SYSTEMATICS AND MOLECULAR PHYLOGENY OF MARINE PRAWNS AND SHRIMPS OF GUJARAT

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Summary

Biodiversity is the basic need for human existence. In order to meet the need for food, health and other necessities of the growing human populations it is of prime importance. There are various biodiversity regions, but out of all, the coastal marine ecosystems are known to be biologically the most diverse, productive and significantly valuable areas. It has been estimated to cover more than double the number of species on land. Man depends on these ecosystems and it's organisms to harness the energy, food, medicine, fuel, fertilizer and many other industrial products. The Indo-Pacific region possesses the world's highest marine biodiversity in terms of species richness and unique ecosystems (Keyse et al. 2014). The Indian marine water is enriched with 13000 species of flora and fauna (Venkataraman and Wafar, 2005) where in the case of fauna, the maximum diversity is recorded for crustaceans (3377 species).

Crustaceans exists to fourth-largest diversity and they are the second most abundant diverse animal group on the planet. They abundantly inhabit the costal marine environment of the tropical and subtropical region. In the marine ecosystem, their distribution ranges to abyssal zone depth of 6000 and up to 2000 meter above mean sea level on mountains. Decapods crustaceans are the most common invertebrates inhabiting in coastal marine habitat. Out of all the decapods crustaceans living in the different marine habitats, one of the best known and most interesting target groups is prawn (includes shrimps). Many of the prawns are pelagic, but the majority of the shrimps are benthic, living on a variety of benthic substratums like rock, mud, and sand.

The prawn and shrimp fauna have been studied extensively for taxonomical and systematic research. So many researchers have compiled a list of shrimp fauna of the world from different time periods. The taxonomy of shrimps is very confusing and many synonymous are available of a single species, so compilation of a valid species was very much needed. A total of 4048 species shrimps have been reported by De Grave and Frensen (2011). They are subdivided into the four major groups viz. are Dendrobranchiata (68 genera, 533 species), Procarididea (2 genera, 6 species), Stenopodidea (12 genera, 71 species) and Caridea (389 genera, 3438 species).

The taxonomy of suborder Dendrobranchiata has not changed much apart from the addition of a few new genera and species (Farfante and Kensley, 1997). According to Martin and Davis (2001) and De Grave et al., (2009), infraorder Stenopodidea currently contains 69 species belonging to 13 genera and 3 families. The classification of infraorder caridea has been revised many times due to the huge diversity of (Chace, 1992; Holthuis, 1993). Bracken et al., (2009) recently questioned super family arrangement within caridea. Christoffersen (1986, 1987, 1988) published a series of papers and suggested changes in caridean systematic study.

The work on Indian prawn and shrimp fauna has a long history. Remarkable work on deep-sea shrimps was carried out during the marine exploratory survey by Royal Indian Marine Survey Ship "Investigator" at the end of the 19th century. Indian scientists have also contributed in the taxonomy of Indian prawn and shrimp fauna (Kunju, 1960; Muthu and George, 1971; Mohamed and Suseelan, 1973; Silas and Muthu 1976; George, 1979; Thomas, 1979; Rao 1984; Ravindranath, 1989). George and Muthu (1968) gave a taxonomic note of genus *Metapenaeopsis* including a new record of *M. barbata* (De Haan) from Indian water. During the period they described a new species of *Solenocera waltairensis* from the east coast of India (George and Muthu, 1968b). This species differs from the other closely related species based without basial spine on the second pereiopod. In 1973, Muthu described a new species *Parapenaeopsis indica* (=*Mierspenaeopsis indica* (Muthu, 1972)) from Kakinada, east coast of India. Suseelan (1996) has recorded 118 species of prawns which are contributing in the commercial prawn fisheries of India. Jeyachandran (2005) recorded the presence of 46 species of Palaemonid shrimps from Indian seas. Kathirvel et al. (2007) recorded 84 species of commercially important penaeid shrimps from India and also added notes on their economic importance and geographic importance. Rath and his coworkers consolidated a check-list of 49 species of prawns of the Godavari estuary, Andhra Pradesh including new records of 6 species of penaeid and palaemonid prawns (Rath et al. 2016).

Most of the commercial species of prawns belongs to the super family penaeoidae. Studies on penaeoidae prawns are more extensive and at present 5 families, 23 genera and 121 species are known from Indian water including the Lakshadweep and Andaman and

Nicobar Island (Radhakrishnan et al. 2012). Currently the prawn and shrimp fauna of India is represented by 364 species and 128 genera (Samuel et al. 2016).

Due to the use of various morphological and molecular tools, in the last few decades, the classification of order decapoda is getting stable. The history of molecular genetics dates back to the early 1950s when F. Crick, J. Watson and M. Wilkins established the currently accepted model of DNA structure (the double helix). Since then, details of structure and function of DNA and genes have been clarified. Methods for DNA cloning, sequencing and hybridization developed in the 1970s, and DNA amplification and automated sequencing, which were developed in the 1980s, led to the development of various classes of DNA markers. The classical molecular technique for studying genetic variation at co-dominant Mendelian-inherited loci is allozyme electrophoresis. The technique was developed in the 1960s and was dominating until the early 1990s. The first population genetic studies based on the analysis of mitochondrial DNA emerged with the work of Avise et al. (1979).

To resolve the taxonomic ambiguity in decapoda crustaceans DNA barcoding technique has been used effectively (Kou et al. 2013; Li et al. 2014; Yang et al. 2015). Mitochondrial 16S ribosomal DNA (rDNA) and cytochrome c oxidase subunit I (COI) genes are often employed and have proven useful for segregate closely related species across diverse animal phyla and marine decapods (Chan et al. 2008). Mitochondrial DNA sequence was used to investigate the identification and phylogenetic relationship among five species of genus Parapenaeopsis Alcock, 1991 using 16S ribosomal DNA (rDNA) and cytochrome c oxidase subunit I (COI) gene (Chowdhury et al. 2018).

In the coastal area, the intertidal zone is considered as the most diverse and productive area because with in the area of a few meters various kinds of flora and fauna are found (Underwood, 2000). The variation in the distribution and abundance of the organisms in a variety of intertidal area and the zonation of particular habitat has provided. Gujarat is the western proximity of the country harbors the longest coastline of 1650 km. The coastline of Gujarat is mainly divided into three coastal regions viz. Gulf of Kachchh, Saurashtra and Gulf of Khambhat.

A total of 25 species of commercially important shrimps belonging to 8 genera, 5 families were reported from Gujarat (Radhakrishanan et al. 2012). Trivedi et al (2015) compiled a

checklist of the crustacean fauna of Gujarat and listed 35 species from Gujarat. It is noteworthy that in all the old studies of prawn fauna of Gujarat was on biology of commercial species, stock and catch assessment and population dynamics (Ramamurthy, 1963; 1967; Deshmukh, 1975; Rao, 1983; Gopalakrishnan et al. 1985; Ghosh et al. 2012). Only a few studies have been carried out on the taxonomy of from Gujarat (George et al. 1963; Dash et al. 2012; Beleem et al. 2019).

The coastal region of Gujarat state supports various kinds of marine habitats like mangroves, coral reef, rocky shore and mudflats. These habitats were studied extensively for the diversity of various marine fauna. Amongst the various fauna studied the prawn fauna especially caridean shrimp is not studied in detail. The Geographical distribution and habitat preference are not described so far for most of the western coastal regions of Indian as well as Gujarat state.

Objectives of the study

1. **To study of Diversity, morphological taxonomy and distribution of marine prawns and shrimps.**
   - I. Systematics of marine prawns and shrimps: A taxonomical approach
   - II. Geographical distribution and habitat preference of prawns and shrimps found in different coastal regions of Gujarat.

2. **To study of phylogenetic relationship among prawns and shrimps.**
   - I. Cladistic analysis of prawn and shrimps species based on morphological characterstics.
   - II. A comprehensive phylogenetic analysis of prawn and shrimp based on mitochondrial COI sequence data.

Methodology

Study area- The coastal area of Gujarat is divided into 3 regions: Gulf of Kachchh, Saurashtra coast and Gulf of Khambhat. The coastline of state is distributed in 15 districts of which, 4 districts are located in Gulf of Kachchh, 6 districts are located on Saurashtra coast and 8 districts are located in Gulf of Khambhat. The sampling sites for each coastal

district were selected on the basis of habitat parameter (e.g. Sandy shore, rocky shore, open and mangrove mudflat etc.) and accessibility of the study site. A total of 55 sites were surveyed along the Gujarat coast during the entire study to assess the diversity, distribution and habitat preference of prawns and shrimps.

Sample collection- Sampling was done using standard methods. For the intertidal species of prawn, handpicking method was adopted. For commercial species the commercial fishing trawlers and the local fish market surveyed. Details of the off shore location of the catch, approximate depth of sample collected, type of gears used etc. all this information is gathered from the fishermen. The fresh specimens photograph was taken immediately after collection for fresh coloration, stored in icebox and brought in the laboratory.

Morphological Identification- All the specimens were sorted out according to their sex. Detailed morphometry of each specimen was carried out. The specimens were identified up to species level by comparing morphological characters with available illustrative keys, research papers and monogram. For further confirmation of species, all the specimens were examined and compared with the images and identification characters available on Marine Species Identification Portal Website (http://species-identification.org/). The recent classification of prawn and shrimp was adopted from the WoRMS website (www.marinespecies.org). All the specimens was preserved using two different methods. These are followed.

1. ***Preservation for Morphological Examination-*** All the specimens were preserved in 10% formalin (v/v) after identification.
2. ***Preservation for Molecular Study-*** For molecular study one or two copy of each species were preserved in 70% ethanol.

All the identified species were deposited into the Zoology museum, Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara with a specific deposition code (e.g. ZL-AR-PR-1: *Metapenaeus affinis* (H. Milne Edwards, 1837)). These codes were allotted to each identified species.

Morphometric analysis- After the identification of penaeidae and caridean shrimp, from each species 3 male samples were taken and female omitted due to the size variation. The morphmetric measurements were taken including TL (Total length) and CL (Carapace length) on the basis of the truss network system (Aktas et al 2006). In this study, there were a significant correlation were observed average measurement of each variable and total length of the species. Therefore, it was necessary to remove the effect of size variation for species from each morphmetric variables; they were standardized according to the Elliott et al (1995).

$$\text{Madj} = \text{M}(\text{Ls}/\text{Lo})^{\text{b}}$$

The Ls/Lo is computed in the MS excel sheet and detecting the b parameter was carried out using the PAST (Paleontological Statistics Software). The cluster analysis is performed UPGMA multivariate method, in order to evaluate the morphometric relationship among all species in each order.

DNA isolation- Genomic DNA was extracted from abdominal tissue or pleopods of prawns and shrimps. The initial weight was approximately 20 mg and extraction was carried out using DNeasy Blood and Tissue kit (Qiagen) and Phenol/ Chloroform Method.

Quantification of genomic DNA- After extraction of genomic DNA, quantification was carried out using QIAxpert (QIAGEN). Purity and concentration of DNA were measured using gel electrophoresis.

PCR amplification- COI gene was amplified in a volume of 20 µl containing 10 µl Taq PCR master mix (HiMedia), 10 pmol forward primer (LCO 1490), 10 pmol reverse primer (HCO 2198), 2 µl template DNA and nuclease-free water(to make up the final desired volume). Amplification was carried out in Thermal cycler (Applied BiosystemsVeriti®).

Table 1: Thermal cycling conditions for LCO 1490 and HCO 2198

Stage 1	**Stage 2 (35 Cycles)**			**Stage 3 (5 Cycles)**			**Stage 4**	
94ºC	94ºC	45ºC	68ºC	94ºC	50ºC	68ºC	68ºC	4ºC

1 min.	1min.	1 min. 30 seconds	1 min. 30 seconds	1 min.	1 min. 30 seconds	1 min 30 seconds	10 min.	∞

Gel electrophoresis- After PCR amplification the amplicons were observed on 2% agarose gel and bands were visualized under UV Gel doc (Bio-Rad).

PCR Purification- Positive PCR amplified were purified suing EXOSAP IT (affymetrix) according to the manufacturer instructions. In a new PCR tube 10 of positive PCR product and 4 µl of EXOSAP were added. A thermal cycler was run.

Table 4: PCR purification condition

Stage I	Stage II	Stage III
37˚C for 15 minutes	80˚C for 15 minutes	4˚C

Sequencing- Sequencing- The PCR amplified product was bidirectionally sequenced by using the same set of the universal primers (LCO 1490 HCO 2198) on the ABI 3730x196 capillary DNA analyzer using Big Dye Terminator v 3.1 sequencing kit at Eurofins, Bangalore.

Sequence Analysis- The qualities of the bi-directional chromatogram of the generated sequences were checked and noisy parts were trimmed at both ends to avoid the noisy part using sequences nucleotide sequence DNA baser assembler 5.15 version. Further, each sequence was NCBI to check for similar sequences through BLASTn. The phylogenetic tree was reconstructed using the Neighbor-joining (NJ tree) method and Kimura 2-parameter model with 1000 bootstrap replicates by using MEGA X version.

Results and Discussion

Systematics of marine prawns and shrimps: A taxonomical approach-

In the present study total 52 species belonging to 27 genera, 11 families and 4 super families were recorded. Family Penaeidae has contributed 25 species belonging to 9 genera. Family Solenoceridae has contributed 3 species belonging to 1 genus to the total list of species reported from Gujarat. Family Callianassidae has contributed 2 species

belonging to 2 genera to the species list. Family Alpheidae contributed 8 species belonging to 3 genera to the species list. Family Plaemonidae contributed 6 species belonging to 4 genera to the species list. Family Hippolytidae contributed 3 species belonging to 3 genera to the species list. Families Lysmatidae, Thoridae, Pandaloidea, Upogebiidae and Spongicolidae have contributed 1 species belonging to 1 genus each to the species list of prawn and shrimps during the present study. Out of these 52 species, *Athanas parvus* de Man, 1910, *Alpheus chiragricus* H. Milne Edwards, 1837 and *Pestarella rotundicaudata* (Stebbing, 1902) are first time reported from India. Among the caridean, the superfamily Alpheoidea dominated in the number of the species (13), representing 25% of the total species.

The detailed morphometry and listing of all the specimens have been carried out. Systematic list for prawn and shrimp species is prepared in which a detailed identification key and characters for superfamily, family, genera and species are described. All the species were photographed and detailed identification plate were prepared.

Table 1 List of the new records of prawns and shrimps species recorded.

New record from India	**New record from Gujarat**
	Parapenaeus fissuroides indicus Crosnier, 1986
Neocallichirus jousseaumei (Nobili, 1904)	*Megokris sedili* (Hall, 1961)
Alpheus chiragricus H. Milne Edwards, 1837	*Megokris granulosus* (Haswell, 1879)
Athanas parvus de Man, 1910	*Alpheus lobidens* De Haan, 1849
New record from West coast of India	*Lysmata vittata* (Stimpson, 1860)
Procletes levicarina (Spence Bate, 1888)	*Latreutes anoplonyx* Kemp, 1914
Microprosthema validum Stimpson, 1860	*Palaemon serrifer* (Stimpson, 1860)
Thor amboinensis (de Man, 1888)	*Palaemon pacificus* (Stimpson, 1860)
Alpheus edwardsii (Audouin, 1826)	*Cuapetes grandis* (Stimpson, 1860)
Synalpheus cf. coutierei Banner, 1953	*Upogebia carinicauda* (Stimpson, 1860)

Geographical distribution pattern and habitat preference of prawns and shrimps -

In the present study, total 52 species of shrimps, representing 2 suborders, 4 Infraorder, 4 superfamilies, 11 families and 27 genera were recorded. The maximum species diversity was reported from Saurashtra coast (43 species) followed by Gulf of Kachchh (31 species) and Gulf of Khambhat (21 species) (Figure 1). Total 16 species were only reported from Saurashtra coast and 5 species from Gulf of Kachchh. In case of site wise diversity, the maximum numbers of the species (6 species) are reported from the Shivrajpur region, located on the Saurashtra coast of Gujarat, which supports various kinds of habitat like coral reef, sandy and rocky shore. Because of the habitat variation, the maximum numbers of the species occur in this region. Gulf of Kachchh supports the growth of marine animals and it's considered as one of the biological richest marine habitat. The intertidal area of Saurashtra and Gulf of Kachchh supports the growth of various marine fauna due to unique kind of marine habitat such as mangrove, sea grasses, coral reef, rocky and sandy shore.

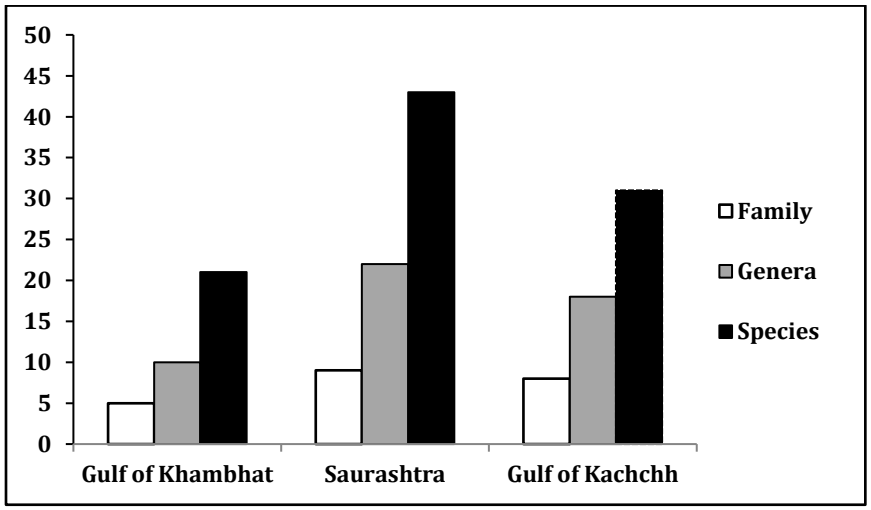

Figure 1 Region wise distribution of shrimp fauna of Gujarat.

Five different microhabitats viz. muddy shore, Sandy shore, Rocky shore, coral reef and benthic. The maximum diversity of prawns was recorded from the benthic region. Species belonging to the suborder dendrobranchiata were absolutely found in the pelagic. The distribution and species diversity dendrobranchiata varies with the depth of the water. In case of intertidal species the maximum species diversity was observed in coral reef followed by the rocky shore, sandy shore and muddy shore (Figure 2). All the macro habitats further divide into eight different microhabitats viz. EnZ (Endozoic), EZ (Epizoic),

H (Hard Substrate), I (Intertidal), M (Mangrove), P (Pelagic), S (Subtidal), SS (Soft Substrate). In the present study microhabitat preference by intertidal species was also studied. The maximum diversity shrimps were recorded from the coral reef. The other microhabitats were supporting the more or less number of species diversity. Families like Alpheidae, which is generally dominantly found in rocky shore, coral reef and under rock boulders. In Endozoic habitat, only a single species *Synalpheus coutierei* was recorded. *Alpheus lobidens* is the only single species present over here, which is found under rock boulders, coral reef, sandy and muddy shore with rock rubbles. All these microhabitats provide the species hiding sites from the predators as well as the algae growing on the surfaces. In case of the Alpheus species crevices provide the best feeding ground.

In the present work, we observed the geographical distribution pattern of prawn species in the different coastal region of Gujarat. The availability of the various microhabitats has played an important impact on the intertidal species diversity of the particular area.

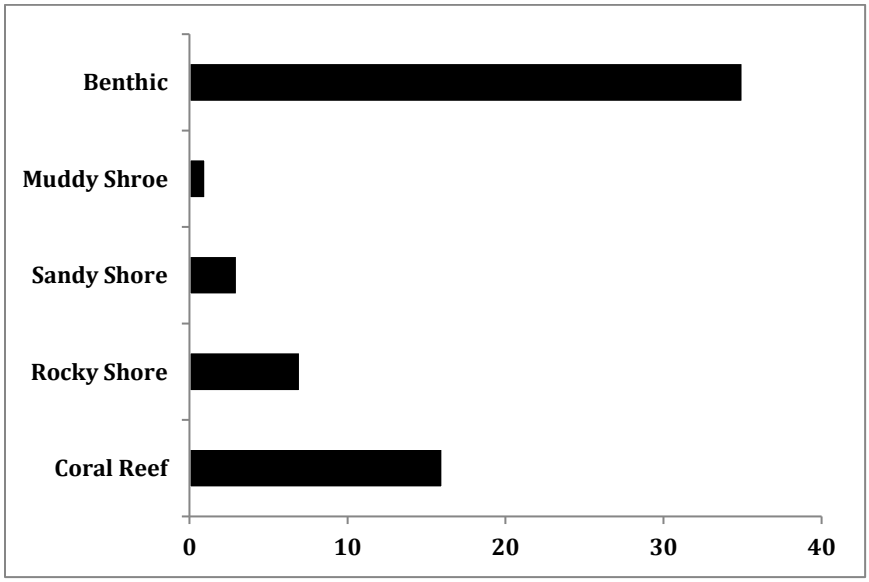

Figure 2 Habitat wise distribution of prawn and shrimp.

Cladistic analysis of prawn and shrimps species based on morphological characters-

In this study, the actual measurements variables converted into the size standardization value. There were a lot of variations between two kinds of value for most of the morphological variables. The size-adjusted values of all the variables of all the species have described as the relationship of species. These values were shows the closeness of the

species. When all these adjusted morphometric values of all the variables are subjected to UPGMA for the cluster analysis. The cluster analysis was performed separately for Dendrobranchiata and Pleocymeta suborders. In UPGMA cluster analysis, the suborder pleocymeta occupied the 4 major clades of infraorders Axiidae, Caridea, Gebiidea and stenopodidae. Most of the discriminative morphometric characteristics or variables for the suborder Dendrobranchiata are rostrum length, total length and second abdominal segment. Where Rajakumaran et al (2014) reported that rostrum length, perpendicular of the third abdominal segment, diagonal of third abdominal segment 2, diagonal of the fourth abdominal segment, and diagonal of fifth abdominal segment 1. In case of the pleocymeta suborders total length, first abdominal segment and first pereiopods length and width. The taxonomical description of a species has commonly depended on the description of unique sets of morphological characters (Rajakumaran et al. 2014). In the present study, the Cladistic investigation of morphological variables was based taxonomical relationship of recorded species by increasing number of variables using truss network system and results supports the taxonomical trait between all the groups reported during this study.

A comprehensive phylogenetic analysis of prawn and shrimp based on mitochondrial COI sequence data-

The total database consists of 21 generated sequences of 5 families. While performing the BLAST most of the generated sequences showed 97-100% similarities with the conspecific database sequence in GeneBank. However, the studies species *Metapenaeus sp.* and *Meatpeanaeus sp.* (2) were identified using the morphological key but the similarity search results in NCBI shows something different from the taxonomic identification. The base 0.1 suggests the nucleotide per sites in the alignment. The analysis of 21 species of prawn and shrimp from Gujarat water showed the two major clades (Figure 3). The first major clade shows the suborder pleocymeta with their respective distance between the suborder dendrobranchiata. It has been clearly seen that the separate clades have been formed for different family and genus. Within the *Metapenaeus* clade, 2 minor clades were identified. The relationship between *M. moyebi, M. monoceros, M. ensis*, *Metapenaeus sp*. and *Metapenaeus* sp (2) form a single. The species *Megokris granulosus* and *Parapenaeus longipes* share the same clade due to the sister genus.

The NJ tree depicted the *Penaeus indicus* and *Penaeus penicillatus* as a sister species. The sequences of *Penaeus monodon* resulted in sister clade with *Penaeus semisulcatus* . The *P. indicus, P. penicillatus* and *P. merguiensis* often posses difficulties at species level identification due to the overlapping of morphological characteristics (Jose and Pillai, 2013).

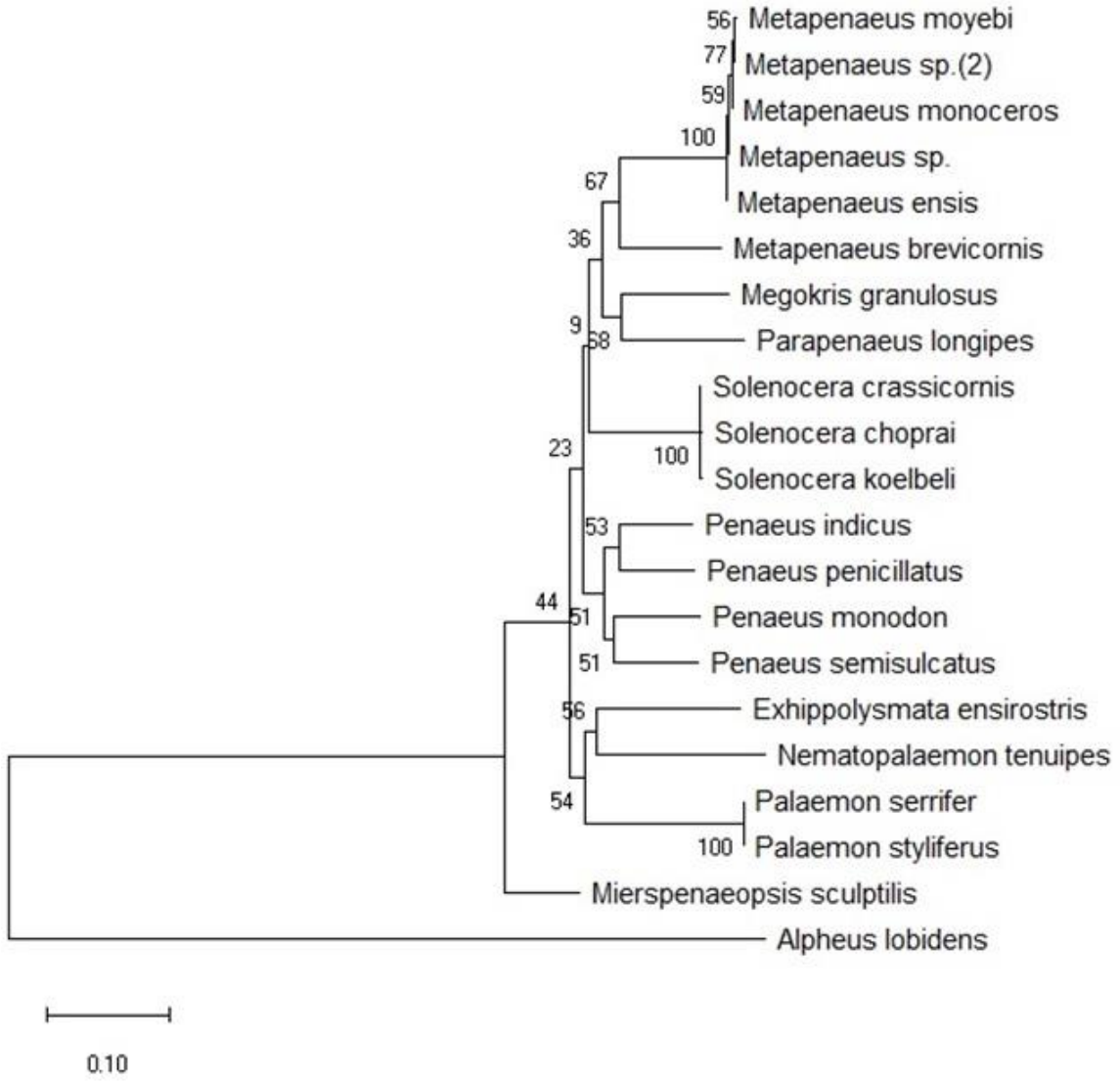

Figure 3 Neighbour-joining (NJ) tree of prawn and shrimps based on COI sequence.

All three species of genus *Solenocera* form a single clade. The present study identifies *P. indicus, P. penicillatus* based on the molecular data. Further, the species *P. monodon* and *P. semisulcatus* form the sister clade based on the molecular data. The overlapping of

morphometric parameters i.e. petasma, rostrum teeth and lack of clear morphological characters lead to misidentification of species (Jose and Pillai, 2013). In family Plaemonidae two minor clades were formed of two genera i.e. *Palaemon* and *Nematopalaemon.* Genus *Exhippolysmata* share the sister clade of genus *Nematopalaemon.* Currently, the *Parapenopsis sculptilis* placed in new genus *Mierspenaeopsis.*

Conclusion-

The major highlights of this study are listed below.

- The present study carried out to access the diversity, distribution and habitat preference of the shrimps and prawns of Gujarat.
- A total 52 species of prawn and shrimps are reported including 3 new records from India, 4 from the west coast of India, 10 from Northwest coast of India and 4 from Gujarat.
- The present study adds sixteen more species to the checklist of prawn and shrimp fauna of Gujarat and three species to the checklist of prawns of India.
- The present work showed that the coastal areas of Gujarat state support a huge diversity of prawns and shrimp fauna.
- In this study, the distribution of shrimp species according to the region wise was observed and which can use as a baseline data for further studies.
- In the present study, the habitat preference of shrimp's species was studied on all kinds of habitat along the Gujarat coast. That can be use as for habitat conservation.
- Shrimps belong to family Callianassidae were found under the burrows.
- In this study the cladistic/phylogenetic tree were developed by UPGMA cluster and NJ analysis, which helps in the systematic, conservation, ecological and evolutionary studies as a primary data.
- From these morphmetrical analyses, it has been concluded that the discriminate function analysis and cluster analysis proved to be an effective procedure for distinguishing and classifying species, and describing the taxonomical relationship than traditional morphological and meristematic traits for Penaeidae and caridean species.

- In this study, the Universal primer LCO1490 and HCO2198 has worked well with 21 species of shrimps, which is necessary to conclude that whether the primer used is species specific.
- The DNA barcodes received matched with previous submissions on the BOLD and BLAST databases allowing us to confirm prawn and shrimp species.
- This study will serve as a framework for future analysis of prawn taxonomy and population structure.

Recommendation for future study-

The coastal area of Gujarat supports different kinds of marine habitats like Rocky shore, muddy, sandy shore, mangrove, coral reef, open mudflat, etc. Although we extensively surveyed Gujarat, several coastal areas, particularly coral reefs of MNP in GoK and extensive mudflats of GoKh and western proximities of Kachchh could not be explored due to time constraints, permissions required and habitat inaccessibility. The results of this study suggested that the Gujarat coast has enormous potential and scope for the taxonomical and ecological studies of prawn and shrimp fauna. The remaining area of Kachchh should be explored for the diversity studies.

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