema planum* (outgroup) showed a separate clade. The first cluster included Uttar Pradesh, second Odisha and third Maharashtra cluster showing genetic similarity.

The results of distance matrix of COI generated by Clustal W software and NJ tree showed genetic variability in all the three populations. COI generated three distinct clusters indicating three sub species of *L. marginalis.* Therefore, the present investigation suggests three sub-species of *L. marginalis* in the water bodies of three different Indian regions. Overall, the sequences of COI gene provided complete insight to the genetic polymorphism present in *L. marginalis*

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