MOLECULAR TAXONOMY OF SELECTED SPECIES OF *MACROBRACHIUM* BATE, 1868 (DECAPODA, PALAEMONIDAE) OF KERALA WATERS.

BY

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THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTERS OF FISHERIES SCIENCE

Faculty of Fisheries

Kerala Agricultural University

2011

DEPARTMENT OF FISHERY BIOLOGY,

COLLEGE OF FISHERIES,

PANANGAD, KOCHI - 682506.

Dedicated to my family and to guide
Dr.K.V. Jayachandran.

DECLARATION

I hereby declare that this thesis entitled -**"MOLECULAR TAXONOMY OF SELECTED SPECIES OF *MACROBRACHIUM* BATE, 1868 (DECAPODA, PALAEMONIDAE) OF KERALA WATERS"** is a bonafied record of research work done by me during the course of research and that the thesis has not formed the basis of award to me for any other degree, diploma, association, or other similar title of any other University or society.

Panangad

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G.JAYARAJ

CERTIFICATE

Certified that this thesis entitled --**"MOLECULAR TAXONOMY OF SELECTED SPECIES OF *MACROBRACHIUM* BATE, 1868 (DECAPODA, PALAEMONIDAE) OF KERALA WATERS"** is a record of research work done independently by **Mr. G. JAYARAJ** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship, or association to him.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my Parents, sister and brother as their care and blessings have immensely contributed in the success till this phase of my life.

I would like to express my gratitude and respect to my major advisor Dr. K. V. Jayachandran, Professor and Head, Dept. of Fishery Biology, College of Fisheries, my first promoter, for his enthusiastic guidance, encouragement, and continuous support throughout this work. With his permanent support, I never lost my eagerness to progress. Truly, without his assistance, this thesis would not have been accomplished within the time frame.

I express my sincere gratitude to The Dean, College of Fisheries, Panangad, for providing facilities for carrying out the present investigation.

I am highly grateful to Dr. A. Gopalakrishnan, Principal scientist, NBFGR, Kochi unit, for his supervision and advice during my research and also providing all necessary facilities and freedom to work in the laboratory. The knowledge I received from the laboratory is immense and as a young researcher I would like to continue the collaboration with this lab in the future also.

I would like to thank Prof. Dr. J. Rajasekaran Nair, Dept. of Fishery Biology, College of Fisheries, for rendering able guidance and support throughout the research work and also for the invaluable co-operation and suggestions during the period of my research work.

I would always remain indebted to Prof. Dr. T.M. Jose, Dept. of Fishery Biology, College of Fisheries, who offered wholehearted support, patience and guidance for my research work and to Prof. Dr. Anna Mercy for her excellent support.

I gratefully acknowledge Dr. P.R. Divya, Scientist, NBFGR, Kochi unit, for her unselfish motivation, creative suggestions and co-operation. I also would like to thank Mr.Rajkumar for all technical work in the laboratory and also acknowledge Dr.V. S. Basheer, Dr. Raja Swaminathan and Mr. Kathirvel, Scientists, NBFGR, Kochi unit for their valuable support for my research work.

Indeed it is my greatest pleasure to acknowledge many of my close associates of mine, namely, Mr. Sabin and his family, Mr. Yuvaraj and Mr. Prem, Mrs. Indira, Mr. Dev, Mr. Ajay, Mr. Mathi, Mr. Suraj, Mrs. Sherine, Mr. Kareem, Mr. John, Miss. Sheeba and Miss. Sajeela for their encouragements. I also take this opportunity to thank all my friends (both seniors and juniors) in the College for their immense support.

Thanks are also due to all the staff members of all Departments and to all staff of Library, College of Fisheries for their kind support during my study.

I am gratefully acknowledging the financial support received from ICAR during the period master degree. It is my innate duty to express my sincere gratitude to Tamil Nadu Government Fisheries Department for the generous support in granting me a long leave to complete my research work successfully.

I express my reverence to God almighty who gives me an enormous Strength to overcome all the adversities to complete this dissertation successfully.

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INTRODUCTION

1. INTRODUCTION

Freshwater prawns of the genus *Macrobrachium* Bate, 1868 belong to Class Crustacea, Order Decapoda and Family Palaemonidae. They are important because they are most diverse, abundant and widespread among the crustacean genera. *Macrobrachium* is distributed globally across the tropical and subtropical regions of the world and comprises over 200 described species (Jayachandran, 2001). From India around 60 species have been reported (Jayachandran, 2010) and from Kerala 20 species (Jayachandran, 2010). These interesting groups of decapod crustaceans are thought to have originated from marine ancestors. Some of which subsequently migrated towards fresh water in more than one wave and hence its members are known to inhabit the entire range of habitats from purely marine areas to inland hill streams and impounded water bodies (Tiwari, 1955b; Shokita, 1979; Jalihal *et al.,* 1993) Although majority of *Macrobrachium* species inhabit fresh water, most of them require estuarine or marine environments for completing life cycle and hence are euryhaline in nature (Atkinson, 1977; Jalihal *et al.,* 1993) and yet others complete their entire life cycle in pure freshwaters (Jayachandran, 2010).

Some land locked species complete their life cycle in freshwater itself. Their larval life cycle is abbreviated. The abbreviated development of larvae in land-locked species was suggested to be a result of selective pressures for

becoming established in freshwater environments (Shokita, 1979; Magalhaes and Walker, 1988) and is a convergent phenomenon overriding phylogenetic relationships even above the generic level (Magalhaes and Walker, 1988). Pereira (1997) carried out the first phylogenetic study based on morphological characters of the family Palaemonidae. The phylogenetic relationships of the genus *Macrobrachium* remain comparatively poorly understood (Liu *et al.*, 2007; Murphy and Austin, 2005). The palaemonids have historically been a taxonomically difficult group because they appear to have characters which are morphologically close (Holthuis, 1950; 1952; Boulton and knott, 1984; Chow and Fujio, 1985; Finchiam, 1987; Jayachandran, 2001). Until recently, all major systematic studies have been based on morphological characteristics alone (Holthuis, 1950; Riek, 1957; Finchiam, 1987; Jayachandran, 2001).

Among the Indian states, Kerala state have a good resource of *Macrobrachium* species and they are distributed in different water bodies such as hill streams, valleys, lower stretches of rivers and estuaries. Many species of *Macrobrachium* exhibit variations in morphology at intra-specific level. Most of the studies are based on morphological comparisons and taxonomic confusion still exist. Therefore advanced molecular studies are needed for confirmation of species and variants of *Macrobrachium.* This will help to establish phylogenetic relationship also.

Realizing the importance of molecular taxonomy, the present study was undertaken with the objectives such as: confirmation of species status of variants of *Macrobrachium canarae* (Tiwari, 1958), *M. idella* (Hilgendorf, 1898), *M. latimanus* (Von Martens, 1868) and *M. scabriculum* (Heller, 1862) and generate genetic data to postulate phylogenetic relationships. The thesis contains the result of molecular analysis of DNA barcoding [Cytochrome C Oxidase I gene (CO I)] and 16S rRNA of genes of mitochondrial DNA of *Macrobrachium canarae*, *M. idella*, *M. latimanus* and *M. scabriculum*. The four species inhabit varied habitats: *M. latimanus* is a hill stream prawn; *M. canarae* inhabits valleys without migration; *M. idella* and *M. scabriculum* inhabits lower stretches of river with estuarine migrations.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Morphological approaches to *Macrobrachium* Taxonomy

The taxonomic studies of palaemonid prawns of India have been carried out by Kemp (1915- 1925), Henderson and Matthai (1910), Tiwari (1947-1963), Tiwari and Holthuis (1996), Jayachandran (1984 - 2010), Jayachandran and Joseph (1982-1992), Jayachandran *et al.* (2007). All these studies have reported about 60 species of *Macrobrachium* from India and 20 species from Kerala. Studies on molecular taxonomy of species under the genera *Macrobrachium* are rare.

2.2. Molecular approaches in Taxonomy

The biological diversity of each country is a valuable and vulnerable natural resource. Sampling, identification and further study of biological specimens are the first steps toward biodiversity studies. Species identification and classification have traditionally been the specialist domain of taxonomist and these are based on morphology, behavioural and species habitat and provide a nomenclatural backbone and key prerequisite for numerous biological studies. Accurate identification of species is fundamental to biodiversity studies and it becomes absolutely essential for conservation programmes. Conventional morphology based taxonomy is very essential and

also provide a lot of advantages for classifying and identifying the living things. But in some cases facing problems are,

a) The specimens are larval and fragmentary or damaged condition,

b) Cryptic members of species complexes,

c) Having same morphological pattern,

d) Lack of morphological keys,

e) Lack of identification by a traditional taxonomist,

f) Showing little evolutionary differentiation,

g) Members of closely related species which could be identified at a particular life stage only.

In such conditions conventional taxonomy has limitations for finding species variants. So, combining morphological, developmental, behavioural and genetic data allows the confirmation of species status and precise delineation of species differences (Palmer *et al.*, 1990; Collins *et al.*, 1996; Collin, 2005; Dayrat, 2005; Desalle *et al.*, 2005).

Molecular techniques have become a major tool for systematic biologists for confirming categories at species level and above. Molecular taxonomy is not meant to be a critique of morphology based taxonomy, but must be firmly anchored within the knowledge, concepts, techniques and infrastructure of traditional taxonomy. The idea of a standardized molecular identification

system emerged progressively during the 1990s with the development of PCR based approaches for species identification.

During the past few years, Nuclear DNA markers are used for species identification and phylogenetic relationship. The availability of vast numbers of nuclear loci does not mean that every phylogenetic problem will be solved easily. For example, finding a large number of nuclear genes that are evolving at the appropriate rate for a given phylogenetic problem can still be challenging, especially for studies at lower taxonomic levels that require rapidly evolving genes. The problem is that rapidly evolving genes are less likely to have conserved primer sites, and so can be difficult to amplify and sequence. Nuclear introns offer one potential solution. Introns are non-coding and thus free to evolve rapidly, but are flanked by exons, which are more conserved. Many primers for nuclear introns are now available that are potentially usable across vertebrates (Lyons *et al.*, 1997; Friesen *et al.,* 1999; Dolman and Phillips, 2004). However, some significant problems still remain. First, whether a given locus will work in a particular clade still seems to be quite hit-or-miss (e.g., because of variation in intron length among clades). Second, even fast-evolving introns may offer limited information in very recent or slow- evolving groups (e.g., turtles). Third, nuclear genes may often retain ancestral polymorphisms that are shared among closely related species which can confuse attempts to reconstruct phylogenies and species limits.

Early studies of molecular taxonomy mainly focused on mitochondrial genes (Avise, 2000). Mitochondrial DNA data have both advantages and disadvantages in systematic studies. On the plus side, mitochondrial genes are relatively easy to amplify and sequence. The mitochondrial genome also has a relatively fast mutation rate in vertebrates, providing an abundance of potentially informative variation, even among closely related species and conspecific populations (Avise, 2000). Furthermore, the mitochondrial genome has a smaller effective population size than the typical nuclear gene. Thus, the mitochondrial genome may be less subject to the problem of retained ancestral polymorphism and so, all other things being equal, the mitochondrial phylogeny may tend to track the phylogeny of the species better than a typical nuclear gene (Moore, 1995). On the other hand, the fast mutation rate of the mitochondrial genome can become a serious disadvantage at deeper phylogenetic levels. When rates of mutation are high, relatively long branches in a phylogeny (those expected to have accumulated many changes) may tend to be erroneously placed together by many phylogenetic methods because they will accumulate many shared traits (Huelsenbeck, 1995; Felsenstein, 2004).

2.2.1. History of DNA Barcoding

One of the first conferences exploring these issues was the DNA taxonomy workshop at the Deutsche Staatssammlung in Munich in April 2002, with participation of some 100 scientists mainly from European countries (Tautz *et al.*, 2002). At the earlier stage, the issues much in the focus were; the most

useful markers for the so called DNA taxonomy (i.e., a universal DNA based classification system across all organismal groups), the difficulties of linking established names to entities within a DNA based system (Tautz *et al.*, 2003), and the implications for nomenclature (Minelli, 2003). With a different viewpoint from the German meeting, a group of scientists lead by Paul Hebert at University of Guelph in Canada developed the use of part of one mitochondrial gene as a universal identification marker for animal species (Hebert *et al.*, 2003a; b). Building upon the idea of the Universal Product Code known as 'barcodes', a few DNA nucleotides (e.g., the sequence of a short DNA fragment) may well provide an immediate diagnosis for species (Brown, 1997).

The use of short DNA sequences for the standardized identification of organisms has recently gained attention under the terms DNA barcoding or DNA taxonomy (Tautz *et al.*, 2003). Among the promising applications of this method are the assignments of unknown life-history stages to adult organisms (Hebert *et al.*, 2004a), the large-scale identification of organisms in ecological or genomic studies (Blaxter, 2004) and most controversially, explorative studies to discover potentially undescribed "candidate" species (Hebert *et al.*, 2004a). Although it is not a fundamentally new technique (Moritz and Cicero, 2004), DNA barcoding is promising because technical progress has made its large-scale, automated application feasible (Tautz *et al.*, 2003; Blaxter, 2004) which may accelerate taxonomic progress (Wilson, 2004).

Since Linnaeus, biologists have used distinguishing features in taxonomic keys to apply binomial species names. Then, as a master key opens all the rooms in a building, the binomial species name accesses all knowledge about a species. From insects to birds, evidence now shows that short DNA sequences from a uniform locality on genomes can also be a distinguishing feature. As a Linnaean binomial is an abbreviated label for the morphology of a species, the short sequence is an abbreviated label for the genome of the species. The barcode of life thus provides an additional master key to knowledge about a species.

2.2.2. DNA Barcode

DNA barcode is a tool used for confirmation of species status, finding out the new species and finding out phylogenetic relationship based on short sequence of mitochondrial gene. A 648-bp region of the Cytochrome C Oxidase subunit I (CO1, COI) of mitochondrial gene has been initially proposed as a potential 'barcode' (Hebert *et al.*, 2003a).

2.2.3. Mitochondrial DNA (mtDNA)

Each mitochondrion has its own DNA, or genome, separate from the DNA in the nucleus. Each mitochondrion is estimated to contain 2-10 mtDNA copies. The mitochondrial genome is a circular molecule of double-stranded DNA, 16,569 base pairs long. A base is a specific component of the DNA and is

made of Adenine, Thymine, Guanine or Cytosine (A, T, G, and C). Within the genome, there is an approximately 1100 base long regulatory region, called the D-loop. Because this region accumulates genetic changes faster than the rest of the genome, it is also referred to as the hypervariable region. The remainder of the mitochondrial genome is coding DNA - it is copied into RNA molecules that perform downstream functions within the cell. The mitochondrial genome codes for 13 proteins (used in energy production by the mitochondria) two ribosomal RNAs (used for protein synthesis) and 22 transfer RNAs (also used for protein synthesis).

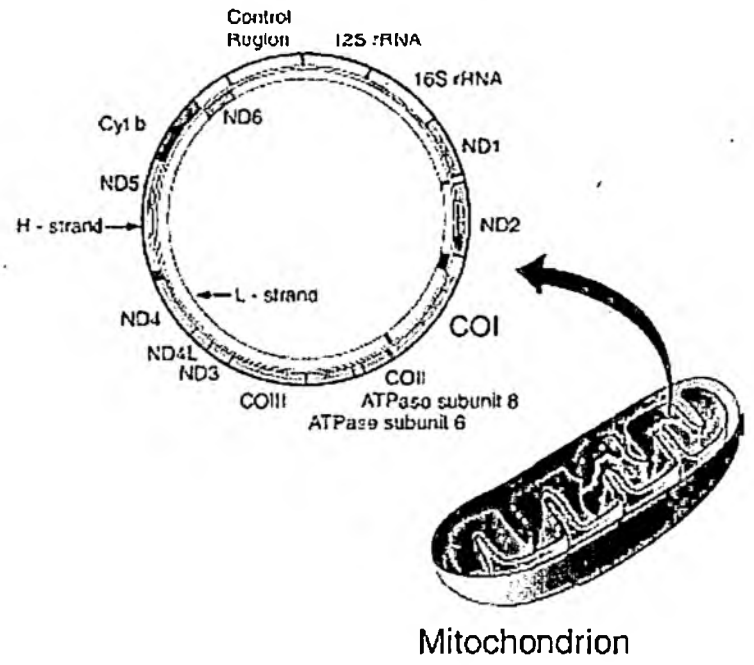

Fig. 1. A diagrammatic representation of a mitochondrial genome based on complete mitochondrial DNA sequences.

2.2.4. Why mitochondrial DNA (mtDNA)?

1. Mitochondrial DNA is inherited only from the mother: the fertilized egg destroys the mitochondria of the sperm. Because of this selective maternal transmission, mitochondrial DNA sequences can be used by population geneticists and evolutionary biologists to shed light on the unbroken genetic line connecting us to our maternal ancestors.
2. The low effective population size and rapid mutation rate in animals makes mtDNA useful for assessing genetic relationships of individuals or groups within a species and also for identifying and quantifying the phylogeny among different species, provided they are not too distantly related.
3. Copy number: while each cell typically contains only 2 copies of nuclear DNA sequences, the same cell encompasses 100-10,000 mitochondrial genomes.
4. Absence of introns: In animals, mitochondrial genes rarely contain introns, which are non-coding sequences interspersed between the coding regions of a gene. Thus, amplification of mitochondrial DNA is usually straight forward.

2.2.5. Why select the barcode sequence from within one gene?

With a few exceptions, animal mitochondria contain an identical set of genes: 13 protein-coding, 2 ribosomal RNA, and 22 transfer RNA genes. While the order of the genes and their polarity differ markedly among animal phyla, sequences from diverse organisms can be easily compared as long as the barcode locality is limited to one gene. Staying within the boundaries of a single gene also eases development of broad range techniques for recovery of barcode sequences from diverse organisms.

2.2.6. Why standardize on COI for animals?

The mitochondrial protein-coding genes generally contain more differences than the ribosomal genes and thus are more likely to distinguish effectively among closely related species. Sequence comparisons among protein-coding genes are easier because they generally lack insertions or deletions. Among candidate protein-coding gene regions, the Cytochrome C Oxidase I (COI) locality contains sequence differences representative of those in other mitochondrial protein-coding genes.

The Cytochrome C Oxidase I (COI) gene does have two important advantages: first universal primers for this gene are very robust, enabling

recovery of its 5' end from representatives of most, if not all, animal phyla (Folmer *et al.*, 1994; Zhang and Hewitt, 1997); Second, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene, its third position nucleotides show a high incidence of base substitutions, leading to a rate of molecular evolution that is about three times greater than of 12S or 16S rDNA (Knowlton and Weigt, 1998). In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closed allied species, but also phylogeographic groups within a single species (Cox and Hebert, 2001; Wares and Cunningham, 2001).

2.2.6.1. Landmarks based on DNA Barcoding

Cytochrome C Oxidase I gene, (COI) has proved to be suitable for the identification of a large range of animal taxa, including gastropods (Remigio and Hebert, 2003), fish (Ward *et al.*, 2005, Lakra *et al.*, 2010), crustacea (Costa *et al.*, 2007) , ringtails (Hogg and Hebert, 2004), butterflies (Hebert *et al.*, 2004a), birds (Hebert *et al.*, 2004b; Kerr *et al.*, 2007), mayflies (Ball *et al.*, 2005), spiders (Greenstone *et al.*, 2005), ants (Smith *et al.*, 2005), and recently diatomea and Protista (Evans *et al.*, 2007).

Cytochrome C Oxidase 1 gene (COI) provides identifications at two levels in the taxonomic hierarchy - orders and species (Costa *et al.*, 2006). The Cytochrome C Oxidase I gene (COI) region is appropriate for discriminating

between closely related species across diverse animal phyla and this has been used for marine and freshwater fishes (Hajibabaei *et al.*, 2005; Steinke *et al.*, 2005; Ward *et al.*, 2005; Hubert *et al.*, 2008; Lakra *et al.*, 2009). Mitochondrial DNA reveals polygenetic structure and cryptic diversity in Australian freshwater macro-invertebrates (Andrew *et al.*, 2004). Molecular data have proven very useful for clarifying the taxonomic relationships and defining species boundaries in morphologically conservative or highly variable groups of freshwater crustaceans based on COI gene. Potential benefits of a COI barcode identification system for crustaceans include the identification of parasitic crustaceans at any developmental stage (Oines and Heuch, 2005) as well as the detection of invasive crustacean species (Armstrong and Ball, 2005). Recently Francis *et al.* (2008) submitted Genbank data on *Fenneropenaeus merguiensis* based on COI gene.

2.2.7. Ribosomal RNA

The advent of molecular technique made possible not only the genetic analysis and also the study of evolutionary relationship among the species or phylogeny. Although there are many ways to study phylogenetic relationships (example amino acid sequence of protein, nucleotide sequence of nucleic acid and presence or absence of enzyme), it is now recognized that ribosomal RNA is important indices of phylogeny. Ribosomal RNA has the characteristics that are

important in studying the evolutionary divergences. These universal characteristics have identical functions in all living organisms. This functional constancy makes rRNA ideal molecular chronometers to measure evolutionary changes. Because rRNA is a small molecule that cannot tolerate much structural changes and still retains its function, its sequences moderately well conserved or constant across phylogenetic lines. Consequently small differences in rRNA sequence can be used to determine evolutionary distance. Among the rRNAs, 16S is used most commonly as phylogenetic tool.

2.2.7.1. Landmarks based on 16S rRNA

The 16S rRNA gene has both fast and slow evolving region and therefore can provide useful information across the broad taxonomic spectrum from the population to the family level. Sequences from the 16S rRNA mitochondrial gene region have been found very useful for studying taxonomic questions and phylogenetic relationship within a number of crustaceans groups (Bucklin *et al.,* 1995; Crandall and Fitzpatrick 1996; Kitaura *et al.*, 1998; Crandall *et al.,* 1999). Murphy and Austin (2002; 2003; 2004; 2005) published a series of results for the phylogeny of *Macrobrachium* species based on the mitochondrial DNA fragment of the large subunit (16S) ribosomal (r)RNA gene marker. Conserved mtDNA genes like 16S and 12S ribosomal RNA have been widely used to study the phylogeny and population genetic analysis of penaeid shrimps (Palumbi and Benzi, 1991; Garcia-Machado *et al.*, 1993;, Millan *et al.*, 2002;

Gusmao *et al.* , 2000). Sequence comparison of mitochodrial 16S rRNA gene was carried out between *Penaeus monodon, Marsupenaeus japonicus* and *Fenneropenaeus indicus* as an approach to find the usefulness of this gene segment for phylogenetic analysis in penaeids (Shekhar *et al.*, 2006). Partial sequence of 16S rRNA gene has been used for resolving taxonomic ambiguities and analyzing phylogenetic relationship in bivalves (Bendezu *et al.*, 2005; Klinburga *et al.*, 2005), fishes (Quenouille *et al.*, 2004; Doiuchi and Nakabo, 2006) and crustaceans (Imai *et al.*, 2005).

2.2.8. The Consortium of the Barcode of life (CBOL)

The Consortium for the Barcode of Life (CBOL) is an international organisation devoted to developing DNA barcoding as a global standard in taxonomy. It comprises more than 120 member organisations from 45 countries and includes museums, herbaria, zoos, research organisations, governmental and intergovernmental agencies as well as other organisations involved in taxonomic research and biodiversity issues. Members agree to submit their DNA barcode sequences and voucher specimen data to a public database. CBOL was launched in May 2004. The Canadian Centre for DNA Barcoding (a member of CBOL) oversees a website for Barcode of Life Data Systems (BOLD) that permits the uploading of COI sequences from the 50 region of the COI gene and returns species level identification when one is possible. At present the site has more than 165,000 sequences from almost 20,000 species and these numbers

are increasing steadily. The site also permits a variety of forms of data analysis for submitted sequences. CBOL works in cooperation with a number of other organisations including the Global Biodiversity Information Facility (GBIF), National Centre for Biotechnology Information (NCBI) and many taxanomic communities and web based projects.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Stock Solutions for DNA Isolation

0.5 M Tris Cl (pH : 8.0)

Tris base - 3.028g

Distilled water - 40ml

Adjust pH to 8.0 by using HCl.

Make up the volume to 50ml, autoclave and store at 4°C.

0.5M Tris Cl (pH: 8.3)

Take above composition, adjust pH to 8.3 using HCl, make up to 50ml, autoclave and to store at 4°C.

0.5M EDTA (pH: 8.0)

EDTA - 9.31g

Distilled water - 40ml

Adjust pH to 8.0 by using NaOH.

Make up the volume to 50ml, autoclave and store at 4°C.

10mM Tris Cl (pH: 7.5)

Tris base - 0.03028g

Distilled water - 20ml

Adjust pH to 7.5 using HCl.

Make up the volume to 25ml, autoclave, and store at 4°C.

RNAase buffer

10mM Tris Cl (pH 7.5) - 10µl

15mM NaCl (0.8766mg/ml) - 30µl

Distilled water - 960µl

Autoclave and store at 4°C.

TBE buffer 10X (pH: 8.0)

Tris Cl (0.9M) - 10.8g

Boric acid (0.9M) - 5.5g

EDTA (0.5M) - 0.75g

Make up the solution to 100ml with double distilled water.

Autoclave and store at 4°C

Bromophenol blue

0.5% Bromophenol blue - 500mg

Make up the solution to 100ml with double distilled water.

Autoclave and store at 4°C

Saturation of Phenol with Tris-HCl (pH: 8.0)

Reagents required

Water saturated Phenol	- 500 ml
0.5 M Tris-HCl (pH: 8.0)	- 1000 ml
0.1 M Tris-HCl (pH : 8.0)	- 1500 ml

Preparation of saturated phenol with tris-HCl

a) 0.1% (500 mg) 8-hydroxyquinoline was added to 500 ml of water saturated phenol.

b) Flask-containing phenol was covered with aluminium foil to avoid light reaction.

c) 500 ml 0.5 M Tris-HCl was added.

d) Solution was stirred using magnetic stirrer for 15 minutes.

e) Phenol was allowed to settle by keeping the solution for 30 minutes.

f) Supernatant was decanted.

g) 500 ml of 0.1 M Tris-HCl was added.

h) Steps 4, 5 and 6 were repeated twice.

i) pH of decanted supernatant was checked using a pH paper. (The final pH should be 8.0).

j) 500 ml of 0.1 M Tris-HCl was added to phenol.

k) Phenol was stored at 4°C in dark bottles covered with aluminium foil.

Chloroform: Isoamyl alcohol - 24: 1 v/v

3M Sodium acetate (pH: 5.2)

pH was adjusted to 5.2 using glacial acetic acid.

TE buffer

Tris-HCl (pH: 8.0) - 10mM

Na_2 EDTA. $2H_2O$ (pH: 8.0) - 1mM

Reagents required for Agarose Gel Electrophoresis

1. Agarose
2. Gel running buffer-1xTBE
3. Ethidium bromide

3.1.2. Mitochondrial DNA (mtDNA) primers

16S rRNA and Cytochrome C Oxidase I (COI) primers from Bioserve Laboratories, India were used to amplify mtDNA regions of the selected *Macrobrachium* species as mentionrd in table 1.

Table 1. Mitochondrial DNA (mtDNA) primers used in the present investigation.

Sl. No	mtDNA Primers		Sequence 5'-3'	No. of bases	Annealing temperature	Reference
1	16S rRNA	L	ACTTGATATATAATTAAAGGGCCG	24	52°C	Daisy *et al.*,.2009
		H	CTGGCGCCGGTCTCAACTCAAATC	24		
2	COI	L	CCTGCAGGAGGAGGAGAGCC	20	50°C	Palumbi *et al.*, 1991
		H	AGTATAAGCGTCTGGGTAGTC	21		

3.2. Methods

3.2.1. Collection of samples

Specimens of *Macrobrachium canarae*, *Macrobrachium idella*, *Macrobrachium latimanus* and *Macrobrachium scabriculum* were collected during the period of 15th November to 12th December, 2010. The specimens of two varieties of *M. idella* and *M. scabriculum* were collected

from chamappakara (9° 57' N latitude 76° 19' E longitude) in Ernakulum district and *M. canarae* and *M. latimanus* were collected from middle stretches of Periyar River at Thattekad stations (76° 40' to 76 ° 15' N latitude and 10° to 11° E' longitude) in Idukki district. These specimens were accurately identified based on work done by Jayachandran, 2001. *M. idella* shows variations within the individuals based on arrangement of teeth pattern on rostrum and those are marked as Type I and Type II. The tissue (pleopods along with muscle) samples of two individuals of each specimen were stored in sterile eppendorf tubes containing 95% ethanol and sealed with Para film and kept at room temperature until further analysis.

3.2.2. Genomic DNA extraction

Total genomic DNA was extracted from the tissue samples following the procedures of Sambrook *et al.* (1989) after few minor modifications. In 2ml centrifuge tubes, 5mm X 5mm of tissue (stored in 95% ethanol) from each specimen was taken and ethanol was evaporated by keeping the tubes open. To lysis the cells, incubation buffer (10mM Tris, 1mM EDTA, 0.4M NaCl, 10% SDS and Proteinase K) was added and incubated at 55^0C for two hours. After incubation, the DNA was purified by successive extraction with phenol: chloroform: isoamyl alcohol (25: 24: 1) and chloroform: isoamyl alcohol (24: 1), respectively, then centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to a fresh tube

and DNA was precipitated by adding $1/10^{th}$ volume of 3M sodium acetate and twice the volume ice-cold absolute ethanol.

The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 minutes. After a wash with 70% ethanol, the DNA was air dried and re-suspended in 20µl TE buffer (10mM Tris, 1mM EDTA, pH 8.0). Concentration and purity of extracted DNA was determined spectrophotometrically (T70 UV/VIS Spectrometer, PG Instruments Ltd.) at 260nm and 280nm. DNA quality was assessed electrophoretically, by running 2 µl of the DNA stock on 0.7% agarose gel (Hoefer-HE33, Mini horizontal submarine unit, Pharmacia Biotech, USA) electrophoresis.

3.2.3. PCR amplification of mitochondrial DNA (mtDNA)

PCR reactions for 16S rRNA, and Cytochrome C Oxidase I (COI) region amplifications were carried out in PTC 200 gradient thermal cycler (M.J. Research, Inc., Watertown, Massachusetts, USA) employing the primers 16S rRNA-L (5'- **ACTTGATATATAATTAAAGGGCCG** -3') and 16S rRNA-H (5'- **CTGGCGCCGGTCTCAACTCAAATC** -3') for 16S rRNA region amplification and COI – F (5'- **CCTGCAGGAGGAGGAGAGCC** -3') and COI - R (5'- **AGTATAAGCGTCTGGGTAGTC** -3') for Cytochrome C Oxidase I region amplification. PCR amplifications were performed in 25 µl reactions containing 1x assay buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 10 p moles/µL of primer mix, 10 mM dNTPs (Genei, Bangalore, India), 1.5 U *Taq* DNA polymerase and

20 ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. The reaction mixture was initially denatured at 95° C for 5 minutes followed by 30 cycles (94° C for 45 seconds, 54° C for 30 seconds and 72° C for 45 seconds). The reaction was then subjected to a final extension at 72° C for 5 minutes. The composition of the reaction mixture is given below in table 2.

Table 2. Preparation of master mix for PCR amplification of mitochondrial DNA

Components	**Vol. per reaction**
De ionized water	18.0µl
10x Assay Buffer	2.5µl
dNTPs	2.0µl
Primer mix	0.5µl
25 mM $MgCl_2$	0.5µl
Taq DNA polymerase	0.5µl
Template DNA	1.0µl
Total volume	**25.0µl**

3 µl PCR product along with marker (100bp DNA ladder, Genei, Bangalore, India) was run on 1% agarose gel with 1x TBE buffer for 30 minutes and stained with ethidium bromide. The gel was visualized under

UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA). The remaining PCR product was cleaned using GeNei™ Quick PCR purification kit (Genei, Bangalore, India).

3.2.4. Sequencing PCR

The amplified PCR products were used as the template for sequencing PCR. The conditions for PCR were as follows; 25 cycles of 95° C for 30 seconds, 50-55° C for 5 seconds and 60° C for 4 minutes and finally stored at 4 ° C. The composition of the reaction mixture is given below in table 3.

Table 3. Preparation of reaction mix for Sequencing PCR

Components	**Vol. per reaction**
BDT [Big Dye Terminator v3.1 Cycle Sequencing kit.]	1 µl
Buffer (Supplied with Big Dye Terminator v3.1 Cycle Sequencing kit.)	1.5 µl
DNA	1 µl (5-20ng)
Primer (forward or reverse)	0.5 µl (6-10pmol)
De ionized water	6 µl.
Total volume	**10.0µl**

3.2.4.1. Preparation of sequenced PCR products for capillary electrophoresis

a) 2µl of 125mM EDTA was added to each tube (PCR tube containing the PCR product) and mix.

b) 73µl H_2O was added to this tube (final volume was adjusted to 100µl)

c) It was transferred to a 1.5ml tube and 10µl 3M sodium acetate (pH-4.6) was added.

d) 250µl of 100% ethanol was added and mixed gently.

e) The tubes were incubated at room temperature for 15 min.

f) Tubes were spun at 12,000 rpm, at room temperature, for 20 min.

g) The supernatant was decanted and 250µl of 70% ethanol was added.

h) Tubes were again spun for 10min and the ethanol was decanted.

i) The above step was repeated again.

j) The supernatant was decanted and the pellet was air dried for 30-45min.

3.2.5. Analysis of Data

The raw DNA sequences were edited and aligned using BIOEDIT sequence alignment editor version 7.0.5.2 (Hall, 1999). Neighbor Joining and Maximum Parsimony Analysis were performed for tree making with MEGA version 3.1 (Kumar *et al.*, 2004) and the resulting phylogenetic trees were subjected to a consensus and bootstrapped with 1000 replicates.

Sequneces of 16S rRNA and COI genes of different species of the genus *Macrobrachium* were downloaded from NCBI genbank for data analysis. Details of species and accession no is given in table 4 and 5.

Table 4. 16S rRNA sequences downloaded from NCBI Genbank with accession no.

Species	**Accession number**	**References**
Macrobrachium idella	AY 858837	Parhi *et al.*, 2004
Macrobrachium scabriculum	GU 987059	Munasinghi, 2010
Macrobrachium latimanus	EU 493143	Chen *et al.*, 2008
Macrobrachium latimanus	DQ 194937	Liu *et al.*, 2006

Table 5. CO I sequences downloaded from NCBI Genbank with accession no.

Species	**Accession number**	**References**
Macrobrachium latimanus	AB235276	Liu *et al.*, 2006
Macrobrachium latimanus	AB235277	Liu *et al.*, 2006

RESULTS

4. RESULTS

4.1. Description of species under investigation

Four species, namely *M. canarae* (Tiwari, 1958), *M. idella* (Hilgendorf, 1898), *M. latimanus* (Von Martens, 1868) and *M. scabriculum* (Heller, 1862) have been utilized for the present investigation.

4.1.1. Hierarchical classification of the Genus *Macrobrachium*

Phylum	:	**Arthropoda** Von Siebold & Stannius, 1845
Class	:	**Crustacea** Brunnich, 1772
Subclass	:	**Malacostraca** Latreille, 1802
Series	:	**Eumalacostraca** Grobben. 1892
Order	:	**Decapoda** Latreille, 1802
Suborder	:	**Pleocyemata** Burkenroad, 1963
Infra order	:	**Caridea** Dana, 1852
Super family	:	**Palaemonoidea** Rafinisque, 1815
Family	:	**Palaemonidae** Rafinisque, 1815
Genus	:	*Macrobrachium* Bate, 1868

4.1.2. *Macrobrachium canarae* (Tiwari, 1958)

(Fig. 2)

Synonymy: *Palaemon canarae* Tiwari, 1958.

Description: Rostrum very long, extending beyond antennal scale by about $1/5^{th}$ its length, slender , narrow, about 1.75 times as long as carapace; distal end upturned; upper margin with 7-10 teeth (usually 8-9), of which 2 are post-orbital; proximal group of 7-8 teeth equidistant, distal 1-2 teeth smaller and separated from proximal group by very wide gap. Ventral margin with 4-7 teeth (usually 4-6), the gap between them progressively increasing towards the tip.

Carapace smooth, antennal and hepatic spines characteristic of the genes present , latter situated below and behind level of former.

Abdomen also smooth, pleurae of segments I-III broadly rounded at posteroventral margin; those of IV –VI backwardly directed.

Telson conical, tip reaching up to or beyond outer lateral spine of uropodal exopod; dorsal surface with pairs of spines, both situated in distal half; distal end also with 2 pairs of spines – outer pair smaller and immovable, inner pair much longer and movable, overreaching tip of telson; three to four plumose setae present between inner pair of spines and 2 pairs of smaller hair –like setae dorsally.

First pair of chelipeds slender, reaching tip of antennal scale when extended; ischium equal to merus; merus about 0.8 length of carpus; carpus slender,

more than twice length of chela; palm inflated, equal to fingers. Tufts of setae present along outer margin of fingers.

Second pair of chelipeds slender, longer than 1st pair, equal similar in both sexes, reaching up to or beyond antennal scale by chela when extended; ischium slender, subequal to merus; carpus the longest segment, cylindrical; chela about ¾ of carpus; palm dorsoventrally compressed and invariably shorter than half of carpus; fingers shorter than palm , 0.72 to 0.88 length of palm, with small proximal gap when closed; movable fingers with 2 minute denticles and immovable finger with 1 denticle in proximal part and with tufts of very short hairs.

Three pairs of non-chelate legs simple; ischium almost equal to carpus; merus almost equal to propodus; dactylus simple.

Pleopods and uropods typical in structure.

Distribution: India (Tamil nadu and Kerala states).

Maximum size: 44 mm (male); 55 mm (female).

Remarks: Very isolated in distribution and abundant.

Fig. 2. *Macrobrachium canarae* (Tiwari, 1958

4.1.3. *Macrobrachium idella* (Hilgendorf, 1898)

Synonymy: *Palaemon* (*Eupalaemon*) *idea idella* Hilgendorf, 1898; *Palaemon* (*Eupalaemon*) *multidens* Coutiere, 1900; *Palaemon multidens* Coutiere, 1901; *Palaemon idea* Henderson et Matthai, 1910; *Macrobrachium idella* Holthuis, 1950 (with complete synonymy); Jayachandran et Joseph, 1985.

Description: Rostrum long , extending up to the distal end of antennal scale, the basal crest not much raised proximal dorsal margin slightly up curved dorsal margin with 12-15 teeth, of which 2 post-orbital. First dorsal situated slightly in front of anterior ¼ length of carapace; 2nd separated from 1st by distinct space; 3rd close set to 2nd; 2nd -10th teeth equidistant; 11th slightly separated from 10th and 12th from 11th by distinct gaps; 12th and 13th teeth smaller and sub distal. In specimen with 12 dorsal teeth, the rostrum is sub distal to antennal scale; 2nd tooth separated from 1st gap; 2nd -9th equidistant; 10th tooth separated from 9th by distinct gap; similarly 11th also separated from 10th by the distinct gap. 11th and 12th teeth smaller and sub distal. Ventral margin with 4-5 teeth situated beneath level of 7th-11th dorsal teeth. Small setae present between teeth of both dorsal and ventral margins.

Carapace almost smooth except for a few minute spinules in the dorsal part. Both antennal and hepatic spines characteristic of the genus present, latter situated below and behind level of former.

Abdomen glabrous. Pleurae of segments I-III typical, of IV and V directed backwards and of VI ending in spine.

Telson basally broad, narrowing distally; tip of telson extending almost to level of distal end of uropod. Dorsal part of telson with tubercles. Two pairs of spines present dorsally, 1st pair about midlength of telson, 2nd pair slightly above midway between 1st pair and tip of telson. Posterior end of telson pointed, with 2 pairs of spines; outer pair smaller and immovable, inner pair longer and movable, reaching beyond tip of telson. A few plumose setae present between inner pair of spines.

Antennules typical and ratio of three segments of peduncles 9:3:4. Antennae also typical. Apophysis of mandible 1.5 times longer than incisor process, latter tridentate.madibular palp 3 segmented of which 1st and 2nd segments equal, 3rd longer. Maxillae and maxillipeds also typical.

Distal end of 2nd segment of endopod of 3rd maxillipeds reaching level of distal end of antennal flagellar peduncle.

First chelate legs slender; ischium inflated, about 1.5 times longer than chela but shorter than merus and carpus; merus shorter than carpus but longer than ischium and chela; carpus longer than all other segments; palm equal to fingers.

Second chelate legs equal sized, normally 1.5 to 1.82 times of total length; ischium flat and stout, shorter of all segments; merus longer than ischium and dactyl but shorter than carpus, propodus and palm; carpus longer than chela; thickness of palm almost equal to thickness of distal carpus; palm about 2

times longer than fingers; fingers equal, slender; movable finger with 2 denticles and immovable finger with 1 prominent denticle in proximal part of cutting edges; immovable finger covered with small hairs only along the cutting edge whereas the movable finger completely pubescent; all podomeres covered with prominent tubercles.

Three pairs of non-chelate legs simple; ischium almost equal to carpus or slightly smaller; merus almost equal to or slightly smaller than propodus; dactyls simple.

Pleopods and uropods typical in structure.

Distribution: Africa (Ponds and rivers Tanganyika; tributary of Onilahy river, W. Madagaskar), India (S. Canara district, Calicut, Palghat, Malabar district, Kottayam Cochin, Trivandrum S.W. India; Pondicherry S.E. India).

Maximum size: 120mm (male), 90 mm (female).

Remarks: The species contains a few variants. During the present two variants have been collected and utilized for molecular taxonomy. The differences in 2nd Pereiopods and rostrum. Based on arrangement of teeth pattern on rostrum showing variation within the individuals and these are marked as a type I and type II for present investigation purpose as shown in fig. 3 and 4.

4.1.4. *Macrobrachium latimanus* (Von Martens, 1868)

(Fig.5)

Synonymy: *Palaemon latimanus* Von Martens, 1868; *Palaemon euryhynchus* Ortmann, 1891; *Palaemon (Macrobrachium) latimanus* De Man, 1892; *Palaemon* (*Macrobrachium*) *singalangensis* Nobili, 1900; *Macrobrachium latimanus* Holthuis, 1950 (with complete synonymy); Jayachandran, 1984.

Description: Rostrum short, deep, extending just in front of antennular peduncle, usually falling short of level of outer spine of antennal scale; distal end sharply pointed and upcurved; upper margin with 6-7 teeth (usually 6), of which first 2-3 teeth post orbital; in specimens with 2 postorbital teeth,3rd tooth situated above orbit; in specimens with 3 post-orbiatl teeth, 4th tooth above orbit; first proximal tooth at about ¼ length of anterior carapace. Ventral margin of rostrum entirely convex, with 2-3 (teeth usually 2). Small setae present between teeth of both dorsal and ventral margins.

Carapace smooth; antennal and hepatic spines characteristic of the genus present, the latter situated below and behind level of the former.

Abdomen glabrous. Pleurse of segments I-III broadly rounded, of IV and V pointing backwards and VI spinous.

Fig. 3. *Macrobrachium idella* (Hilgendorf, 1898) Type I- rostrum showing pattern of arrangement of teeth.

Fig. 4. *Macrobrachium idella* (Hilgendorf, 1898) Type II- rostrum showing pattern of arrangement of teeth.

Telson robust, tip of telson reaches as far as level of outerspines of exopod of uropod; dorsal margin with 2 pairs of spines, of which proximal pair midlength of the telson and distal pair about midway between proximal pair of spines and posterior tip of telson; distal end of telson also with 2 pair of spines; outer pair smaller and immovable, inner pair longer and movable, overreaching tip of telson; a few plumose setae present between inner pair of movable spines.

First pair of chelate legs slender, reaching in front of antennal scale and up to distal end of carpus of 2nd chelate leg; ischium inflated: merus shorter than carpus; carpus longer than ischium, merus and chela; palm equl to fingers. Tufts of setae present at tips of fingers.

Second pair of chelate legs strong, equal, shorter than total body length; ischium stout, slightly flattened; merus longer than carpus, distal end reaching level of outer spine of antennal scale; carpus smaller than merus and chela, triangular in structure; palm inflated and longer than fingers; fingers equal, without gap when closed and with several denticles on cutting edges; movable finger with a few (usually 5) equal denticles situated in proximal half of fingers; distal denticles at about 2/3 length of fingers and rest of cutting edge entire; immovable finger also with several denticles, mostly smaller in size; distal denticle the largest, situated midlength of finger and rest of cutting edge entire; both fingers densely pubescent. All podomeres covered with small tubercles on all sides.

Three pairs of non chelate legs equal; ischium shorter than carpus; merus shorter than propodus; dactyl simple.

Pleopods and uropods typical in structure.

Distribution: Indo-west pacific: Japan, Ryukyu Islands, Formosa, Bonin Islands, Sri Lanka, India (Kerala).

Maximum size: 100mm (male); 75(female).

Remarks: The species is purely freshwater in habitat and occupies hill streams.

Fig. 5. *Macrobrachium latimanus* (Von Martens, 1868)

4.1.5. *Macrobrachium scabriculum* (Heller, 1862)

(Fig. 6)

Synonymy: *Palaemon scabriculus* Heller, 1862, 1865, Koelbel, 1892; Henderson, 1893; Henderson and Matthai, 1910; Kemp, 1915; Panikkar, 1937; Rajyalakshmi, 1960; *Palaemon dolichodactylus* Coutiere, 1901; Henderson and Matthai, 1910; Stebbing, 1910; Panikkar, 1937; Nataraj, 1942; *Palaemon (Parapalaemon) dolichodactylus* Hilgendorf, 1898; Coutiere, 1900; 1902; Nobili, 1903; Vatova, 1943; Barnard, 1950; *Palaemon (Parapalaemon) scabriculus* Nobili, 1900; 1903; *Palaemon dubius* Henderson et Matthai, 1910; *Macrobrachium scabriculum* Holthuis (1950, with complete synonymy).

Description: Rostrum very short, extending only up to the tip of antennular peduncle; upper margin convex, with 12-15 teeth, of which 5 teeth post-orbital; teeth equidistant. Ventral margin with 1-3 teeth, usually 2. Small setae present between teeth of both dorsal and ventral margins.

Carapace scabrous with hepatic and antennal spines and with minute spinules.

Telson robust, extending almost to same level as outer lateral spine of uropodal exopod; dorsal surface with 2 pairs of spines, of which proximal pair situated about midlength of telson; distal end also with 2 pairs of spines;

outer pair smaller and immovable, inner pair longer and movable, overreaching tip of telson. A few plumose setae present pair of spines.

First chelate legs typical in structure; ischium inflated, slightly shorter than merus; merus shorter than carpus; carpus slightly more than twice the length of chela; palm slightly inflated and equal to fingers.

Second chelate legs strongly unequal; either the right or left longer; larger cheliped with ischium shorter than merus; merus almost equal to carpus; carpus almost equal to palm or slightly longer; fingers slender and distinctly longer than palm; fingers with more than 20 denticles distributed along entire length; entire palm and basal part of fingers with thick pubescence. Smaller 2nd cheliped with ischium smaller than merus ; merus slightly smaller than carpus; carpus smaller than chela but distinctly longer than palm; palm shorter than fingers; fingers double the length of palm ; cutting edges of the fingers with 5-6 small denticles, present in proximal part only. Stiff setae present on the surface of 2nd pair of chelipeds and all podomeres spinulose.

Three pairs of non chelate legs progressively increasing in length from 1st to 3rd; ischium almost equal to carpus or slightly shorter; merus longer than propodus; dactyl simple.

Pleopods and uropods typical in structure.

Distribution: Juba, S.Italian Somaliland; Region des grands Lacs; Zanzibar; Mozambique; N.W. Madagascar: E.Madagascar; Kotri, near mouth of Indus river, Pakistan ; India, Sri langa and Sumatra.

Maximum size: 65mm (male); 47mm (female).

Remarks: This species exhibit wide range of variations in morphological characters.

Fig. 6. *Macrobrachium scabriculum* (Heller, 1862)

4.2. DNA analysis

4.2.1. Isolated genomic DNA

The DNA was isolated from two samples each from each selected species of *Macrobrachium* by the phenol-chloroform method (Sambrook *et al.*, 1989). The extracted DNA was electrophoresed through 0.8% agarose gel containing ethidium bromide (Fig. 7).

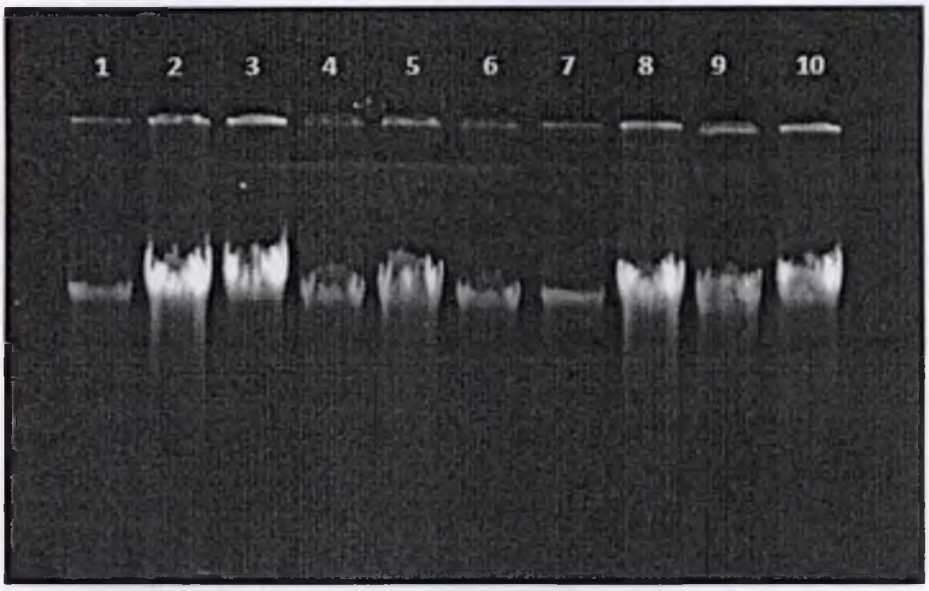

Fig.7. Genomic DNA of selected species of *Macrobrachium* (Lane no. 1 to 10). 1&2 (*Macrobrachium idella* Type I), 3&4 (*Macrobrachium idella* Type II), 5&6 (*Macrobrachium canarae*), 7&8 (*Macrobrachium scabriculum*), 9&10 (*Macrobrachium latimanus*)

4.2.2. Mitochondrial DNA (mtDNA) Analysis

The results are given below

4.2.2.1. Sequence characteristics and genetic divergence based on 16S rRNA and Cytochrome C Oxidase I (COI) genes

The 16S rRNA and COI haplotype of selected *Macrobrachium spp.* (Fig. 8 and 9) were aligned using BIOEDIT sequence alignment editor version 7.0.5.2 (Hall, 1999) and were used to estimate genetic divergence values and for constructing phylogenetic trees (Neighbor Joining 'NJ' and Maximum Parsimony 'MP') using MEGA 3.1 (Molecular Evolutionary Genetics Analysis) (Kumar *et al.*, 2004). 'Indels' (insertions/deletions) or 'gaps' during aligning the sequences were treated as "fifth character". Kimura-2-parameter method was identified as the mutational model in the present study to accommodate higher rate of transitions that is generally observed.

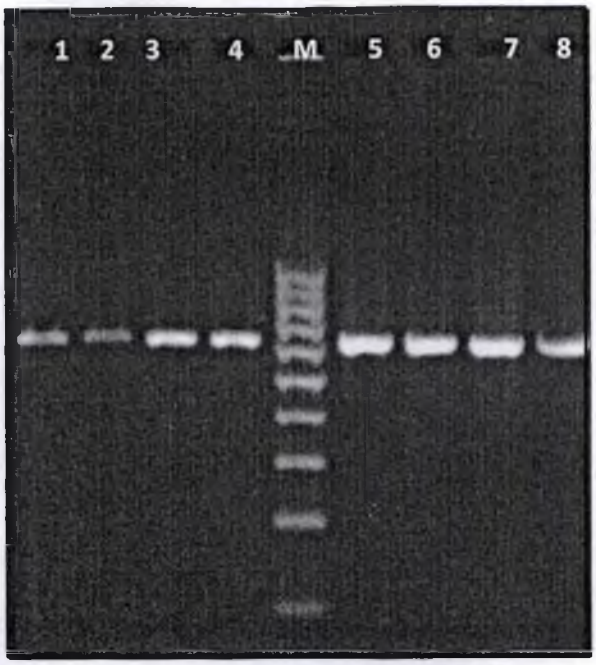

Fig. 8. mtDNA profile of 2 individuals each of *Macrobrachium idella* with 16S rRNA primer (lane 1 to 4); with COI primer (lane 5 to 8); M - marker (100 bp DNA ladder).

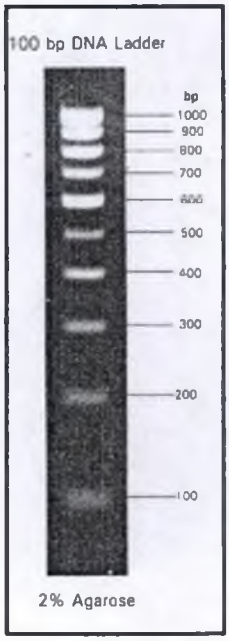

Fig. 9. Marker used in mtDNA (100bp DNA ladder) analysis.

4.2.2.1.1. 16S rRNA

A total of 518 base pairs of aligned sequences of 16S rRNA gene were studied in selected *Macrobrachium* spp. Between the different *Macrobrachium* species, 403 sites (78 %) were constant; 108 bases (21 %) exhibited variation. Among the substitutions 20 were transitions and 16 were transversions; the average pair wise ratio of transitions (Si) vs. transversions (Sv) was 1.3. Between *M. idella Type I and Type II,* 504 sites (98.8 %) were constant; 4 bases (1.2%) exhibited variation. Among the substitutions, the average pair wise ratio of transitions (Si) vs. transversions (Sv) was 1.2. The transitions and transversions changes occur only in C↔T (1). The mean genetic divergence value based on 16S rDNA sequences between *M .idella types* were 0.47 %. The pair-wise divergence based on 16S rRNA gene of selected species of *Macrobrachium* with other *Macrobrachuim* spp. downloaded from NCBI for data comparisons is shown in table 6.

The Maximum parsimony (MP) and the Neighbour joining (NJ) analyses using Kimura 2 parameter yielded trees with identical topology with high bootstrap support values. Well-supported highly divergent lineages were identified corresponding to two types *of M. idella, M. canarae, M. scabriculum and M. latimanus.* The Maximum parsimony (MP) and the Neighbour joining (NJ) tree based on 16S rRNA gene are shown in fig. 10 and 11 respectively.

Table 6. Pair-wise genetic divergences of selected species *Macrobrachium* with other *Macrobrachium* spp. from NCBI based on 16S rRNA gene sequences.

Samlpe ID	*M. idella* I(1)	*M. idella* I(2)	*M. idella* II(1)	*M. idella* II(2)	*M.idella* NCBI AY 858837	M.canarae 1	*M.canarae* 2	*M.scabriculum* 1	*M. scabriculum* 2	*M. scabriculum* NCBI GU 987059	*M.latimanus*	*M.latimanus* NCBI EU 493143	*M. latimanus* NCBI DQ 194937
M. idella I(1)													
M. idella I(2)	0.0000												
M. idella II(1)	0.0040	0.0040											
M. idella II(2)	0.0040	0.0040	0.0040										
M.idella NCBI AY 858837	0.0000	0.0000	0.0040	0.0040									
M.canarae 1	0.0898	0.0898	0.0900	0.0898	0.0898								
M.canarae 2	0.0898	0.0898	0.0900	0.0898	0.0898	0.0020							
M.scabriculum 1	0.0614	0.0614	0.0616	0.0614	0.0624	0.1043	0.1043						
M. scabriculum 2	0.0569	0.0569	0.570	0.0569	0.0569	0.0994	0.0994	0.0040					
M. scabriculum NCBI GU 987059	0.1019	0.1019	0.1021	0.1019	0.1019	0.1489	0.1489	0.0398	0.0397				
M.latimanus	0.0953	0.0953	0.0956	0.0953	0.0953	0.1210	0.1210	0.1098	0.1048	0.1498			
M.latimanus NCBI EU 493143	0.0947	0.0947	0.0925	0.0947	0.0947	0.1182	0.1182	0.0943	0.0894	0.1272	0.0705		
M. latimanus NCBI DQ 194937	0.0924	0.0925	0.0903	0.0925	0.0925	0.1161	0.1161	0.0992	0.0873	0.1250	0.0615	0.0080	

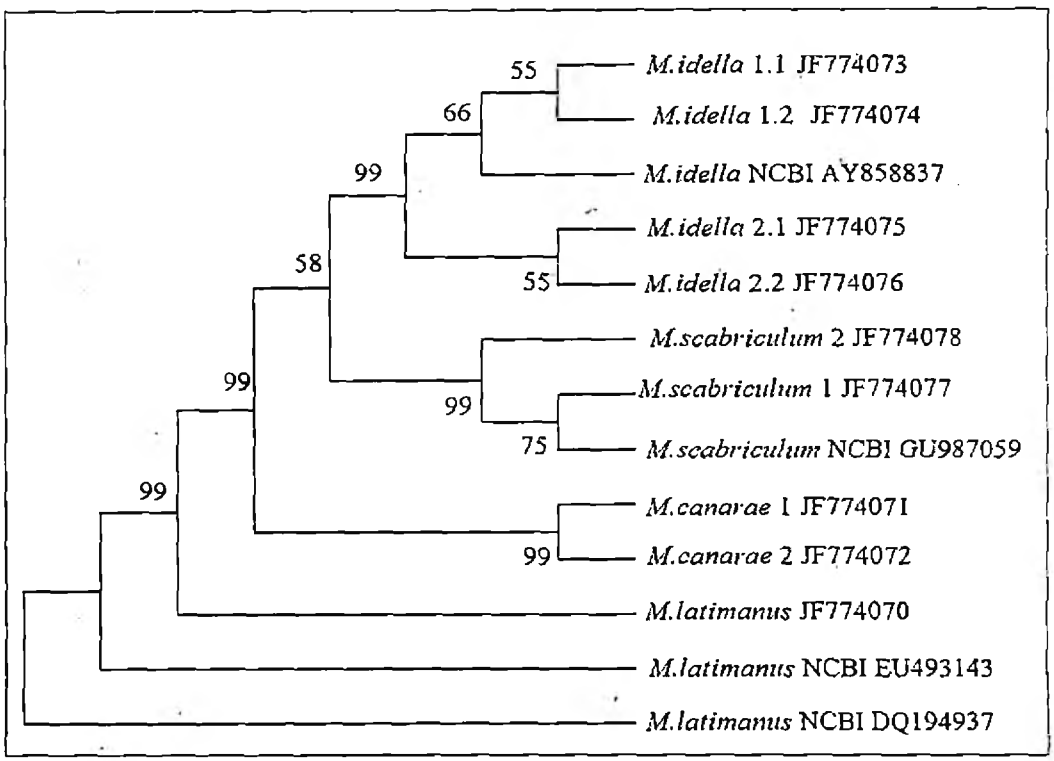

Fig. 10. Maximum Parsimony tree from 16S rRNA gene sequence data of of selected species of *Macrobrachium* and other *Macrobrachium* spp. from NCBI. The numbers above branches are bootstrap values. Scale indicates branch length as number of base changes between branches

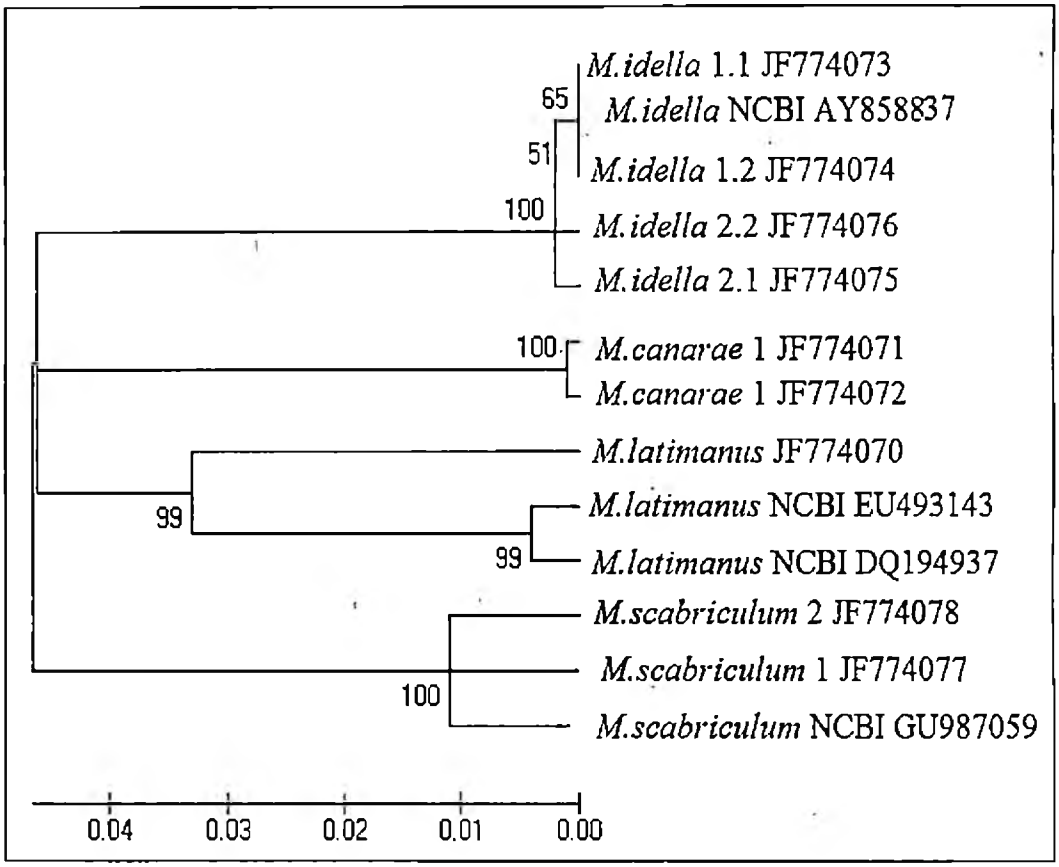

Fig. 11. Neighbour Joining tree from 16S rRNA gene sequence data of selected species of *Macrobrachium* and other *Macrobrachium* spp from NCBI The numbers above branches are bootstrap values. Scale

4.2.2.1.2. Cytochrome C Oxidase I (COI) gene

A total of 604 base pairs of aligned sequences of COI gene were studied in selected *Macrobrachium* spp. Between the different *Macrobrachium* species, 462 sites (76 %) were constant; 141 bases (23 %) exhibited variation. Among the substitutions 46 were transitions and 23 were transversions; the average pair wise ratio of transitions (Si) vs. transversions (Sv) was 2. Between *M. idella Type I and Type II,* 571 sites (95 %) were constant; 30 bases (4.7 %) exhibited variation. Among the substitutions, the average pair wise ratio of transitions (Si) vs. transversions (Sv) was 7.7. The transitional changes were 14 and transversional changes were 2. The mean genetic divergence value based on COI sequences between *M .idella types* were 4.7 %. The pair-wise divergence based on COI gene of selected species of *Macrobrachium* with other *Macrobrachuim* spp. downloaded from NCBI for data comparisons as shown in table 7.

The Maximum parsimony (MP) and the Neighbour joining (NJ) analyses using Kimura 2 parameter yielded tree s with identical topology with high bootstrap support values. Well-supported highly divergent lineages were identified corresponding to two types *of M. idella, M. canarae and M .latimanus.* The Maximum parsimony (MP) and the Neighbour joining (NJ) tree based on COI gene is shown in fig. 12 and 13 respectively.

Table 7. Pair-wise genetic divergences of selected species *Macrobrachium* with other *Macrobrachium* spp. from NCBI based on COI gene sequence.

Sample ID	*M. idella* I (1)	*M.idella* I (2)	*M. idella* II(1)	*M.idella* II(2)	*M.canarae* 1	*M.canarae* 2	*M.latimanus* NCBI AB 235276	*M.latimanus* NCBI AB 235276
M. idella I (1)								
M.idella I (2)	0.0050							
M. idella II(1)	0.0486	0.0466						
M.idella II(2)	0.0118	0.0118	0.0413					
M.canarae 1	0.1978	0.1970	0.1781	0.1877				
M.canarae 2	0.1998	0.1989	0.1800	0.1897	0.0101			
M.latimanus NCBI AB 235276	0.1785	0.1778	0.1598	0.1733	0.1826	0.1823		
M.latimanus NCBI AB 235277	0.1762	0.1755	0.1576	0.1711	0.1826	0.1823	0.0033	

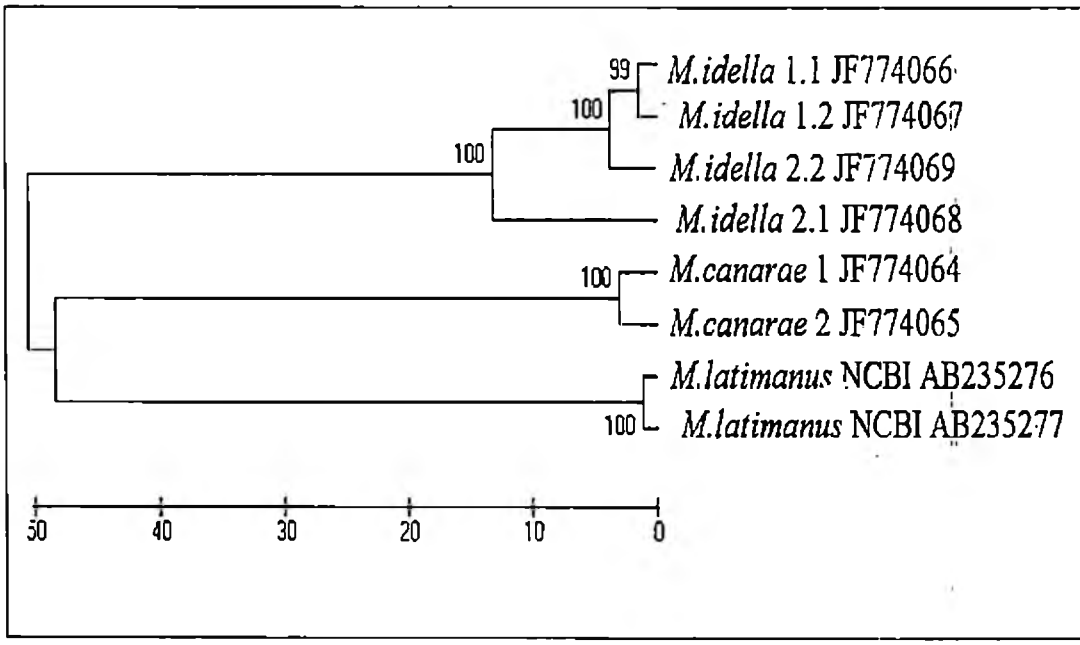

Fig. 12. Maximum Parsimony tree from COI gene sequence data of of selected species of *Macrobrachium* and other *Macrobrachium* spp from NCBI. The numbers above branches are bootstrap values. Scale indicates branch length as number of base changes between branches

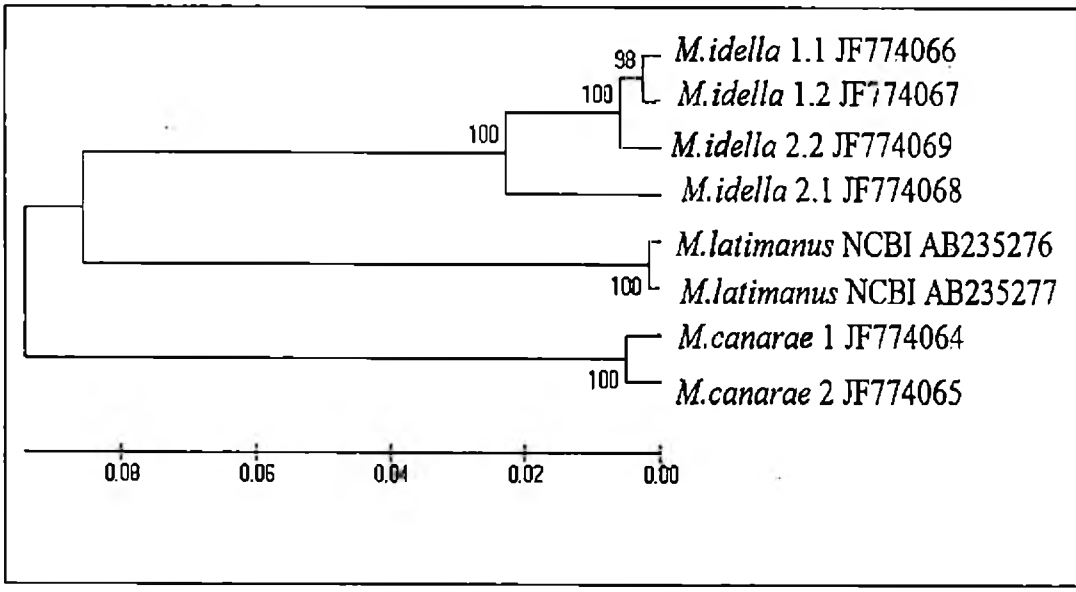

Fig. 13. Neighbour Joining tree from COIgene sequence data of selected species of *Macrobrachium* and other *Macrobrachium* spp from NCBI The numbers above branches are bootstrap values. Scale indicates genetic divergence

4.2.3. Submission of sequence data to NCBI

The sequences of 16S rRNA and COI were submitted to NCBI (National Centre for Biotechnology Information) Genbank and received the accession numbers for selected species of *Macrobrachium* as mentioned in table 8. The sequences of 16S rRNA and COI are shown in fig.14 and 15 respectively.

Table 8. · Accession numbers of 16S rRNA and COI Sequences for selected species of *Macrobrachium* obtained from NCBI.

Species	mtDNA gene	Accession number	Web site
Macrobrachium idella Type I (1)	16S rRNA	JF774073	http://www.ncbi.nlm.nih.gov
Macrobrachium idella Type I (2)	16S rRNA	JF774074	
Macrobrachium idella Type II (1)	16S rRNA	JF774075	
Macrobrachium idella Type II (2)	16S rRNA	JF774076	
Macrobrachium canarae (1)	16S rRNA	JF774071	
Macrobrachium canarae (2)	16S rRNA	JF774072	
Macrobrachium scabriculum (1)	16S rRNA	JF774077	
Macrobrachium scabriculum (2)	16S rRNA	JF774078	

Macrobrachium latimanus	16S rRNA	JF774070
Macrobrachium idella Type I (1)	COI	JF774066
Macrobrachium idella Type I (2)	COI	JF774067
Macrobrachium idella Type II (1)	COI	JF774068
Macrobrachium idella Type II (2)	COI	JF774069
Macrobrachium canarae (1)	COI	JF774064
Macrobrachium canarae (2)	COI	JF774065

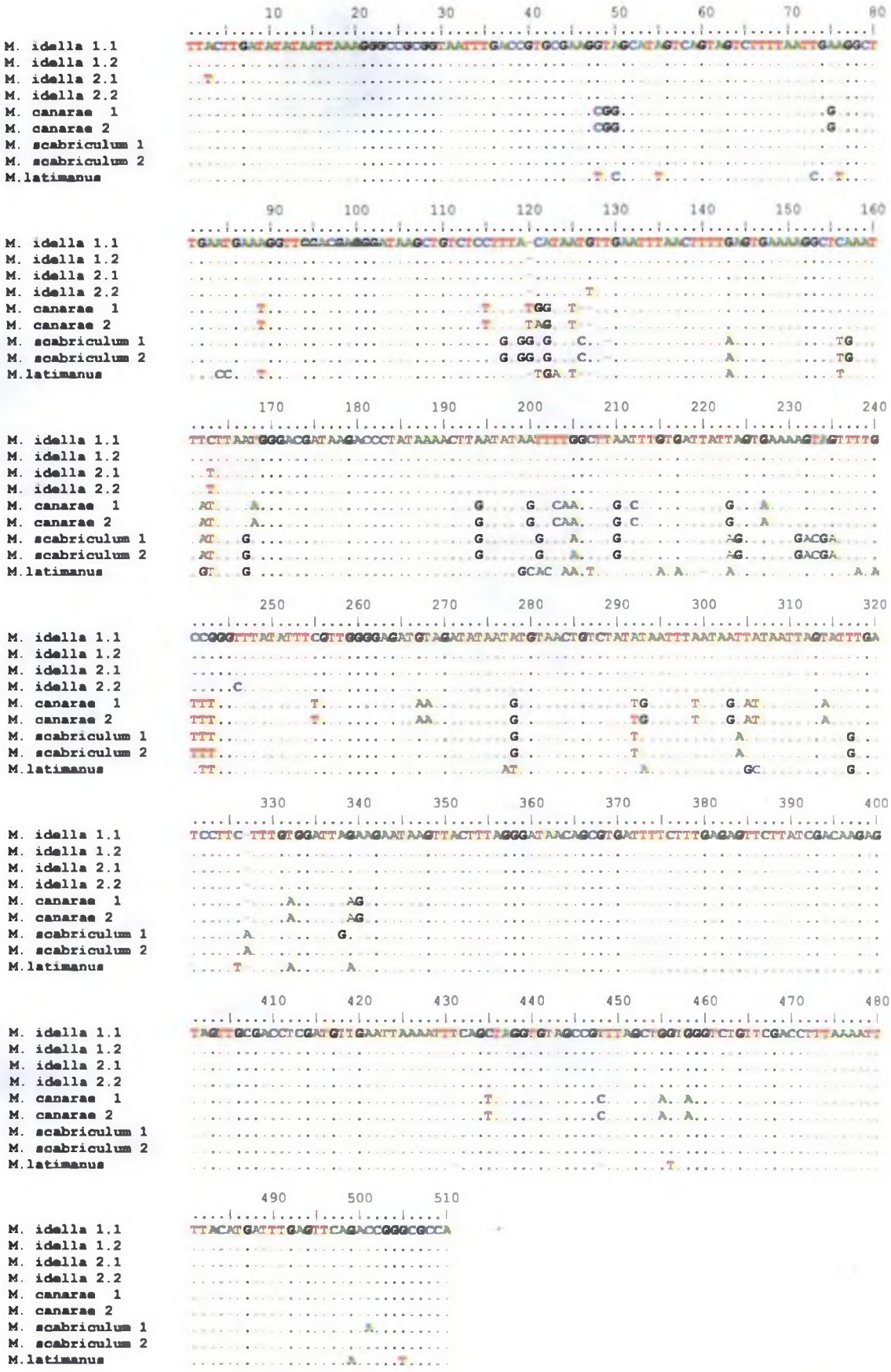

Fig. 14. CLUSTAL W Multiple alignment of DNA sequences for 510 bp of the 16SrRNA gene from selected *Macrobrachium* species. Identity with first sequence denoted by dots. Insertions/deletions of specific nucleotides indicated by dashes.

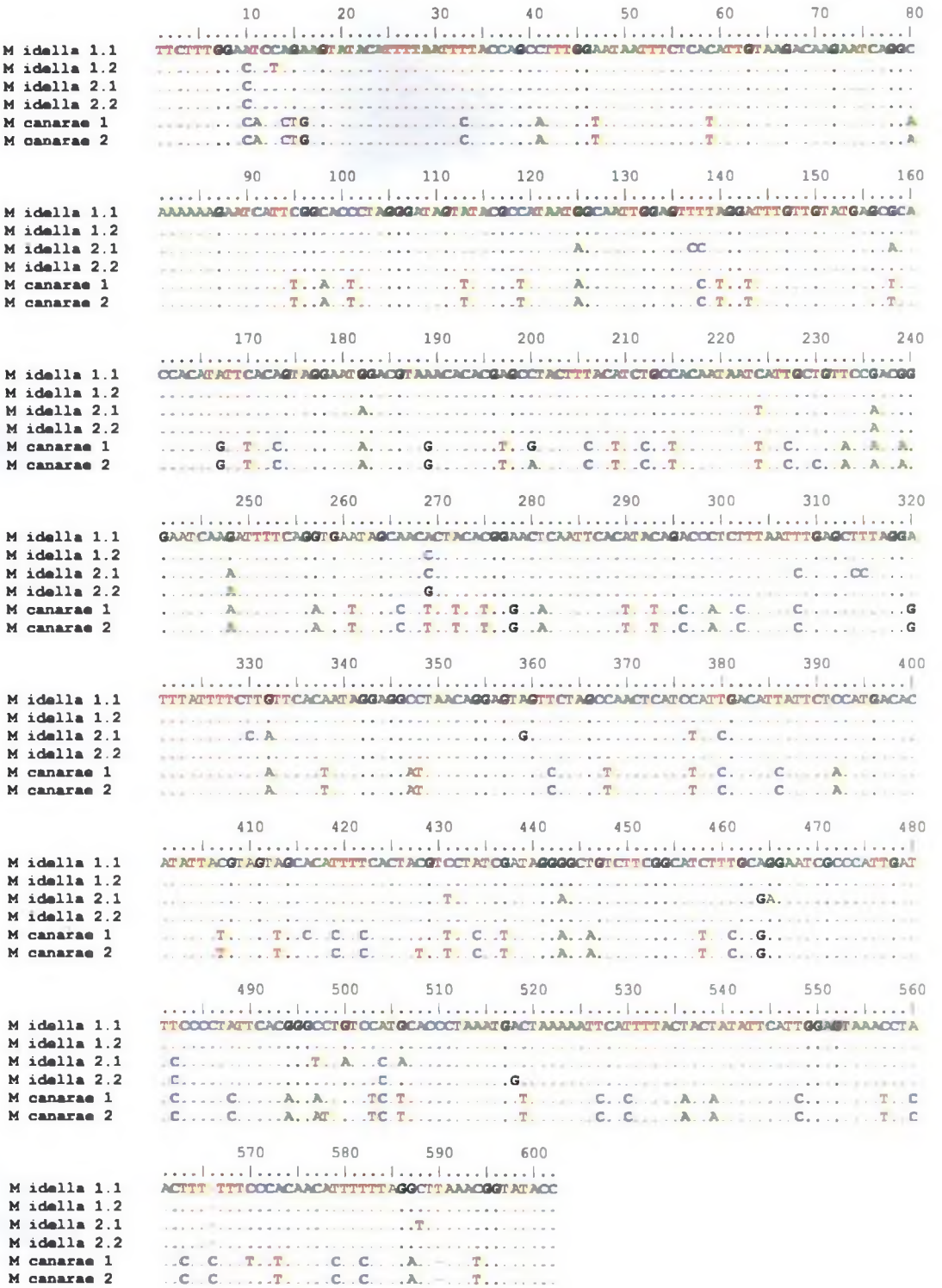

Fig. 15. CLUSTAL W Multiple alignment of DNA sequences for 602 bp of the COI gene from selected *Macrobrachium* species. Identity with first sequence denoted by dots. Insertions/deletions of specific nucleotides indicated by dashes.

DISCUSSION

5. DISCUSSION

Conventionally, morphological data has been the strength to identify the species. Developments in the field of molecular biology have identified several nuclear and mitochondrial markers, which are important tools to study the precise genetic relationships from taxa to individual levels. At present, both morphological and molecular data are used to investigate the confirmation of species status and construct the phylogenetic relationships of selected species of animal taxa.

Members of the genus *Macrobrachium* are distributed globally across the tropical and subtropical regions and comprises over 200 described species (Jayachandran, 2001) based on morphological data. About 60 species are reported from India and from Kerala 20 species (Jayachandran, 2010). Pereira (1997) carried out the first phylogenetic study based on morphological characters of the Family Palaemonidae. Palaemonids are highly conservative and difficult to identify accurately based on morphology. They are distributed in different water bodies such as, marine, brackish water and freshwater including hill streams. Confusion exists regarding the phylogenetic relationships. More over majority of them show migrant populations too. The use of DNA based methods to infer phylogenetic relationship can potentially overcome some of the difficulties encountered through the use of morphological characters. Several workers have

successfully used these mitochondrial genes for evolutionary studies in fresh water prawn species (Nicholas *et al.*, 2004; Daisy *et al.*, 2009; Leonardo *et al.*, 2010).

At molecular level, all organisms are subject to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation. In conjunction with selection and genetic drift, there arise genetic variation within and among individuals, species, and higher levels of taxonomic categories. In many organisms, some genes in the mtDNA also seem to accumulate mutations more rapidly than do nuclear genes. In other words, it provides markers with greater variability and sensitivity to drift, and is therefore more likely to show differences between populations or species. This makes mitochondrial gene attractive for both systematic and population genetic studies. Mitochondrial DNA (mtDNA) based techniques are gaining more attention and has been extensively used to understand, in a large variety of organisms, the molecular relationships among individuals, populations and species due to maternal inheritance and fast mutation rate (Brown *et al.*, 1979).

An appropriate target gene like Cytochrome C Oxidase I gene (COI) is conserved enough to be amplified with broad range of primers and divergent enough to allow discrimination of closely allied species. Cytochrome C Oxidase I gene, (COI) has proved to be suitable for the identification of a large range of

animal taxa, including Gastropods (Remigio and Hebert, 2003), Fish (Ward *et al.*, 2005; Lakra *et al.*, 2010) and Crustacea (Costa *et al.*, 2007). Sequences from the 16S rRNA mitochondrial gene region also have been found very useful for studying taxonomic questions and phylogenetic relationship within a number of crustaceans groups (Bucklin *et al.*, 1995; Crandall and Fitzpatrick 1996; Kitaura *et al.*, 1998; Crandall *et al.*, 1999). The 16S rRNA gene has both fast and slow evolving region and therefore can provide useful information across the broad taxonomic spectrum from the population to the family level. Murphy and Austin (2002; 2003; 2004; 2005) published a series of results for the phylogeny of *Macrobrachium* species based on the mitochondrial DNA fragment of the large subunit (16S) ribosomal (r) RNA gene marker.

The present study is based on both morphological and molecular data to classify the taxonomic ambiguity of two populations of *M.idella* and of *M.canarae, M.latimanus, M.scabriculum* and to construct the phylogenetic tree of the selected species of *Macrobrachium.*

Species of *M. canarae* are reported only from South-West coast of India and is showing restricted distribution (Tamil Nadu and Kerala) (Jayachandran, 2001). Sequences of 16S rRNA and COI of two individuals were generated for the first time and submitted to NCBI (Accession No: JF774071; JF774072 and JF774064; JF774065). Within the *M. canarae* individuals the divergence percentage was 0.2% and 0.1% using 16S rRNA and COI respectively. Within

each of the *Macrobrachium* species studied, levels of intra-specific variation were low and this was reflected in the lowest number of haplotypes (one to two for each species). This may be due to the high proportion of the identified haplotype in the samples or the limited number of individuals (2 each) collected for the present study (*M. canarae* were found to be genetically close with *M.idella* compared to *M.latimanus).*

M. idella is distributed in fresh and brackishwaters and also reported from ponds and river of Tanganyika; tributary of Onilahy River; W. Madagaskar and South East and South West coast of India (Jayachandran, 2001). This species is a highly variant group and contains few morphological variants. The present study generated 16S rRNA and COI sequences of two types of four individuals *M.idella* (Type I; Type II) and were submitted to NCBI (Accession No: JF774073; JF774074; JF774075; JF774076 and JF774066; JF774067; JF774068; JF774069). Two individuals of *M. idella* Type I species showed no divergence value while type II individuals showed 0.4% divergence. 16S rRNA sequence of M. *idella* available at NCBI (AY 858837) was compared with *M.idella* TYPE I and II. It was found that *M idella* type I was showing no variation and with type II individuals 0.4% divergence was showed. Based on COI gene, *M. idella* type II was showed 4.86 % divergence with *M. idella* type I. This confirms the divergence between the types of *M .idella.*

M. latimanus is distributed purely in freshwater habitat and are reported from Japan, Ryukyu Islands, Formosa (Taiwan), Bonin Islands, Sri Lanka and India (Kerala). 16S rRNA sequences of *M.latimanus* were generated and submitted to NCBI (Accession No: JF774070). Available sequence of 16S rRNA from NCBI (Accession No: EU 493142 and DQ 194937) were compared with sequences of *M.latimanus* (Accession No: JF774070) and it showed 6.15 and 7.05 % divergence respectively. This is an important finding and it require further investigation to separate the populations of *M.latimanus* from Rijukju Islands and from India.

M. scabriculum like *M.idella* exhibits wide range of variation in morphological characters and is distributed in Juba, S. Italian, Somaliland, Zanibar, Mozambique, Pakistan, India, Sri Lanka and Sumatra. Sequence of 16S rRNA was obtained from two individuals of *M. scabriculum* and were submitted to NCBI (Accession No: JF774077; JF774078). Between the two individuals of *M. scabriculum* the divergence value was just 0.4%. 16S rRNA sequence (GU 987059) was downloaded from NCBI, which is showing 3.9% divergence value with present species of investigation.

Transitions outnumbered transversions in the present study in accordance with the previous studies of mitochondrial DNA in fish (Garcia *et al.*, 2000). Generally for mtDNA, much excess of transitions related to transversions is typically observed (Page and Holmes, 1998). Hence further analysis and tree

construction, "Kimura-two-Parameter mutational model" is generally adopted (Hall, 2004). Kimura-two-Parameter method accommodates higher rate of transitions *i.e.*, this method allows differential weighing of transition and transversion probabilities (Hall, 2004). In the present study also, for estimating genetic divergence Kimura-two-Parameter method was adopted.

Phylogenesis from molecular data are often computed by pair-wise genetic distance based (numerical) methods like Neighbor Joining (NJ) tree, with branch lengths that are proportional to the amount of divergence, making it possible to estimate the relative times of separation. NJ tree making method is a widely used distance–clustering algorithm that allows unequal rates of divergence among lineages. Phylogenetic trees are also made based on "discrete methods" that operate directly on sequences like the Maximum Parsimony (MP) tree. MP chooses the tree (or trees) that require the fewest evolutionary changes (*i.e.*, it makes trees from sequences exhibiting smallest evolutionary changes). In variant characters (bases) those that have the same state in all taxon are obviously useless (phylogenetically uninformative) and are ignored by the MP method. Both the numerical (NJ) and discrete (MP) tree making methods are used in the analysis in most of the species (Hall, 2004) as in the present study.

Phylogenetic analysis using NJ and MP method with COI data identified two lineages among two types of *M.idella* species, supported by moderate to high bootstrap values. Both the trees generated using 16s r RNA and

COI gene produced the same pattern, forming two distinct clades of *M.idella;* though the level of divergence revealed through 16S r RNA was negligibly low. The results also indicate the different *Macrobrachium* species are genetically distinct and a closer genetic relationship exists between the two types of *M.idella* species. Estimates of genetic divergence for COI using Kimura-2 Parameter model was 4.86% between two types of *M. idella* species. The higher genetic divergence values (4.86%) compared to that of 16S rRNA(0.4%) between the two types of *M.idella* species in the present study indicates usefulness of COI data in discriminating recently evolved species especially when sequence information of 16S rRNA often failed to do so. The natural extension of the present study is to examine the finer scale of taxonomy of these two *M.idella* types with more intensive sampling from different geographic localities using fast-evolving markers. The morphological variations between the two populations of *M.idella* are meagre (fig.3 and 4). Hence clubbing morphology with confirmatory tool like barcoding (COI) along with 16S rRNA result with mixture of sample size from different regions would be a best proposal in molecular taxonomic works.

SUMMARY

6. SUMMARY

Freshwater prawns of the genus *Macrobrachium* Bate, 1868 belong to Class Crustacea, Order Decapoda and Family Palaemonidae. They are important because they are most diverse, abundant and widespread among crustacean genera. *Macrobrachium* is distributed globally across the tropical and subtropical regions of the world and comprises over 200 described species (Jayachandran, 2001). From India around 60 species have been reported and from Kerala 20 species (Jayachandran, 2010). These interesting groups of decapods crustaceans thought to have originated from marine ancestors, some of which subsequently migrated towards fresh water in more than one wave, hence its members are known to inhabit the entire range of habitats from purely marine areas to inland hill streams and impounded water bodies. Although majority of *Macrobrachium* species inhabit fresh water, majority of species require estuarine or marine environments for completing life cycle and hence are euryhaline in nature and yet others complete their life cycle in pure freshwaters (Jayachandran, 2010).

Among the Indian states, Kerala state having good resource of *Macrobrachium* and are distributed in different water bodies such as, hill streams, valleys, lower stretches of rivers and estuaries. Many species of

Macrobrachium exhibit variations in morphology at species level. Hence there is a great deal of confusion exist among species of *Macrobrachium*. Most of the studies are based on morphological comparisons. Therefore advanced molecular studies are needed for confirmation of species status and variants of *Macrobrachium*. This will help to establish phylogenetic relationship also.

Realizing the importance of molecular taxonomy, the present study was undertaken with the objectives such as: confirmation of species status of variants of *Macrobrachium canarae* (Tiwari, 1958), *M. idella* (Hilgendorf, 1898), *M. latimanus* (Von Martens, 1868) and *M. scabriculum* (Heller, 1862); generate genetic data to postulate phylogenetic relationship. The thesis contains the result of molecular analysis of DNA barcoding [Cytochrome C Oxidase I gene (COI)] and 16S rRNA of genes of mitochondrial DNA of *Macrobrachium canarae*, *M. idella*, *M. latimanus* and *M. scabriculum*. The four species were collected from different water bodies and inhabit varied habitats: *M. latimanus* is a hill stream prawn; *M. canarae* inhabits valleys without migration; *M. idella* and *M. scabriculum* inhabits lower stretches of river with estuarine migrations.

All the four species including the varieties of *M.idella* were accurately identified based on literature (Jayachandran, 2001).

The specimens were photographed and immediately preserved in 90% ethanol for further analysis. Total mitochondrial DNA was extracted from pleopods and muscles by the standard procedure designed by phenol-chloroform

method (Sambrook *et al.*, 1989). The extracted DNA was subjected to PCR amplification.

Mitochondrial DNA regions of 16S rRNA and COI were further subjected to PCR sequencing.

The PCR sequenced 16S rRNA and COI have been aligned in BIOEDIT version 7.0.5.2. (Hall, 1999) and phylogenetic study done in MEGA version 3.1 (Kumar *et al.*, 2004). Thus 15 sequencing data have been constructed, which are, two individuals of *M. idella* Type I, *M. idella* Type II, *M. canarae, M. scabriculum* and individual of *M. latimanus* based on 16S rRNA gene and two individuals of *M.idella* Type I, *M.idella* Type II, *M.canarae* based on COI genes. These obtained sequences were submitted to NCBI and received the accession numbers

COI gene revealed 4.86% divergence between *M.idella* type I and type II species. Pair-wise sequence divergence and mean genetic distance computed between *M. latimanus* reported from Kerala waters with the published sequence of *M.latimanus* from Rijuku Islands was 6.15-7.05%. This is an important finding and it opens further investigation to separate the populations of *M. latimanus* of Rijuku Islands and from India. *M.scabriculum* is also shows significant variation with published sequence from NCBI.

Phylogenetic trees of the *Macrobrachium* species based on Parsimony analysis and neighbor joining method using the COI and 16S rRNA sequence data resolved in the study and it revealed that the genetic relationship is clearly shows between two types of *M.idella* and other selected species of *Macrobrachium* from Kerala waters. The thesis also contains morphological description of species under study. Hence, combining morphological and molecular data is very effective to finding species variants and accurate identification of species.

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7. REFERENCES

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LIST OF ABBREVIATIONS

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Abbreviations / Symbols		Descriptions
A	:	Adenine
bp	:	Base pairs
BOLD	:	Barcode of Life Data Systems
C	:	Cytosine
CBOL	:	The Consortium for the Barcode of Life
COI	:	Cytochrome C Oxidase subunit I
°C	:	Celcius
Conc.	:	Concentration
Cyt b	:	Cytochrome b
DNA	:	Deoxyribo Nucleic Acid
dNTP's	:	Deoxynucleoside triphosphates
EDTA	:	Ethylene Diamine Tetra Acetic acid
Fig.	:	Figure
G	:	Guanine
g	:	Gram
GBIF	:	Global Biodiversity Information Facility
HCL	:	Hydrochloric Acid
l	:	Litre
M	:	Molarity
K-2-P	:	Kimura-2-Parameter
MEGA	:	Molecular Evolution of Genetic Analysis
Mol. Wt.	:	Molecular Weight

MP	:	Maximum Parsimony
mg	:	Milli gram
MgCl2	:	Magnesium Chloride
ml	:	Milli litre
mm	:	Milli meter
mM	:	Milli mlolar
min	:	Minutes
mtDNA	:	Mitochondrial DNA
ng	:	Nano gram
nm	:	Nano meter
No.	:	Number
nDNA	:	Nuclear DNA
NAOH	:	Sodium hydroxide
NCBI	:	National Centre for Biotechnology Information
NJ	:	Neighbor Joining
PCR	:	Polymerase Chain Reaction
pM	:	Pico Moles.
RNA	:	Ribo Nucleic Acid
RNase	:	Ribonuclease
rRNA	:	Ribosomal RNA
Rpm	:	Revolutions per Minute
SDS	:	Sodium Dodecyl Sulphate
T	:	Thymine
Taq	:	Thermus aquaticus

TBE	:	Tris Borate EDTA
TE	:	Tris EDTA
Tris	:	Tris (hydroxyl methyl) amino methane
µg	:	Micro gram
µl	:	Micro Litre
%	:	Percentage

ABSTRACT

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Freshwater prawns of the genus *Macrobrachium* are a conspicuous and important prawn constitute one of the most diverse, abundant and widespread crustacean genera. They are distributed in different water bodies of the tropical and subtropical regions of the world and comprises over 200 described species (Jayachandran, 2001). The palaenonids have taxonomically difficult groups because they appear to be morphologically higher conservative and also greater confusion among the evolutionary status. The present study was carried out both on morphological and molecular data to investigate the confirmation of species status and finding phylogenetic relationship of selected species of *Macrobrachium* in kerala waters. The species of *M. canarae, M. idella, M. latimanus* and *M. scabriculum* were collected from different water bodies of kerala, such as hill streams, rivers and brackish water. The four species inhabit varied habitats: *M. latimanus* is a hill stream prawn; *M. canarae* inhabits valleys without migration; *M. idella* and *M. scabriculum* inhabits lower stretches of river with estuarine migrations. These species were accurately identified based on morphology of earlier literature done by Jayachandran, 2001 and it shows variations within the individuals of *M. idella*. The use of DNA based methods to infer phylogenetic relationship can potentially overcome some of the difficulties encountered through the use of morphological characters. In molecular part, 16S rRNA and COI genes of mitochondrial DNA were analysed. Sequences of 16S

rRNA and COI were obtained and submitted to NCBI. Based on sequence information, *M. canarae* shows few minor variations within the individuals. *M. idella* is shows significant variations within the two types of individuals. *M. latimanus* is shows separate clades of other country species. *M. scabriculum* is also shows significant variation with NCBI sequence of *M. scabriculum*. Phylogenetic relationship is clearly shows two types of *M. idella* based on NJ and MP tree. Hence, both morphological and molecular data is very effective for finding species variants and phylogeny.