International Journal of Advance Research in Science and Engineering
Vol. No. 5, Issue No. 08 , August 2016
www.ijarse.com
IJARSE
ISSN 2319 - 8354

A COMPREHENSIVE REVIEW ON MORPHOLOGICAL, MOLECULAR AND PHYLOGENETIC TAXONOMY OF ZOANTHIDS

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ABSTRACT

Zoanthids, benthic Anthozoans are found in nearly all marine environments. Despite their relative abundance, Zoanthids have been overlooked by scholars, because of the intrinsic difficulty in establishing a sound taxonomy based on external morphological criteria and internal structure due to the presence of sand and detritus in their body. In nature these cryptic organisms presents high grade of morphologic diversity especially as colour morphs within a species. This review provides a general introduction to Zoanthids, their ecological and pharmaceutical importance and two different methods of taxonomy i.e. morphological and molecular. Congeneric status of Zoanthids is discussed here through phylogeny. Several Molecular techniques and tools used for phylogenetic studies are summarized that are used by researchers in different biological disciplines.

Key Words: DNA barcoding, DNA sequencing, phylogenetic analysis,Zoanthids

I. INTRODUCTION

Amongst all the animals dwelling in marine environment, few groups have so far not been studied well. The hexacorallian order Zoantharia (Family –Zoanthidea), which are found in shallow water, may also make up a considerable component of some deep-sea coral communities have received very little attention (Sinniger*et al.* 2013; Burnett*et al.* 1997). Over the last decade, deep sea corals have received a considerable attention particularly on seamounts (Miller *et al.*, 2009). Within coral reef communities other than corals mostly benthic molluscs have been studied in details. Even though more in diversity as well as abundance the sponges, zoanthids and actinarians are over looked by scientists. However, due to their significance in relation to bioactive compound release they are now gaining importance in studies.

Zoanthids have ecological and pharmaceutical importance. Zoantharia have been playing an important role in ecosystem as bio-builders that act as habitats for diverse invertebrate and fish communities (Sinniger*et al.* 2013). It has also become an interest of pharmaceutical research as they have some bioactive compounds secreted from their body (Behenna*et al.* 2008) like,Palytoxin, the marine toxin from the zoanthid*Palythoa spp.* (Moore *et al.* 1971); alkaloids of norzoanthamine group from *Zoanthus* spp. etc. These have shown promise in the treatment of osteoporosis (Fukuzawa*et al.* 1995). The latest, Zs Green (zFP506) and Zs Yellow (zFP538),

the respective coloured fluorescent proteins extracted from the zoanthid*Zoanthus spp.* (Stewart, 2006)having important role in biotechnological revolutionary tools. A family of protein named Green Fluorescent Proteins (GFP) - like proteins, initially isolated from marine organisms, started a trend in biotechnological research, which is expanding gradually. Han *et al.* (2006) isolated two prostaglandins, PGA2 and PGB2, from the Okinawan zoanthid *Palythoakochii*.Wilke*et al.* (2010) have obtained a mixture of two cytotoxic LAAs from the zoanthid*Protopalythoavariabilis* that can act as anticancer agent.

In spite of having ecological and economical value, zoanthids are less studied because of their challenging taxonomy and due to that they have been become recent interests in the field of scientific study (Burnett *et al.* 1997;Ryland & Lancaster 2004). Taxonomic reviews have historically been confused by high levels of morphological variation within and between supposed species (Burnett *et al.* 1997). Zoanthids do not have distinct morphological characters for species level identification; a high level of intra-specific variation in terms of colour (Lwowsky 1913), number of tentacles (Herberts 1972) and trapping of sand and detritus material in the mesoglea makes their structure very complicate. Therefore, taxonomy of zoanthids is badly in need of revision.

Total 302 species of zoanthids have been reported from the world (http://www.gbif.org/species/1433). Scientist from different countries like Japan (Reimer *et al.* 2012), UK (Ryland & Lancaster 2004), Australia (Burnett *et al.* 1997), Canada, USA (PhilippandFautin 2009) and India (Pandya *et al.* 2014; Parikh *et al.* 2015) are recently working on these animals. From India, first time in recent year zoanthids has been reported by Bhattji*et al.* (2010) from the Gulf of Kuchchh, Gujarat. It has been also reported from the coast of Saurashtra, Gujarat (Pandya and Mankodi 2012) and the coast of Goa (MythiliandGophane 2013). As we entered in the field of molecular identification of zoanthids, comprehensive literature was collected and based on that a stream line approach of the work is established. This review paper is the comprehensive documentation of such literature.

II. MORPHOLOGICAL TAXONOMY

Zoanthids are radiallysymmetricalhexacorallian organisms of class Anthozoa, phylum Cnidaria (Fig: 1). These animals are characterized by colonies of soft bodied polyps forming mats or solitary in nature and carrying two rows of tentacles (Sinniger*et al.,* 2005) and contain nematocysts (Ryland and Lancaster, 2004). Overall, zoanthids are composed of two germ layer i.e. diploblastic; ectoderm and endoderm. The mesoglea fills the space between germ layers and hence is not true coelomate.

Morphological classifications of Zoanthids is studied through different parameters viz., (1) Colony: macroscopic morphology, number of polyps etc., (2) External morphology: the number of tentacles, capitular ridges, height and diameter of polyps, colour patterns of tentacles, oral disk colour [Fig: 2], column, coenenchyme, host association etc. (Reimer &Fujii 2010, Mythili&Gophane 2013). Besides these, the internal structures include: (1) Actinopharynx (stomadeum or gullet) is a colon or a tube-like structure lined by endodermic cells, and protrudes itself into the gastro-vascular cavity or coelenterons of the organism, (2) Siphonoglyph (sulcus) is the highly glandular region of the actinopharynx having a thick covering of cilia. The Siphonoglyph ascertains the dichotomic alignment inside a polyp. This symmetrical alignment is further delineated due to the existence of 'Mesenteries' and (3) Mesenteries (septa) are radially-arranged layers of tissues, present 'all' or 'portion' of the

distance between the body wall to the central tube or actinopharynx. These are arranged in cycles of six. The distribution of mesentries and the type (complete or incomplete) of the fifth pair of mesentery with respect to the dorsal injunction decides the further division of the taxonomic order into the sub-orders: Brachycnemina (incomplete fifth pair) and Macrocnemina. These sub-orders are further segregated into families – Sphenopidae and Zoanthidae (Brachycnemina); Epizoanthidae, Abyssoanthidae and Neozoanthidae (Macrocnemina) based on relative features.

For example in *Zoanthusaff.vietnamensis*(Pax& Mueller 1957)polyps erect and smooth, often open in daytime, polyps not embedded ("intermediae" or "liberae") in coenenchyme, oral disks always purple or pink, often with white oral opening, outer surface of polyps somewhat lighter in color than *Z. sansibaricus*(Reimer 2010). Where as in *Zoanthusaff.pulchellus* (Duchassaing and Michelotti 1860)polyps are of approximately 4–6 mm in diameter with a slightly larger oral disk, polyp height 4 to over 30 mm, 50 to 60 short tentacles, with a brightly coloured oral disks of green or pink and encrusting colonies with a lamellar coenenchyme found mostly in shallow water, has a more developed coenenchyme than Z. sociatus (Reimer *et al*., 2012). Both have pink oral disk and mostly found in shallow water.

III. MOLECULAR TAXONOMY

To overcome uncertainty in morphological features of species the identification and phylogeny established at a molecular level has been gaining. The molecular markers like mitochondrial DNA cytochrome oxidase subunit I (mtCOI), the most conserved DNA, mt 16SrDNA, mt 12SrDNA and ITS-rDNA are used for molecular level identification and phylogeny of Zoanthids.

IV. DNA BARCODING AND MOLECULAR PHYLOGENY WORKFLOW

Zoanthids are present in abundance but due to their complicated morphology they have been unattained (Reimer *et al.* 2010, Reimer &Fujii 2010). DNA barcoding is a new technique (Herbert *et al.* 2003), which reassesses conservation priorities (Myers *et al.* 2000) to increase taxonomic knowledge. Molecular approaches using allozymes (Burnett et al. 1997) and DNA marker, DNA sequencing and phylogenetic analysis (Sinnigeret al. 2005, Reimer et al. 2006b, and Reimer et al. 2007b) have begun to reassess the diversity.

DNA sequencing and phylogenetic analyses are used to confirm the taxonomical classification. Generally partial cytochrome oxidase subunit I (COI) sequences are used for barcoding. However, it has been observed that COI is well suited for birds (Hebert *et al.* 2004b) and insect (Hajibabaei*et al.* 2006) but not suitable for Anthozoans. Barcodingis potentially very problematic for Anthozoa, due to the high conservation in anthozoan mitochondrial genes (Shearer *et al.* 2002). Hebert *et al.* (2003) tested only 17 cnidarians for COI barcoding and concluded that this marker was not suitable for Anthozoa. Huang et al. (2008) demonstrated that utilizing only COI may not be useful for identifying anthozoans.

Zoanthids have also slow evolutionary rate that is 10-20 times slower than other bilateral organism counterparts, and therefore, for barcoding, the multi region barcoding should be carried out. For barcoding of zoanthids three markers are mostly targeted i.e. Mitochondiral 16S ribosomal DNA (mt 16SrDNA), cytochrome oxidase subunit

I (COI) gene and internal transcribed spacer region of ribosomal DNA (ITS rDNA) (Parikh *et al.* 2015, Reimer &Fujii 2010, Mythili&Gophane 2013, Sinniger*et al.* 2010, Burnett *et al.* 1997). Sinniger*et al.* (2008) reportedCOI and/or 16S barcode are more accurate methods of species identification in this group.

Zoanthids are in symbiotic relation with zooxanthallatealgaeand for that it is required to identify that, which symbiont is associated with the zoanthids. For that due to high evolutionary rate of ITS-rDNA in *Symbiodiniumsp.*, it has been used as a marker at the species level and subsequent lower taxa (Coleman & Mai 1997, Hunter *et al.* 1997). Reimer (2008) reported multiple intra-genomic copies of ITS-rDNA and multiple types of *Symbiodinium spp.* due to niche specialization within the species are the most likely reasons behind high micro variation in *Z. sansibaricus.* Chen *et al.* (1995) used the 5' end of 28S rDNA to investigate relationship between Anthozoa and Zoantharia.

Barcoding requires the assembly of tissue samples and the subsequent isolation and archiving of genomic DNA. For that, different techniques are used by various scientists.For extraction of DNA, sample collection is done by hand in intertidal area or by SCUBA or snorkelling from numerous sub marine sites (Reimer &Fujii 2010, Reimer *et al.* 2012). Such samples are collected in 75-95% analytical grade ethanol (Reimer &Fujii 2010, Mythili&Gophane 2013, Reimer *et al.* 2012). The small tissue of zoanthid was taken and various protocols were used to isolate DNA: Like spin-column Dneasy Blood and Tissue Extraction protocol (Qiagen, Santa Clarita, CA, USA), DNeasy Tissue Kit for animals (Qiagen, Tokyo, Japan) (Reimer &Fujii 2010**,** Reimer *et al.* 2007), Chloroform: Phenol: Isoamylalcohol extraction protocol (Mythili&Gophane 2013), DNeasy Plant Minikit (Qiagen) (Sinniger*et al.* 2005), guanidine extraction protocol (Reimer *et al.* 2012, Sinniger*et al.* 2010).

After isolation the amplification of targeted gene or molecular markers should be carried out and for that primers are available for particular gene. Amplified PCR products were run on agarose gel electrophoresis with the concentration of 1.5% - 2.0% according to the size of amplified DNA sequence. Different sequencers are used for analysis of the sequence and various software are utilized for further analyses. After getting sequence, it is submitted to NCBI data base or Genebank and compare with available sequence. DNA sequences of interest can be retrieved from NCBI database (Mythili&Gophane 2013) or similar search tools. Sequence with E-value less than 10-5 are homologs and that should be taken for further phylogenetic analysis.

The evolutionary relationship among the species is called a phylogeny that can be represented by a phylogenetic tree. The basic steps in any phylogenetic tree are to assemble and align a dataset, build (estimate) phylogenetic trees from sequences using computational methods and stochastic models, and statistically test and assess the estimated trees (Linder &Warnow 2005, Durbin *et al.* 1998, Liò& Goldman 1998).

Once sequences are selected and retrieved, multiple sequence alignment is created.There are different methods and tools available for alignment of the sequences. For multiple Sequence alignment, tools like Clustal Omega, Clustal W, Clustal X (Reimer &Fujii 2010,Reimer *et al.* 2010), Kalign, MUSCLE, MView, T-Coffee, WebPRANK, LALIGN etc. are available. Clustal W is currently the most mature and widely used software tool (Baldauf SL 2003, Hall BG 2004, Linder&Warnow 2005).

For phylogenetic tree construction different methods are available. In that distance based method includes UPGMA (Unweighted Pair Group Method with Arithmetic Mean), neighbourhood joining and Buneman trees; while character based methods includes maximum parsimony and Maximum likelihood. Reimer &Takishita (2006) used maximum likelihood tree for S*ymbiodinium sp.*ITS-2 analysis and concluded presence of subclade

C1 and closely related S*ymbiodinium spp.* associated with *Palythoaspp.*Sinniger*et al.* (2005) used maximum parsimony and Bayesian tree for Hawaiian Gold coral and Zoanthids, and observed that all those species appear related to zoanthids of the genus *Savalia* as well as to the octocoral-associated zoanthid *Corallizoanthustsukaharai*, suggesting a common ancestor to all octocoral-associated zoanthids. Sinniger*et al.* (2005) used maximum parsimony and Bayesian phylogenetic inference for the analysis of zoanthids. Reimer *et al.* (2007) observed close relationship between *P.tuberculosa(Esper, 1791)* and*P.mutuki* during analysis of maximum likelihood tree of mtDNA, 16S rDNA. They have also reported first time potential evolution in Playthoa during analysis of ML tree of ITS-rDNA sequence. Reimer *et al.* (2012) carried out analysis of Maximum likelihood (ML) tree of COI, mt16S rDNAandITS-rDNA and observed *Sphenopus*phylogenetically positioned within the genus *Palythoa* substrate and also mentioned that the unique characters of *Sphenopus*have evolved recently within *Palythoa* and only in the *Sphenopus*lineage by preforming ancestral state reconstruction utilizing maximum likelihood (ML) and maximum parsimony (MP) methods traced on an identical ML tree of mitochondrial 16S ribosomal DNA. There are several software available like Paup*, PAML, PHYLIP that is used to create phylogenetic tree by using alignment methods (Durbin *et al.* 1998, Ming-Chang *et al.* 2000). At last bootstrapping analysis is carried out that gives a way to judge the strength of support for nodes on phylogenetic tree. A number is presented by each node, which reflects the percentage of bootstrap trees which also resolve that clade. Bootstrapping values are typically presented from 1000 repeated calculations.

V. MORPHOLOGY VS MOLECULAR TAXONOMY

After evolution of the Barcoding system, question comes to the mind that why taxonomic method is required for the identification of the species when there is gene level information is available??? Most of the studies published during recent years have been reviewed here and there is no sign that traditional taxonomy is being replaced by DNA barcoding. Mitochondrial DNA barcodes cannot provide enough information about taxa to help make decisions regarding species status and the technicians doing the barcoding do not have expertise with all the new taxa and it's the sequence. Tapas *et al.*, 2014 mentioned that barcoding with COI has not 100% accuracy; it seems 2% error rate. Even though Mitochondrial DNA Barcodes would not hint at the fundamental mechanisms of evolution revealed by these classic studies, its role in identifying specimens to a species level is an important aid for taxonomic workflow. Traditionally, the identification of zoanthid species was based on morphological characters such as polyp shape and size, colony color and shape, as well as locality (Ryland and Lancaster 2003). According to the most recent estimates, 193 species of *Palythoa* have been formally or informally named in the literature (Fautin 2006). However, due to *Palythoa* species heavy sand encrustation (up to 45% of total body weight; Mueller and Haywick 1995), as well as large intraspecific variations in morphology (e.g., polyp shape and size, colony shape, and color), species diversity, identification and taxonomy within this genus remain very difficult and confused (Muirhead and Ryland 1985, Ryland and Muirhead 1993, Burnett et al. 1994), and it is likely that many described zoanthid species are invalid due to inadvertent redescription (Burnett et al. 1997). Specifically, both the methods are appropriate for application however the traditional system of morphological and morphometric helps in justifying species and taxa present in nature. Visual observation can help in determining such characteristics and till date up to species level organisms are

identified with this method. Molecular taxonomy can help us when there is very close and non-distinct similarities at species level and through morphology the one cannot be distinguished. Molecular taxonomy or identification in form of barcodes can be useful to authenticate species identification done in the field and can also serve as a purpose of perfect documentation of information when specimen is maintain in laboratory or museum. We can say that morphology and molecular taxonomy both work in symbiotic relation for identification of a Zoanthid species and molecular techniques provide support to taxonomic methods for the identification of the species.

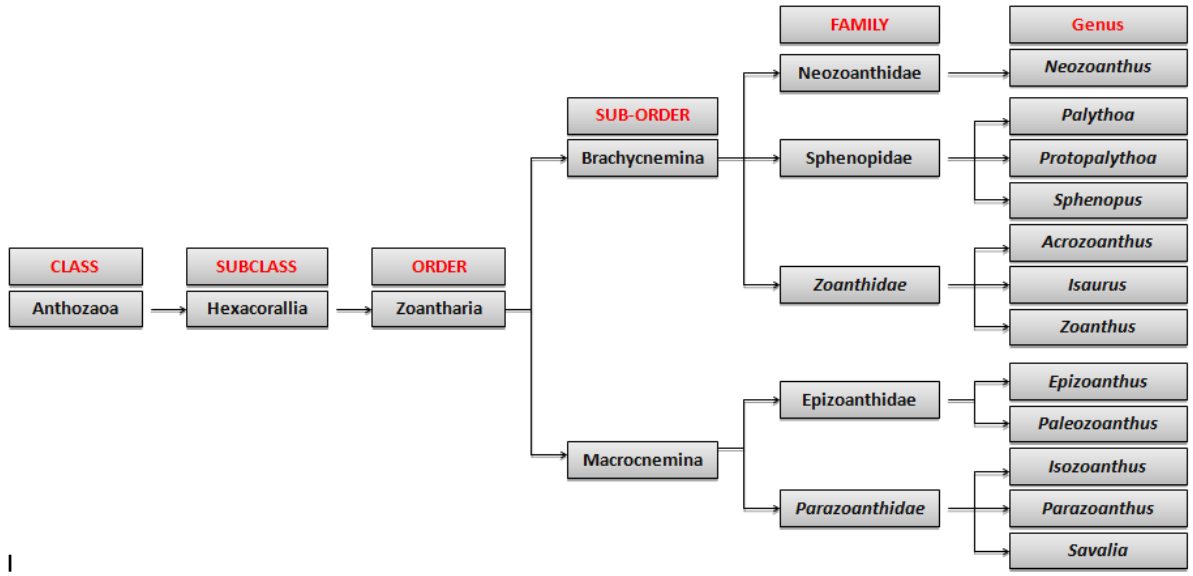

Fig: 1. Classical Organization of order Zoantharia. (Sinniger*et al.*, 2005)

Fig: 2. Colour Morphs of Zoanthids of Saurashtra Coast [Photographs-self taken]

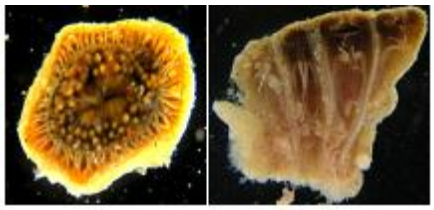

Fig: 3. Sand Encrustation in Zoanthids [Photographs-self taken]

Fig: 4. *Zoanthuspulchellus* which previously identified as *Zoanthusvietnamensis* because of pink colouration [photographs-self taken]

VI. CONCLUSION

Although Zoanthids present in ample density, due to their complicated morphology and dissimilarities within species they have been unattained. The variation within species is may be due to the symbiotic algae they possess in their body. Molecular techniques and phylogenetic analyses have been used to confirm the taxonomic classification. In our study also we had morphologically identified the specimen as *Zoanthusaff. vietnamensis* but by molecular we had confirmed it as *Zoanthusaff. pulchellus*(Fig: 4). As this designation is based on only mt 16S rDNA and morphology/ecology, it is hoped that future in-depth investigation with more specimens and phylogenetic data will confirm this identification.However, for identification and application of Zoanthids both the methods i.e. morphological and molecular methods are required to conclude the appropriate species. The phylogeny among species can be established through computational methods and stochastic models. We envisage in the near future Zoanthids will play an important role in ecosystem and in the field of health due to pharmacological reasons and this molecular phylogeny method will play a key role in taxonomic identification making it easier and more confirmative than morphological classification.

VII. ACKNOWLEDGEMENT

Authors are thankful to Head, Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara for providing necessary facilities and Gujarat State Biotechnology Mission [GSBTM], Gandhinagar for the financial support for this research work. Authors are thankful to Dr.Khushali Pandya for her support in the work on ecology and identification of the specimens.

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