2. Taxonomy of *Babylonia spirata*

2.1. Introduction

18S rRNA is the structural RNA for the small component of eukaryotic cytoplasmic ribosomes, and thus one of the basic components of all eukaryotic cells. The 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening. In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers (Meyer *et al.*, 2010). Their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in the smallest organisms. The 18S gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings. 18S sequences were studied by Field *et al.*, (1988) for largescale phylogeny of the animal kingdom. In animals, especially in the marine gastropods, 18S rRNA is extensively used to evaluate genetic diversity and population structure in most invertebrate organisms. The various gene regions have been employed for DNA bar-coding. However, consensus by the scientific community is essential with respect to those most suitable genes that allow robust, repeatable amplification and sequencing to provide unequal resolution to identify a boras spectrum of organism. Generally, DNA bar-coding can be used either for identification of already described species or the discovery of undescribed species. Whatever may be the application but certain criteria should be followed before using DNA bar-coding.

Apparently, DNA isolation seems to be problematic in gastropods (Skujiene and Soroka (2003) and Williams (2007) as it hampers the application of modern sequence analysis techniques and impend the study of their evolution. The storage of the tissue in
different preservatives results in different amplification success rates. It entirely depends on the preservative used as well as on the storage duration Schander and Halanych (2005). The partial 18S rRNA sequences (covering approximately 900 nucleotides and 4 molluscs) suggested either molluscan monophyly Ghiselin (1988) or polyphyly (Field et al., 1988) or paraphly Patterson (1989) and Lake (1989) depending on the tree construction methods used.

Due to the emergence of these new techniques, manoeuvrability would be definitely reduced to a great extent in the field of taxonomy. Moreover, these recent technological developments would aid the researchers to give an apparent view and suggestions while describing any taxa. This study has thrown light on the employed of new strategy in identification of *B. spirata* and preservation of its valuable genetic resource for the future prospects. Classification tree of family Buccinidae is given below.
2.1.1. Classical Taxonomy of Neogastropoda: Buccinidae

Taxonomy is an integral part of any biological study. Scientific studies involving a taxon must have an accurate identification of that taxon. In the past, morphology alone sometimes fails as an effective identifier of species (Packer et al., 2009). In the cases of morphologically similar species, or where the specimens are derived from larval or juvenile life stages, the usefulness of traditional methods of comparative morphology could be limited Gossner and Hausmann (2009). Furthermore, traditional taxonomy is heavily dependent on specialists whose knowledge is usually lost when they retire, thus there is a need for a novel scheme that would preserve taxonomic information and knowledge and make these more accessible (Tautz et al., 2003).

Buccinoidea is one of the generally accepted monophyletic groups within neogastropods. It is an abundant and diverse group of carnivorous marine gastropods, which encompasses about 1000 species (Taylor et al., 1980). Up to seven families are usually included in this superfamily: Buccinidae, Fasciolariidae, Nassariidae, Melongenidae, Buccinulidae, Columbellidae and Colubrariidae. In an extreme view, Ponder and Warén (1988) classified all but one, Columbellidae, as subfamilies of Buccinidae. Thomas and Himmelman (1988) have depicted in detail about the variation in the shell morphology, shell height, influence of predatory characteristics like shell thickness and then the shell aperture of three different populations in the Atlantic coast in Canada.

The order Neogastropoda is one of the most abundant and diverse groups of marine gastropods, encompassing more than 5200 species (Taylor, 1980). It is generally accepted that evolution of the order, as well as that of other caenogastropods, was determined mainly by the changes in the anatomy of the digestive system (Kohn, 1983),
while the similarity of shells is often the result of convergence. Much attention has been paid to the anatomy of the digestive system (Ponder, 1974) and current phylogenetic analyses of the higher classification of Neogastropoda have been based mainly on anatomical characters of the digestive system (Kantor, 1996; Kantor and Taylor, 2002), especially on the foregut. Similarly, discrimination of the families within the Neogastropoda has traditionally been based on radula and other foregut characters. The stomach of neogastropods remains a poorly studied structure. Only two publications (Smith, 1967; Medinskaya, 1993) are dedicated entirely to stomach morphology and its comparative analysis.

Considering the importance of molecular identification there are no attempts to study genetic status of Indian Babylon species, despite of its economic importance and significant contribution in marine fishery. The 18S rRNA is currently one of the most widely used gene for phylogeny, systematic and the identification of species. For gastropod tissues, however there is only very few information available on the amplification success of the 18S gene fragment. Therefore, in this chapter the morphological and taxonomic ambiguity of *B. spirata* was carried out to find out the phylogenetic relationships with other gastropod species through the 18S rRNA gene sequences.

### 2.2. Review of Literature

The three actually most common species, *viz.* *Babylonia areolata*, *B. japonica* and *B. spirata*, have continuous ranges. The other species have strikingly distinct ranges or are known from only one or a few localities. Some of the obvious distributional disjunctions are partly removed by adding records of fossil occurrences to the map, indicating that extinctions have occurred in large areas.
Bouchet and Rocroi (2005) described that according to the current classification of Gastropoda, *B. spirata* belongs to order Neogastropod. The order Neogastropoda is a highly diversified group of predatory marine shelled gastropods with more than 16,000 living species of many well known ecologically significant superfamilies including Buccinoidea, Muricoidea, Olivoidea, Pseudolivoidea, Conoidea and Cancellarioidea. Heulsken (2008) observed the genus Babylonia belongs to the family Buccinidae of super family Buccinoidea. Buccinidae is one of the most diverse families of Neogastropoda. Therefore the phylogenetic status of *B. spirata* mains quite ambiguous.

Hellberg and Morrissey (2011) reported that the DNA is more informative than protein and can be easily extracted from small traces of organic material. Ramussen and Morrissey (2008) described that the PCR based methods are extremely sensitive, often more rapid than other techniques and are widely used in the fishery industry. Ravindra kumar *et al.*, (2009) reported that the genetic characterization of fish and shellfish species that are particularly threatened or economically important species is usefull for planning their conservation strategies for safeguarding biodiversity.

Arularasan *et al.*, (2014) studied the species diversity of *B. spirata* in Tamil Nadu coast, less genetic diversity even though distinct population structure. He also described that highly informative for characterization of diversity in this gastropod species. Skujiene and Soroka (2005) observed the 18S rRNA is currently one of the most widely used gene for phylogeny, systematic and the identification of species for gastropod tissue; however, there is only little information available on the amplification success of the 18S rRNA gene fragment as a function of preservation storage media, storage duration or DNA extraction.

Saitou and Nei (1987) introduced the Neighbor- Joining (NJ) method and it has become the most widely used methods for building phylogenetic tree distances. The
Neighbor-Joining method is a greedy algorithm, which attempts to minimize the sum of all branch-length on the constructed phylogenetic tree. Conceptually, it starts out with a star formed tree where each leaf corresponds to a species and it relatively picks two nodes adjacent to the root and joins them by inserting a new node between the root and the two selected nodes (Thomas Mailund et al., 2006). Studies of using the 18S gene sequence of the ribosomal gene to evaluate the relationships among the various bivalve subclasses have been conducted on a small number of Heterodonta species Adamkiewicz et al., (1997) and Canapa et al., (1999a).

2.3. Materials and Methods

2.3.1. Sample of Babylonia spirata (Linnaeus, 1758)

Live animals of B. spirata were collected from Therespuram coast, Tuticorin, Tamilnadu, India (80° 48’N; 78° 94’ E). Immediately after the collection, samples were stored in the ice box and brought to the laboratory. Then, the species were identified based on their morphological characters.

2.3.2. Materials required for the dissection of species

For dissection, hammer, forceps and scissors were used as the major instruments. Hammer was used to break the animal shell while the, forceps and scissors were used to cut and collect the required amount of tissue for the study.

2.3.3. Morphological examination of Babylonia spirata

A morphological characteristic of the shell was also examined.

2.3.4. Tissue collection

For DNA extraction, small piece weighed about 100 mg of the tissue were excised using clean scalpel- blade and stored in a plastic vial containing 95% ethanol. Then, the vials were sealed with paraffin and kept at room temperature for further studies.
2.3.5. DNA isolation

The genomic DNA was isolated using the phenol- chloroform method followed by Oana Paula Popa et al., (2007) in gastropod species. The concentration of isolated DNA was estimated using a UV spectrophotometer.

a) Tissue sample

1. Tissue sample was placed in a centrifuge tube containing 100µl of lysis buffer. Then, complete homogenization was performed using the glass rod.

2. The homogenized contents were then made up to 200µl using lysis buffer and incubated for one hour at 55 °C (under a controlled environment).

3. To that, proteinase K 4µl of 20 mg/ml was added and incubated at 55 °C for 3h. It was followed by RNAase (2 µl of 10 mg/ml) and incubated at 37 °C for 20 min.

4. The samples were then treated with equal volume of phenol- chloroform at a ratio 25:25 and centrifuged at 12000 rpm for 12 min at 4 °C.

5. The supernatant was transformed to a new vial to this 1/10th volume of sodium acetate and twice the volumes of absolute ethanol were added to precipitate the nucleic acid contents.

6. Precipitated nucleic acid was pelleted out of by centrifuging at 12000 rpm for 15 min at 40 °C.

7. Pellet was retained and air dried pellet was dissolved in 20µl of T.E buffer and stored at -20 °C for further use.

8. The qualities of DNA were analyzed in 0.8% agarose gel electrophoresis stained with ethidium bromide.
b) Agarose gel electrophoresis

Reagents:

1. TAE buffer (Stock solution 50 X)
   - Tris base 242g
   - Acetic acid glacial 57.1ml
   - EDTA 0.5M
   - Working concentration 1 X

2. Gel Loading Buffer
   - Bromophenol blue 25mg/ml
   - Sucrose 4g/ml
   - Agarose 0.8%
   - Ethidium bromide 20 mg/ml

Procedure

1. Primarily, 50 ml of 1X TAE was added to the pre-weighed agarose. It was then melted to a clear solution by heating.

2. It was allowed to cool until it reached bearable temperature. 2.5µl of ethidium bromide stock solution was added.

3. It was then poured on to a for solidification.

4. After that comb was taken out carefully.

5. Now, the casted gel has placed an electrophoresis tank and then 1X TAE buffer was added until the gel was completely submerged.
6. DNA samples were then mixed with the gel loading buffer and loaded into the lanes of the well along with the control.

7. The samples were then electrophoresed at 50V until the run have reached 2/3rd of the gel.

8. After that, the gel was finally viewed under UV transilluminator.

c) Estimation of DNA by Bio-Photometer

1. The biophotometer and the UV lamp were switched on.

2. The wavelength was set at 260 nm and 280 nm.

3. The instrument was set at a zero absorbance with T.E buffer or sterile water as blank.

4. 5 or 7µl of the sample was taken in a quartz cuvette and made up to 50 µl TE buffer or sterile water.

5. Absorbances of the solution with the samples were recorded.

6. The concentration of DNA in the sample was calculated using the given formula.

\[ A_{260} \times 50 \mu g \times \text{Dilution factor} \]

d) Purity of the DNA

\[ A_{260}: A_{280} \text{ ratio} = \frac{A_{260}}{A_{280}} \]

- 1.8: pure DNA
- 1.7-1.9: fairly pure DNA (acceptable ratio for PCR)
- Less than 1.8: pressure of protein
- Greater than 1.8: presence of organic solvent
e) Polymerase chain reaction

Master Mix components

Milli Q Water - 10.8µl

dNTP MIX (10Mm) - 2µl

Taq buffer (10X) - 2µl

Forward PRIMER (10 Pmol) - 2µl

Reverse PRIMER (10Pmol) - 2µl

DNATemplet (100ng/µl) - 1µl

Taq DNA polymerase (5U/µL) - 0.2µl

-------

Total 20µl

-------

Table 1. Universal 18S rRNA gene primers (Giribet et al., 1996) were selected

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Produced size (Base pairs)</th>
<th>Gene targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>18SIF</td>
<td>TACCTGGTTGATCCTGCCAGTAG</td>
<td>45 °C</td>
<td>600</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>18S2R</td>
<td>GAATTACCGCGGCTGCTGG</td>
<td>45 °C</td>
<td>600</td>
<td>18S rRNA</td>
</tr>
</tbody>
</table>

An automated eppendorf Germany thermal cycler was used to perform the PCR in which the temperature and time duration are automated and which is given in the table below. The amplified products were checked on 1.5% agarose gel electrophoresis and the molecular weight was checked using a molecular weight marker (100bp RNA ladder).
<table>
<thead>
<tr>
<th>S.No</th>
<th>Process involved</th>
<th>Required temperature</th>
<th>Time duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial duration</td>
<td>94 °C</td>
<td>3 mints</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94 °C</td>
<td>1 mints</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>48 °C</td>
<td>1 mints</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>72 °C</td>
<td>1 mints and 20 sec</td>
</tr>
<tr>
<td>5</td>
<td>Final extension</td>
<td>72 °C</td>
<td>7 mints</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4 °C</td>
<td></td>
</tr>
</tbody>
</table>

Number of cycles 32

2.3.6. Sequencing the amplified product

The amplified DNA products were then subjected to bidirectional sequencing at Synergy Scientific Services, Chennai. The sequence analyzer (Applied biosystem) was used for the targeted sequence analysis.

2.3.7. Sequence data analysis

The electropherogram data generated by automated DNA sequencer and were read using the tool chromas Pro (Vl. 42) and were carefully checked for misscalls and base spacing.

2.3.8. BLAST analysis

The sequence obtained from electropherogram was subjected to BLAST analysis from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Weighed with other similar sequence in the NCBI genome database. Based on the similarity searches of barcoding sequences, the sequence of *B. spirata* was compared with the sequences of *B. zeylanica*, *B. lutos*, *B. areolata*, *Hemifusus colosseus*, *H. tematanus*, *Rapana rapiforms*, *R. venosa*, *Bathyoma tippetti*, *Nassarius conoidalis*, *Conus textile*, *C. aristophanes*, *C. quecinus*, *Chicoreus asiancs*, *Murex troscheli*, *Pterynotus triformis*, *Neptunea lyrata*, *Buccinum pemphisus*, *Bullacta exarata* and *Nordotis discus*. Each BLAST report was extracted via FASTA format from NCBI gene bank.
2.3.9. Pairwise alignment of DNA Sequence

Clustal X 2.0.6 was used to align the nucleotide sequences (Thomson, 1997). Clustal X seems to be the widely used system for aligning any number of homologous nucleotide or protein sequences. It could be freely downloaded from www.uk.plbio.kvl.dk/bioinfo.htm for multiple sequence alignments, by progressive alignment techniques. The sequences in FASTA file format were loaded into the tool through the option ‘Load sequence’ in the file menu. Then, it was completely aligned through the option ‘Do Complete Alignment’ in the Alignment menu. After computation scores values were obtained.

2.3.10. Molecular phylogenetic analysis

After the pairwise alignment, the aligned sequences were subjected to molecular phylogenetic analysis using the software MEGA 4.1β3. It was used to construct the phylogenetic trees via Neighborhood Joining method using kimura2 parameter and calculated the genetic distance for the given set of sequences in each codon position.

2.4. Results

2.4.1. Morphological description of the *Babylonia spirata*

The whelks were categorized under the order of Neogastropod and family Buccinidae. They are mostly carnivorous and scavengers. In India *B. spirata* were observed to be landed, mostly in the bottom trawls and rarely in crab net.

**Habitat**

It thrives well on muddy bottom of marine environment. Where as Salinity below 19 ppt was observed to be lethal. The *Babylonia* species mostly prefered in coars sand (Umapandi, 2013).
Food

It is a carnivore and readily accepts calm meat as food.

Growth

The laboratory studies indicated that the settlement after a day old juvenile have attained are average shell length 1.58 mm, average total weight 0.009g attain of an average shell length of about 34.3 mm and the total weight of 9.137g in 200 days (Shanmugaraj et al., 1994).

Head

The body is externally divided into three regions viz, head, foot and visceral mass. The head was observed to be bilaterally symmetrical with a pair of black coloured eyes at the base of the lateral side. The colour of the head is brown with blackish pigmentation.

Tentacles

Two pairs of contractile cephalic tentacles are present on either side of the snout which measure about 3 mm and 5 mm in length. Its depends upon the size of the animal.

Shell

The shell of *B. spirata* was observed to be thick, void with a horny surface. The shell shows a characteristic colour pattern and consisted of regular spiral rows of round bright brownish patches on a white background. The apex of the shell the oldest part and showed a reverse coiling. The umbilicus was found to be either closed or open. It has a smooth surface. Its apex (spire) was observed to be well elevated with conical shape and the inflated whorls are inflated. The whorls are six or seven in number and lie very close to each other (Figure.2a,b).
Foot

The foot is a muscular organ with a broad ventral creeping sole occurring on the ventral side of the head. It has reddish brown pigmentation and is capable of expansion and concentration. The animal having the shell length of about 60mm possessed of about 32mm. Where as it measures about 50-70mm in length and 40-50mm in width during contraction (Figure. c).

Mantle

The mantle encircles the neck region of the animal like a collar. It was observed to be broad dorsally and its width narrows down rapidly towards the posterior region, where it forms a slight ridge-like structure. Posteriorly, it possesses a continuous wall continuous with the visceral mass. Whereas, anteriorly the dorsal side of the mantle is drawn out into a siphon, which extends thorough the siphonnal canal exteriorly. Their edge involves in the secretion of the shell and plays a major role in their glandular and sensory activities (Figure. 1c).

Operculum

The operculum was found to be at the posterodorsal region of the foot. It is oval in shape horny and chitinous in nature with a terminal nucleus. The concentric rings called lines of growth surround the nucleus. The operculum has 8mm to 10mm concentric bands. The numbers of bands are lesser and appear to be a transparent in the immature forms. The adult B. spirata operculum has a maximum length and width ranged from 32mm and 18 mm with clean line growth (Figure. 2d).

Visceral complex

The fag end of the hind region of the body is occupied by the visceral mass; which contains the stomach, the digestive diverticula and gonads. The stomach is irregular in shape, elongated in antero-posterior direction, embedded in the antierior region of the midgut gland. The midgut gland occupies the major part of the visceral
mass. The gonad covers the stomach on the dorsal side and it exhibits different colours in accordance with the reproductive phase of the animal.

Reproduction

Sexes are separate. Snails of about 20 mm shell length are considered as of matured adults. The sex ratio between males and females is 1:1. Sexes cannot be distinguished from size and shell colour. The testis is yellow or orange and the ovary is dark brown in colour. Fertilization is internal (Yulianda, 2001).

Figure 2. a, b-Dorsal view and Ventral view of *Babylonia spirata*, c) Digestive system of *B. spirata*, d) Anterior and Posterior view of operculum of *B. spirata*
2.4.2. Systematic position

Based on the morphological observation the test gastropod was placed under the following systemic position:

- **Phylum**: Mollusca
- **Class**: Gastropoda
- **Order**: Neogastropoda
- **Family**: Buccinidae
- **Genus**: *Babylonia*
- **Species**: *spirata*

2.4.3. DNA sequence data analysis

Molecular taxonomy is becoming increasingly popular tool proof methodology to identify the test animal. Hence in this study, 18S rRNA gene of *B. spirata* was successfully amplified by the primer (Figure. 3). After the final alignment about 543 base pairs were sequenced and submitted in gene bank (Accession number: KP7597769). In order to figure out the evolutionary relationship among the gastropods, sequence data obtained from the 18S rRNA regions were weighed against the published sequencing data. A total of 22 sequencing of 18S rRNA gene belonging to various species were retrieved from NCBI gene bank.

The mitochondrial 18s RNA gene sequence analysis revealed that the average nucleotide frequency was A=26.15%, T=23.57%, G= 25.97% and C=24.31% (Table. 2). The mitochondrial genome is GC content 50.28% and AT content 49.72%. The Kimura2 parameter (K2P) genetic distance in 2 species is given (Table. 4). The very low evolutionary divergene (0.010) was exhibited between *B. spirata* and *B. zeylanica*. 
Table 2. Percentage of nucleotide composition of 18S rRNA gene of *B. spirata*

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Number</th>
<th>Mol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>142</td>
<td>26.15</td>
</tr>
<tr>
<td>G</td>
<td>141</td>
<td>25.97</td>
</tr>
<tr>
<td>T</td>
<td>128</td>
<td>23.57</td>
</tr>
<tr>
<td>C</td>
<td>132</td>
<td>24.31</td>
</tr>
</tbody>
</table>

Figure 3. Amplification of 18S rRNA of *B. spirata*
2.4.4. Phylogenetic tree

The data were analyzed using distance methods of Neighbourhood Joining (NJ) by the tree building method. Maximum composite likelihood using both complete deletion and pairwise deletion was investigated. The consensus trees produced, had some topology and statistical support of internal branches. The strict consensus trees derived using pairwise deletion gave better statistical support of complete deletion. Therefore, pairwise deletion phylogenetic relationships are presented in this investigation (Table. 3 and Figure. 4). In cluster tree of *B. spirata* has high similarity percentage (98%) between the *B. spirata* and *B. zeylanica*. The sister position of the species *Hemifuscus* has shown maximum similarly by showing 93% with *B. spirata*. Similarly the other two sister group of *B. spirata* have shown 68% (*Rapana* sp.) and 81% (*Conus* sp.). The bootstrap value however strongly supported to the two sets of closely related *Babylonia* sp and poorly supported the other species like *Bullacta* sp, *Nordotis* sp, *Nassarius* sp and *Bathytoma* sp.

2.4.5. Barcode sequence chromatogram of *Babylonia spirata*

```
CCATGCATGTCTAAGTTCACACCCTCGTACGGTGAAACCGCGAATGGCTCATTAATCAGTCAGGGTTCTTCTAGATGATCCAAATTTACTTGGGATAACTGTGGAATTCTAGAGCTAATACGTAGCAGCAGGTGTTGCTGCCCCTCCGGGGGAAAAGATCATATGCTGCCCACTCGAGCACGATGCTACTGGATCGACGCCGATCACCTCTTTCAATGTCTGCCCACTTGAGCAGATGGTACGTGATCTGCCCCACCCTGTTACACACCGGGGATACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCATGAGAAACGGCTACCACCCAAGGAAGGCAGCAGGCGCGCAACTTACCCACTCCTGGCACAACGGGTAGCAGGGGAATCAGGGGTTCGATTCCCGAGAGGGAGCATGAGAAGC
GGCTACCACCCAAGGAAGGCAGCAGCGCCGCAACTTACCCACTCCTGGCACAACGGGTAGTGTGACGGGAAAATAACAAATACGGGAACCTGGATATTGGATAGGCTCCCGTAATTGGAAATGAGTACACTTTAAACCCTTTAACGAGGATCTATTTGAGGGCTTTGCC
```

26
Table 3. Summary of 18S rRNA gene of some gastropod species used in this study

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Species name</th>
<th>Access number</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Bullacta exarata</td>
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</tr>
<tr>
<td>2</td>
<td>Buccinum pemphigus</td>
<td>HQ834008</td>
</tr>
<tr>
<td>3</td>
<td>Chicoreus asianus</td>
<td>HQ834013</td>
</tr>
<tr>
<td>4</td>
<td>Cantharus cecillei</td>
<td>EU236270</td>
</tr>
<tr>
<td>5</td>
<td>Morula granulate</td>
<td>HQ834015</td>
</tr>
<tr>
<td>6</td>
<td>Rapana rapiformis</td>
<td>HQ834012</td>
</tr>
<tr>
<td>7</td>
<td>Rapana venosa</td>
<td>HQ834011</td>
</tr>
<tr>
<td>8</td>
<td>Babylonia aerolata</td>
<td>HQ834021</td>
</tr>
<tr>
<td>9</td>
<td>Babylonia lutosa</td>
<td>HQ834022</td>
</tr>
<tr>
<td>10</td>
<td>Babylonia zeylanica</td>
<td>KF779496</td>
</tr>
<tr>
<td>11</td>
<td>Babylonia spirata</td>
<td>Present study</td>
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<tr>
<td>12</td>
<td>Nassarius conoidalis</td>
<td>HQ834028</td>
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<td>Neptunea lyrata</td>
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<td>19</td>
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<tr>
<td>20</td>
<td>Murex troshchel</td>
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</tr>
<tr>
<td>21</td>
<td>Conus textile</td>
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</tr>
<tr>
<td>22</td>
<td>Pterynotus triformis</td>
<td>HM486919</td>
</tr>
</tbody>
</table>
Figure 4. (Neighbour Joining) NJ tree showing the phylogenetic relationship of the studied Gastropod species based on 18S rRNA gene sequence.
Table 4 K2P genetic distances between the studied Gastropod families based on 18S rRNA gene sequence

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Bullacta_exarata_AF188675</th>
<th>Buccinum_pemphigus_HQ834008</th>
<th>Chicomurus_asianus_HQ834013</th>
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<th>Rapana_rapiformis_HQ834012</th>
<th>Rapana_venera_HQ834042</th>
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2.5. Discussion

In the classical taxonomy a family is more precise than orders but less precise
the genera. Organisms belonging to the same family would have evolved from the same
ancestors and share relatively common characters. According to Ponder and Linberg
(1997) the family Buccinidae belongs to the subclass Orthogastropoda which has a
characteristic outer dextral shell with eight whorls. Due to this noticeable characteristic
feature the test gastropod, is placed under to the family Buccinidae.

*Babylonia spirata* have been considered as an important resource of Indonesia
and India. In Indonesia, investigations have been made on the growth by Yulianda and
Dhanakusumah (2000) and reproductive biology (Yulianda, 2001) of this resource.
While focusing the light on the molecular biology of *B. spirata*, DNA seems to be
more informative than its protein and could be extracted easily from a trace amount of
organic material. Besides, PCR- based methods were observed to be extremely sensitive
and rapid while compare with it with other techniques, which were widely used in the
fishery biology. In these recent days, numerous DNA – based detection methods have
been widely applied for the identification and characterization of different types of
animal and plant. Hebert *et al.*, (2003a) and Hellberg and Morrisy (2011) have
suggested that a single gene sequence would be sufficient to differentiate all or at least
the vast diversity of animal species. They have also suggested the mitochondrial DNA
gene 18S rRNA gene could be effectively used as a universal bio identification tool.

Bouchet *et al.*, (2005) have reported that the Neogastropoda have adapted well to
almost every marine environment commencing with an explosive radiation during the
cretaceous period. Ponder *et al.*, (2008) and Rasmussen and Morrissey (2008) have
accepted the monophyly of the group based on their notable several synapomorphies,
which mostly related with the anatomy of their digestive system.
According to the observation of Cunha et al., (2009) the phylogenetic relationships of Neogastropod were based on their morphological characters remains to be unclear, still toady. Whereas, Huelsken et al, (2008) have placed the genus *Babylonia* sp under the family Buccinidae of Buccinoidea (super family). Buccinidae seems to be the one of the most diversified families of Neogastropoda. Therefore the phylogenetic status of *B. zeylanica* remains quite ambiguous until today.

Traditionally systematic classification has been done on the basis of their morphological traits, but the of molecular data analysis have recently paved the way to reconstruct the phylogenies and are likely to be know as molecular phylogenetic analysis. The usesage of molecular methods have brought a new insight into the phylogenetics and systematic science of the selected fauna. In eukaryotes, nuclear ribosomal DNA typically exhibits a strong concerted evolutionary pattern in which the several hundred of DNA sequences within the species might be diverged from their lineage.

Zou et al., (2011) have reported that the genus *Babylonia* belongs to the family Buccinidae, which was catagorized under the super family Buccinoidea. The Buccinoidea and Muricoidea were been never recorded as monophyletic and they formed major monophyletic clade, sometimes together with Pseudolivoidea and Olivoidea (Zou et al., 2011). Recently, Oliverio and Modica (2010) have claimed that the Buccinoidea as monophyletic, which were grouped together with Pseudolivoidea, Olivoidea and Muricoidea. The monophyly of Murricoidea has not been recorded either in previous morphological and molecular studies (Ponder et al., 2008).

The use of molecular techniques seems to have a profound impact on systematic zoology and phylogentic research. Many studies have depicted that the molecular phylogenetic analysis is a powerful tool for elucidating the evolution of the molluscs fauna (Colgan et al., 2000).
In this present study to determine the molecular evolutionary relationship of the *Babylonia spirata* with the other related species like, *Babylonia* sp, *Hemifuscus* sp, *Rapana* sp, *Conus* sp, *Nordotis* sp, *Bucciumu* sp, *Cantharus* sp, *Murex* sp, *Chicoreus* sp, *Bullacta* sp, *Nassarius* sp, *Bathyoma* sp, *Morula* sp, *Pterynotus* sp and *Neptunea* sp helped to obtain a fool-proof identification of the species. Phylogenetic study was performed by the Neighbour Joining (NJ) method. The cluster analysis comprised of 22 species in total and among them, the *B. spirata* exhibits the highest similarity percentage (98%) with *B. zeylancia*. The sister position of the species *Hemifuscus* sp. have shown a maximum similarly of 93% with *B. spirata*. Similarly, the other two sister group of *Babylonia spirata* have shown the similarity of 68% (*Rapana* sp.) and 81% (*Conus* sp). These results have been supported by the reports of Vaithilingam Ravitchandirane *et al.*, (2013) in which, they have commanted on the phylogenetic status of *Babylonia zeylanica* (Babyloniidae) based on 18S rRNA gene. Further more, they have reported that the Babyloniidae is clusters closely with Columbellidae the super family of Buccinoidea.

The bar-coding profile of the present study clearly reveals that the barcode region of *B. spirata* from Tuticorin coast a stable DNA with appropriate G+C and A+T content which are as follows; Adenine (26.15%), Thymine (23.57%), Guanine (25.97%) and Cytosine (24.31%). Nowadays the aquaculture of this species was observed to be gradually developing. But, it surely requires more molecular markers for genetic resource management and for conducting breeding programmes, interestingly, the above mentioned could be made possible by the usage of micro satellite markers. It might hope fully leads to produce a good quality product, which could be easily marketed in the future. Moreover, from the morphological observations; the test gastropod occupy the family Buccinidae (Morphological description of *B. spirata*). Moreover, seems to the
molecular phylogenetic studies provides a better resolution of linearity among the gastropods when compared with the classification based on morphology and other traits.

Similarly Park and Foighi (2000) have reported that the phylogenetic relationships were made on the basis of the morphological features remains uninvestigated till today. Therefore, this molecular phylogenetic analysis was carried out on the base of ribosomal gene seems to be a valuable tool for this investigation regarding the evolutionary relationships among the gastropod families. Based on this 18S rRNA gene sequence analysis, Babylonidae have been observed to be clustered closely with Hemifuscus sp of super family of Buccinoidea. Today, additional genetic information and increased sampling seems to be trustworthy and provides a new insight into this study of phylogenetic relationship between all Indian species of Neogastropoda.