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Revision of *Isaurus sp.* diversity through molecular investigation along the Saurashtra coast, Gujarat, India

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Abstract
Zoanthids (Order-Zoantheria, Class-Anthozoa) are benthic cnidarians which occupies the larger extent of rocky littoral zone of the tropical seas. The current paper deals with the relationship and phylogenetic confirmation of different *Isaurus spp.* (Anthozoa: Zoathidae) within the genera. *Isaurus sp.* are having, non-erect, recumbent polyp and are having tubercles on their body surface except *Isaurus cliftoni.* This genera is known from both the Atlantic and Indo-Pacific regions and is generally found in intertidal areas. In India, *Isaurus spp.* are believed to be very rare and have been reported from only a handful of locations like Veraval from a previous survey and Okha, Sutrapada and Vadodara Jhala in the present study. Total 07 specimens of the species were collected. Two species of *Isaurus* viz., *Isaurus tuberculatus* and *Isaurus maculatus* were observed and identified morphologically, however upon doing molecular phylogeny using separate genes like COI, 12s rRNA and 16s rRNA, *Isaurus tuberculatus* was the only species confirmed. The phylogenetic tree was constructed using 16s rRNA gene. Phylogenetic relationship of the confirmed species also showed 98% similarity with *Isaurus tuberculatus* of Japan, South Africa and Florida.

Keywords: *Isaurus*, molecular phylogeny, similarity

1. Introduction
The order zoantharia (Anthozoa: Hexacorallia) comprise oftwo zooxanthallate families; the sand encrusted Spenopidae and the non-encrusted Zoanthidae. Species from both the families are often found in subtropical and tropical shallow coral reef and littoral habitats worldwide [1, 2]. The Gujarat coast with the longest coastline in the Indian subcontinent mainly features the rocky areas which richly favors the growth of Zoanthids [3]. The substratum and sedimentation in the Saurashtra region is favorable for the growth of Zoanthids has been observed to be increasing in past few decades [4, 5, 6].
The family Zoanthidae is represented by three genera: Zoanthus, Acrozoanthus and Isaurus. *Isaurus sp.* which are known from both the Atlantic and Indo-Pacific regions, and are generally found in intertidal or shallow subtropical and tropical waters [7]. *Isaurus sp.* occurs in varied colors and forms and are found as individual polyps attached to the rocks. The polyps, during daytime remains closed and receive their nutrition via photosynthetic processes with the help of symbiotic algae present in them while during night, they open up and feed directly [8]. This genus is found mainly from Western Central Atlantic and Southwest Pacific regions [7]. Three species of the genus *Isaurus* have been described are: *I. tuberculatus* (Gray, 1828) [9]. *I. maculatus*and *I. cliftoni* (Gray, 1828) [9], although 22 species have been historically listed in the literature [10]. In India *Isaurus* have been reported from very less places as its occurrence is less. However, *Isaurus tuberculatus* and *Isaurus maculates* were recorded from Veraval, Gujarat [3].
In India, there has been no record on molecular aspects of this genus hence we tried to work on its diversity based on morphological characteristics as well as its molecular aspects. *Isaurus* has been distinct with the presence of tubercles on the polyps (although *I. cliftoni* does not have tubercles), but shares many morphological characters like there is no sand encrustation, presence of zooxanthellae, colonial in nature, generally individual polyps not attached with each other by stolon like Zoanthus. These characteristics makes phylogenetic placement of this genus as separate to Zoanthus.
DNA barcoding is a technique in which a short nucleotide sequence of a mitochondrial genome will act as DNA barcode for species identification of animals and has proven to be a rapid and enhanced tool for precise identification of animal species [11]. DNA barcoding works under the principle that inter species variations are greater then the intra species variations. Allowing one to differentiate the species using nucleotide sequences [12]. Molecular approaches using DNA sequencing and phylogenetic analysis [13, 14] have begun to reconsider the identification of biotic diversity. The DNA sequencing and analysis have often resulted in taxonomic revision of species [15] and genera [16].

2. Materials and Methods
2.1 Sample Collection
Samples of *Isaurus sp.* were collected from several different sites of Gujarat, India i.e. Okha, Sutrapada and Vadodara jhala and were stored in 100% ethanol at -20˚C. Photographs were taken in situto assist in identification. The documentation of morphological data (polyp size, tubercle size, arrangement of tubercles, Capitalum/Scapus) were recorded on site. Unlike *Zoanthus sp.* and *Palythoa sp.*, during collection *Isaurus* polyps in situ were always observed to be closedthus no

tentacle count data were obtained.

2.2 DNA extraction/PCR amplification

Total genomic DNA was extracted from the whole body by using lysis buffer with proteinase K (LBWPK) method [17] and gel electrophoresis was done using 0.8% agarose gel to visualize the DNA. All extracted products were stored at -20˚C. The mitochondrial COI, 12s rRNA and 16s rRNA were amplified using the primers LCO1490 and HCO2198 to obtain a sequence of 658 bp [18], 12S1a and 12S3r to obtain a sequence of ~670bp and 16Sant0a and 16SbmoH, to obtain a sequence of ~750 bp [19] respectively (Table: 1). The PCR amplification was carried out using 1X final concentration of Ready Mix™ Taq PCR Reaction Mix (Sigma) and template DNA (50 ng/µl). PCR reactions of 20 µl contains: 10 µl master mix; 1µl of each 100pm primer, 2µl of DNA template and the remaining volume is of Milli Q water. The reaction was carried out in Thermal cycler (Applied Biosystems Veriti©) with 5 min denaturation step at 94˚C and 35 cycles of 94˚C for 30 sec, 40˚C for 45 sec and 72˚C for 1 min, followed by a 7 min extension at 72˚C for COI amplification and with 2 min denaturation step at 94˚C and 40 cycles of 94˚C for 30 sec, 52˚C for 1 min and 72˚C for 2 min, followed by a 5 min extension at 72˚C for 16s rRNA gene and 12s rRNA gene. The amplified genes were analyzed by electrophoresis in 2% (w/v) agarose gels in tris-acetate buffer and the gel bands were visualized by Gel documentation system (Applied Biosystems).

Table 1: Primer sequences used for the study

Gene	Primer used	Sequence
COI	LCO1490	5′-GGT-CAA-ATC-ATA-AAG-ATA-TTG-G-3′
	HCO2198	5′-TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-CA-3′
16s rRNA	16Sant0a	5’-GAA-GTA-GGC-TTG-GAG-CCA-GCC-A-3’
	16SbmoH	5’-CGA-ACA-GCC-AAC-CCT-TGG-3’
12s rRNA	12S1a	5’-TAA-GTG-CCA-GCM-GAC-GCG-GT-3’
	12S3r	5’-ACG-GGC-NAT-TTG-TRC-TAA-CA-3’

2.3 DNA Sequencing and data analysis

Purification of amplified PCR products were done using Exo SAP-IT© of affymatrix using following reaction mixture of 10 µl of PCR product and 4 µl of Exo SAP-IT©. Clean up was carried out in Thermal cycler (Applied Biosystems Veriti©) for 15 min at 37˚C and 15 min at 80˚C. The purified PCR products were directly sequenced sequencing analysis version 5.4 (Applied Biosystems) and Bio Edit, Biological Sequence Alignment Editor [20]. The consensuses sequences were deposited in Gen Bank. For investigation of phylogenetic relationships, 16S coding sequences of *Isaurus tuberculatus*, were obtained from the Gen Bank. Furthermore, retrieved sequences were aligned using Clustal W for phylogenetic analysis. For reconstruction of phylogenetic tree, maximum likelihood method was used with Kimura 2- parameter model and bootstrap method with 1000 replicates were used to know statistical support. The phylogenetic and evolutionary analysis were done using MEGA 7 [21].

3. Results and Discussion

3.1 Results

3.1.1 Morphological Analysis

Total 07 samples for both the species were collected from different sites of Saurashtra coast i.e., Okha, Vadodra Jhala and Sutrapada (Figure: 1). Identification of collected species through morphology reveals two species i.e., *Isaurus maculatus* and *Isaurus tuberculatus*. The morphological characteristics are given below in Table: 2.

Table 2: Morphology of two species of *Isaurus*

Polyp color	Tubercle arrangement	Tubercle number	Capitalum/Scapus	Morphologically identified species
Red, brown	Large, in longitudinal series	Less in number	Separated by crown tubercles	*Isaurus tuberculatus*
Grey, dark brown	Small, in circular series	Numerous in number	Not separated by crown tubercles	*Isaurus maculatus*

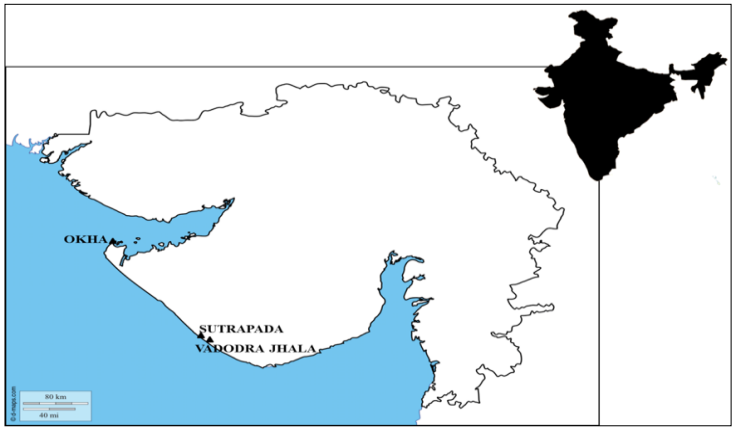

Fig 1: Map showing selected collection sites of *Isaurus* specimens

But as both the species were closely similar to each other morphologically, we used the molecular techniques to emphasize on the actual and critical identification of the species. Because of so much closeness in their morphology, only COI was not enough to reveal the species correctly so DNA sequencing approach was based on three different genes viz., COI, 16s rRNA, 12s rRNA (Table: 3) to resolve the identification issues and explain the authentic species.

Table 3: Showing accession number of all 3 different genes of all 7 specimens (Voucher Specimens are deposited in Bio Gene – GSBTM facility, Gandhinagar)

Sample ID	Species	16s rRNA	12s rRNA	COI
I01	*Isaurus tuberculatus*	KP176687	KP176692	-------
ZDI01	*Isaurus tuberculatus*	KP090267	-------	-------
23	*Isaurus tuberculatus*	KX528542	-------	KX911434
28	*Isaurus tuberculatus*	KX528547	-------	KX911439
30	*Isaurus tuberculatus*	-------	-------	KX911441
32	*Isaurus tuberculatus*	KX528550	KY026136	KX911466
34	*Isaurus tuberculatus*	KX528551	-------	KX911445

3.1.2 Phylogenetic Analysis

The summarized form of the Maximum likelihood tree of 16s rDNA gene sequences of the 13 different *Isaurus* specimens is shown in Figure. 2. The first major clade shows the family Zoanthidae with their respective distances between the species of the Sphenopidae family. The families of Zoanthidae and Sphenopidae distantly shows their relative closeness in characters. Further, species of family Zoanthidae is forming a clade at the upper end of the tree which shows their maximum distances from the others.

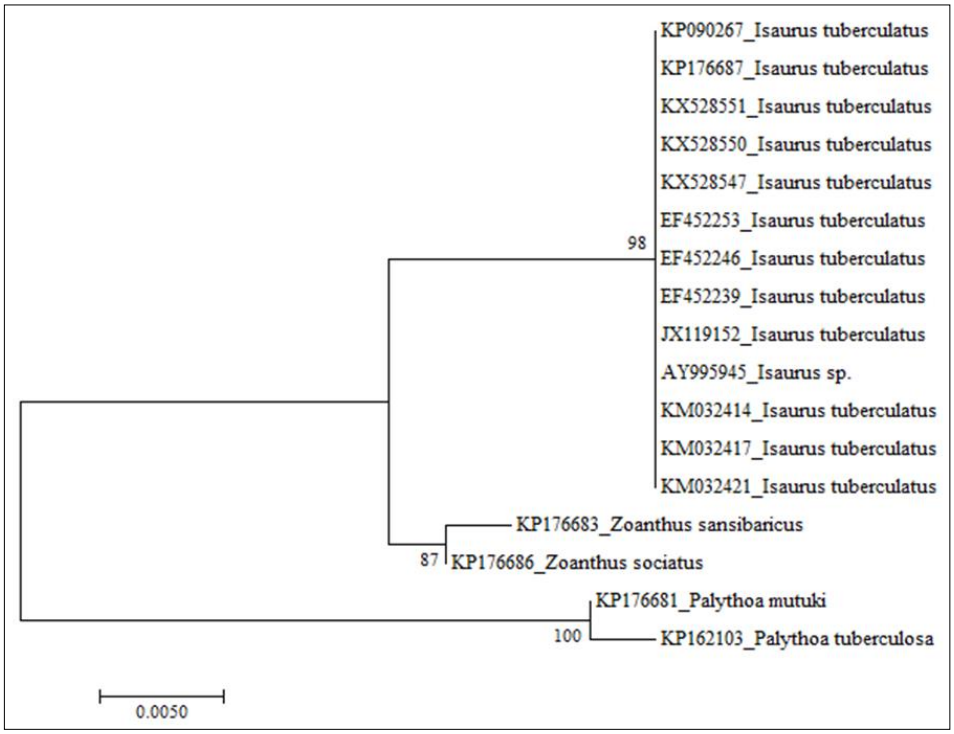

Fig 2: Maximum likelihood tree of mitochondrial 16S ribosomal DNA (mt 16S rDNA) sequences. Values at branches represent ML bootstrap probabilities. Sample names with Accession Numbers are from present study and GenBank (Table 4)

The obtained sequences which are 750 bp of 16s rRNA gene, were analyzed in terms of nucleotide composition. The evolutionary analysis was conducted in MEGA7 [20]. The sequences show over 98% similarity between five specimens of the present study as well as specimens from other countries i.e. Japan, South Africa and Florida obtained from Genbank. The inference was carried out for evolutionary history by using the Maximum Likelihood method based on the Kimura 2-parameter model [22]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 387 positions in the final dataset. The analysis involved 17 nucleotide sequences and those are from present study as well

as obtained from Genbank (Table: 4).

Table 4: The data of present study and retrieved from GenBank for this study

Species	Location	Country	Accession number	Reference
Isaurus tuberculatus	Sutrapada	India	KP090267	Present Study
Isaurus tuberculatus	Okha	India	KP176687	Present Study
Isaurus tuberculatus	Vadodra jhala	India	KX528547	Present Study
Isaurus tuberculatus	Okha	India	KX528550	Present Study
Isaurus tuberculatus	Okha	India	KX528551	Present Study
Isaurus tuberculatus	Yakushima	Japan	EF452239	Reimer *et al.*, 2008[7]
Isaurus tuberculatus	Otsuki	Japan	EF452246	Reimer *et al.*, 2008[7]
Isaurus tuberculatus	Tosashimizu	Japan	EF452253	Reimer *et al.*, 2008[7]
Isaurus tuberculatus	Florida	USA	JX119152	Reimer *et al.*, 2012
Isaurus tuberculatus	Aquarium trade	Indonesia	AY995945	Sinniger *et al.*, 2005
Isaurus tuberculatus	Clansthal	South Africa	KM032414	Risi and Macdonald, 2014
Isaurus tuberculatus	Isipingo	South Africa	KM032417	Risi and Macdonald, 2014
Isaurus tuberculatus	Sodwana	South Africa	KM032421	Risi and Macdonald, 2014
Zoanthus sociatus	Dwarka	India	KP176686	Present Study
Zoanthus sansibaricus	Okha	India	KP176683	Present Study
Palythoa tuberculalosa	Dwarka	India	KP162103	Present Study
Palythoa mutuki	Dwarka	India	KP176681	Present Study

The DNA sequences obtained from the Genbank suggests that there is not much genetic differences between the specimens. The species of Zoanthus and Palythoa are taken as outgroup. It is clearly seen that separate clade has been formed for different families.

3.2 Discussion

The phylogenetic results as well as morphological data obtained suggest that though there are wide variations in certain morphological characteristics, all *Isaurus spp.* specimens examined in this study are conspecific and belongs to only one species i.e., *I. tuberculatus*. Furthermore, the morphological data shows expected *Isaurus maculatus* specimens have morphological characteristics similar to *I. tuberculatus* (excepting tubercle size and arrangements see Tables: 2) make the case of conspecificity even stronger. These results are very similar to previous works [7, 8, 23], who observed large amounts of intraspecific variation in *Isaurus*, besides also an intercolony variation of polyps due to different habitats. As seen in Figure 3, we also observed large amounts of intra-colony polyp variation in coloration and size even in small colonies. The *Isaurus* specimens examined here from Saurashtra coast (I01, ZD-MSU-I01, 23, 28, 30, 32, 34) (Figure 3) also had identical mt 16S rRNA sequences to *Isaurus* specimens from Japan, South Africa and Florida, supporting the hypothesis that *I. tuberculatus* is known to be distributed over a wide range [23]. Previously, *Isaurus sp.* and *Zoanthus sp.* were considered to be the same, however in 1985, Muirhead and Ryland brought out the difference between both these species morphologically. Results obtained in our study also support the differences in both these species by the separation of clades.

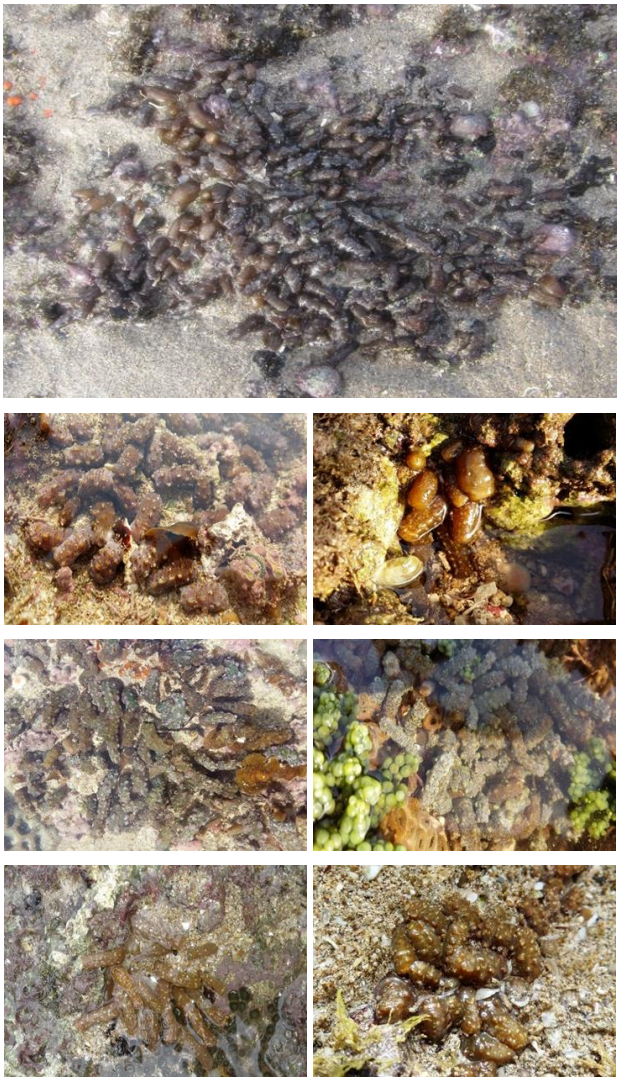

Fig 3: Images showing specimens of *Isaurus tuberculatus*

4. Conclusion

The present study involvs both the morphological and molecular data that suggests all *Isaurus spp.* examined are belonging to only one species i.e. *Isaurus tuberculatus*. We did observe high levels of morphological variations (in particular coloration, polyp size and tubercle size) between all *Isaurus* specimens of individual colonies (Figure 3). Although it remains to be conclusively shown that characters exhibited by *Isaurus maculates* specimens are in fact the same as *Isaurus tuberculatus*. It was concluded from this study and

also with the support from previous research [8, 23, 7] that *I. tuberculatus* has considerable intraspecific morphological variations. Our results also support the above conclusion that *I. tuberculatus* have too much variations in their morphological features particularly in external coloration, tubercle size and arrangement.

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