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Research Article

IDENTIFICATION AND INTRA SPECIES DELINEATION OF ORNAMENTED FLYING FISH *CHEILOPOGON SPILONOTOPTERUS* (BLEEKER, 1866) WITH DNA BARCODES

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ABSTRACT

Efficiency of barcoding is definitively dependent upon species description, splitting decreases while the protuberance increases both intraspecific variation and interspecific divergence. In the present study was Cheilopogon spilonotopterus sequenced for its 700 bp region of cytochrome oxidase subunit I (COI) gene to test its efficacy in identifying the species and also to demonstrate its intra species variations within the barcode region. The present study addresses this issue by examining the patterning of COI diversity in the subphylum Vertebrata, the most ancient and structurally diverse group of family Exocoetidae. The sequences were analyzed for their species identification using Barcode of life database (BOLD's) identification engine. The BOLD's identification engine was also used. The COI sequences of from different geographical regions were extracted from NCBI for intraspecies variation analysis. All sequences were aligned using Clustal W. Phylogenetic tree was constructed with bootstrap test with 500 replicates. The barcode profiling studies clearly revealed that the barcode region of *C.spilonotopterus* from different waters had high cytosine content whereas guanine content was found least. Interesting results were obtained in case of hypothetical barcode protein profiling, as the percentage of leucine was found higher in barcode proteins.

Keywords: Hypothetical, intra species variations, barcode, Cytochrome oxidase.

INTRODUCTION

Fish are the largest group of vertebrates, which exhibit a higher diversity of morphological attributes and biological adaptations. Species are typically circumscribed based on the presence of fixed diagnostic morphological characters which distinguish them from other species . But for fishes, there are a large number of intraspecific invariants or interspecific overlappings, so fish identification is challenging for taxonomists when facing rich biotas (Jun-Bin Zhang and Robert Hanner, 2011). The idea of a standardized molecular identification system emerged progressively during the 1990s with the development of PCR-based approaches for species identification. Molecular identification has largely beenapplied to bacterial studies, microbial biodiversity surveys and routine pathogenic strains diagnose (Maiden et al., 1996; Sugita et al., 1998;Wirth et al., 2006) due to a need for culture independent identification systems. PCR-based methods have also been frequently used in fields related to taxonomy, food and forensic molecular identification (Teletchea et al., 2008) and for identification of eukaryotic pathogens and vectors. Several universal systems formolecular-based identification have been used for lower taxa but were not successfully implemented for broader scopes (LiseFrezal and Raphael Leblois , 2008). The term “DNA barcode” was proposed to suggest thatthe characteristics of nucleotide sequences can be used to represent a species in much the same way as the 11-digit Universal Product Codes in labeling retail products. The central concept of this useful tool is the characteristic of a standard sequence that corresponds to asinglehomologous gene region which can be amplified by a polymerase chain reaction (PCR) with “universal primers”, and distinguishes a species from similar ones across a broad range of taxa. The suggested method can be a powerful tool for identifying larval forms of an organism and even for incomplete specimens on which a morphological diagnosis cannot be performed (Ekrem et al., 2007).For instance, the suggested method was applied to various biological fields and showed a promising ability to differentiate closely related fish species (Sharks:Holmes et al., 2009; Sardine: Quilang et al., 2011; Salmons: Rasmussen et al., 2009; Catfishes: Wong et al., 2011; Scombrids: Paine et al., 2007). It is also used in ornamental fishes and seafood identification, due to its simplicity and accuracy (Steinke et al., 2009; Lowenstein et al., 2010inverting the tubes. Following incubation the samples were chilled on ice for 10 minutes and about 250µl of solution 2 (6M NaCl) was added to it and mixed wellby inverting the tubes several times. Tubes were then chilled on ice for 5 minutes. Then the tubes were centrifuged at 8000 rpm for 15 minutes and following centrifugation, 500 µl of clear supernatant was collected in a 1.5 ml tube. Equal volume of (1ml) of 100% analytical grade ethanol was added to precipitate the DNA. A thin hair like precipitate was observed after addition of ethanol. After 30 minutes the tubes were allowed to spin at 11,000rpm for 5 minutes. The supernatant was removed and partially dried in room temperature. The DNA pellets were washed thrice with 70% cold ethanol. The pellets were suspended in 100 µl of sterile distilled H2O.

MATERIALS AND METHODS

Sample collection and preservation:

The fish samples were collected from Parangipettai (South east coast of India) fish landing centre in live condition and the tissue samples for DNA extraction was excised from the lateral side and cut into small pieces in order to permit ample fluid penetration during preservation in fresh 95% ethanol. After preservation the tubes were stored under refrigerated condition.

DNA extraction:

Salting out procedure was adapted to extract DNA from tissues. The preserved tissue in ethanol was washed four to five times with sterile distilled water to get clear of the ethanol content. The ethanol free tissues was transferred in to 1.5 ml tube and grounded in micropestle with 500µl of solution 1 (500mM Tris-HCL, 20mM EDTA and 2% SDS). After homogenizing the tissues were added with 5µlof Proteinase K (20mg/ml). The tubes were incubated at55ºC in water bath for 2 hours with occasional mixing by Haq et al. 27 inverting the tubes. Following incubation the samples were chilled on ice for 10 minutes and about 250µl of solution 2 (6M NaCl) was added to it and mixed well by inverting the tubes several times. Tubes were then chilled on ice for 5 minutes. Then the tubes were centrifuged at 8000 rpm for 15 minutes and following centrifugation, 500 µl of clear supernatant was collected in a 1.5 ml tube. Equal volume of (1ml) of 100% analytical grade ethanol was added to precipitate the DNA. A thin hair like precipitate was observed after addition of ethanol.After30 minutes the tubes were allowed to spin at 11,000rpm for 5 minutes. The supernatant was removed and partially dried in room temperature. The DNA pellets were washed thrice with 70% cold ethanol. The pellets were suspended in 100 µl of sterile distilled H2O.

Quantitation of DNA by Spectrophotometric method:

10µl of DNA solution was diluted with 990µl of TE. Mixed well and absorbance at 260nm and 280nm was measured. The absorbance at 260nm can be used to calculate the concentration of DNA as follows: Calculations PCR amplification Primers The primer set LCOFw and HCORw designed in the conserved region was used for the amplification of the COI region of the test organisms and the primer sequences are;

Target	Primer Name	Direction	Sequence (5' → 3')
COX1	LCO	Forward	GGTCAACAAATCATAAAGATATTGG
	HCO	Reverse	TAAACTTCAGGGTGACCAAAAAATCA

A 1.0µl of Sample DNA (approximately 100 ng/µl) was added to PCR Mixture containing 100mM TrisHCl (pH8.3), 500mM KCl (pH 8.3),2.5µl MgCl2 (25mM), 2.0µl dNTP's (2.5mM), 1.0µl Primer Forward & Reverse (each of 10pm/µl) and 1u /µl of Taq Polymerase(BioserveMake) & the final volume made to 25 µl with nuclease free water. The thermal profile consisted of 35 cycles at 94 ºC for 50 s, 54 ºC 50 s and 72 ºC for 1 min. QIAGEN QIAquickTMkit was used for sequencing reaction. The samples were precipitated and suspended in 40µl of loading solution provided with the kit. Sequencing was done with MegaBace sequencer- Bioserve Hyderabad, India

BOLD's identification engine:

BOLD (Barcoding of life database) is an online workbench that aids in collection, management, analysis, and use of DNA barcodes. Identification engine is the one of the important components of BOLD database which consists of large volume of barcode sequences for both plants (intranuclear spacer gene) and animals (cytochrome c oxidase subunit gene). BOLD-IDS provide a species identification tool that accepts DNA sequences from the barcode region and returns taxonomic assignments to the species level when possible. The BOLD identification system (IDS) accepts sequences from the 5' region of the mitochondrial gene cytochrome oxidase subunit I and returns species-level identification when one is possible. This identification engine was accessible online through http://www.barcodinglife.org/views/ idrequest. php. The sequences were given in FASTA file format in the query box and results were obtained similar to that of BLAST search.

Profiling the barcode region:

The molecular weight of the single stranded barcodeDNA was calculated as the sum of the monophosphate forms of each deoxyribonucleotide deducting one water molecule each. One water (18 Da) was added at the end to represent the 3' hydroxyl at the end of the chain and one more hydrogen atom at the 5' phosphate end. Nucleotide composition summaries and plots were obtained by choosing "Nucleotide Composition" form the "Nucleic Acid" submenu of the "Sequence" menu. Bar plots showed the Molar percent of each residue in the sequence. The degenerate nucleotide designations were added to the plot wherever they are encountered

Barcode protein profiling:

DNA to Protein:

The online software at www.insilico.ehu.es was used to extract hypothesized amino acid sequences from the COI region of *Cypselurus* spilonotopterus. This software allowed modeling and modifications of already existing techniques, as well as new theoretical approaches. Standard genetic code translation

was used. DNA sequences were fed in to the query box in FASTA format. Minimum size of protein sequence for Open Reading Frames (ORF) is customizable and they were trimmed to MET-to-Stop.

CLUSTAL W:

Clustal W is a general purpose global multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be seen via viewing Cladograms or Phylograms.

Phylogenetic tree construction using MEGA:

Neighbourhood joining (NJ) method of phylogenetic tree

Construction was preferred for accurate establishment of phylogenetic relationship and to trace out the presence of phylogenetic signals in the DNA sequences (Nei et al., 2000). The distance was calculated between every pair of sequences and these were used to construct the phylogenetic tree which guided the final multiple alignment. The scores were calculated from separatepair wise alignments.

MEGA (Molecular Evolutionary Genetic Analysis) version 6:

MEGA is an integrated tool for conducting automatic and manual sequence alignment, inferring phylogenetic trees, mining the web base data bases, estimating the rates of molecular evolution, and testing evolutionary hypothesis (Tamura et. al., 2007)

Bootstrapping:

One of the most commonly used tests of the reliability of an inferred tree is Felsenstein's (1985) bootstrap test which is evaluated using Efron's (1982) bootstrap resampling technique. If there are m sequences, each with n nucleotides (or codons or amino acids) a phylogenetic tree can be reconstructed using the same tree building method. From each sequence nucleotideswere randomly chosen with replacements, giving rise to mrows of ncolumns each. These now constitute a new set of sequences. A tree is then reconstructed with these new sequences using the same tree building method as before. Next the topology of this tree was compared to that of the original tree. Each interior branch of the original tree that was different from the bootstraptree the sequence it partitions is given a score of 0 all other interior branches was given the value 1 was noted. This procedure of re-sampling the sites and the subsequent tree reconstruction was repeated several hundred times and the percentage of times each interior branch was given a value of 1 was noted. This is known as the bootstrap value. The multiple aligned sequences from Clustal X were loaded into MEGA through Create New Alignment option in Alignment menu.

The sequences were trimmed for their conserved regions and saved in MEGA format forphylogram construction. Bootstrap test for phylogeny was preferred to detect the reliability of each branch in phylogram. As a general rule if the bootstrap valuefor a given interior branch is 95% or higher than the topology of that branch then the value is considered "correct" (Neiand Kumar, 2000

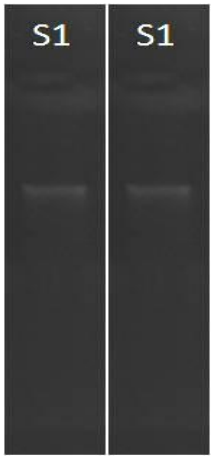

Figure 1: Quantitation of DNA by electrophoresis

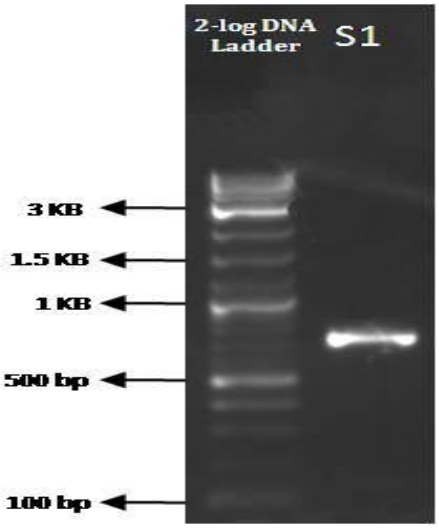

Figure 2: Genomic DNA amplification by PCR

RESULTS

Quantitation of DNA by electrophoresis:

A thick band was seen above the 300kb band of λHind III. This indicates high molecular nature of genomic DNA(Fig1). In the electropherogram the bands of the size ~500bp (for sample S1) was observed against 100bp DNA ladder (Fig 2). There was no overlapping of thebands in the case of test organisms and that way the bands were clear.

Top 10 Sequences Producing Significant Alignments from NCBI:

The sequences were checked for considerable alignments from NCBI. About 10 sequences showed significant alignments of which the maximum identity ranged from 95% to 100%. The maximum score ranged from 1149 to 961. The query coverage was found to be as 100%. The summary of the results is depicted in Fig 3.

Description	Max	Total	query	E-Value	Ident	Accession
Cheilopogonspilonotoperus isolate A.C.Rat001 cytochrome oxidase subunit 1 (CO1)gene, Partial cds; Mitochondrial	1149	1149	100%	00	100%	KT030205.1
Cheilopogonspilonotopterus voucher PGN68 Cytochrome Oxidase subunit 1(CO1) gene, Partial cds; Mitochondrial	1133	1133	100%	00	99%	KF14914.1
Cheilopogonfurcatus voucher ADC11.1162#1 Cytochrome Oxidase subunit 1(CO1)gene, Partial cds;Mitochondrial	1000	1000	99%	00	96%	KF489537.1
Cheilopogonnigricans voucher smith 1163# cytochrome oxidase subunit1 COI gene, Partial cds; Mitochondrial	1000	1000	99%	00	96%	JF493131.1

HirundichthysspMys.shop 2 cytochrome oxidase subunit 1 COI gene partial cds; Mitochondrial	972	972	100%	00	95%	KT719284.1
Cypseluruscallopterus cytochrome oxidase subunit COI gene parialcds; Mitochondrial	970	970	99%	00	95%	KU323667.1
Cheilopogoncyanopterus voucher ADC11_116#1Cytochrome oxidase subunit (COI) gene Partial cds; Mitochondrial	966	966	99%	00	95%	KF489536.1
Cheilopogonatrisignis Mitochondria, Complete genome	965	965	99%	00	95%	KU360729.1
Cypselurushiraii Mitochondria DNA Complete genome	965	965	99%	00	95%	AB182653.1
Hirundichthysoxycephalus voucher HTB 1 Cytochrome Oxidase subunit 1CO1 gene partial cds; Mitochondrial	961	961	99%	00	95%	JF952756.1

Figure 3: Top 10 Sequences Producing Significant Alignments from NCBI

BOLD's search:

Identification summary (Fig 4) showed the probability of placement (100%) along with taxonomic level and taxonassignment. The distance summary is illustrated in Fig 5. A species level match was made. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. The bolds search showed top 20 specimen similarity with 95.31% to 99.51%. (Fig 6). The COI species database tree confirmed that the study organism belongs to the order Beloniformeswhich resembled much similarity with *C. Spilonotopterus*(Fig. 8). Accession numbers of sequences closely related to the Test organism used in the analysis & their locations The test organisms were reviewed for close relations to the test organism (KT 030205.1) for which the accession numbers were cross checked from the database. The accession numbers were KF 714914.1, KF 489537.1, JF 493131.1 and KT 719284.1

Identification Summary:

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Chordata	100
Class	Actinopterygii	100
Order	Beloniformes	100
Family	Exocoetidae	100
Genus	Cheilopogon	100

Figure 4: Results of the BOLD search

Similarity Scores of Top 99 Matches:

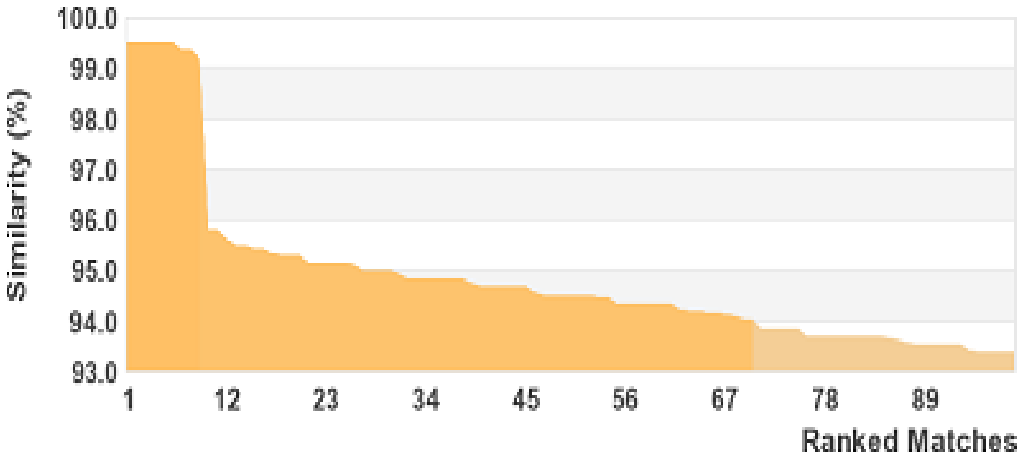

Figure 5: Similarity scores of the top 100 matches

Phylum	Class	Order	Family	Genus	Species	Sy (%)	Status
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Spilonotopterus	99.51	Early release
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Spilonotopterus	99.51	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Katoptron	99.51	Private

Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Atrisignis	99.51	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Spilopterus	99.51	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Spilonotopterus	99.51	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Atrisignis	99.31	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Suttoni	99.35	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Spilonotopterus	99.19	Early release
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	exsiliens	95.79	Published
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	exsiliens	95.79	Published
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	exsiliens	95.79	Published
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Dorsomacula	95.47	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Dorsomacula	95.47	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Cyanopterus	95.42	Early release
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Exsiliens	95.42	Early release
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Cyanopterus	95.32	Private
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Exsiliens	95.31	Published
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	Sp	95.31	Early release
Chordata	Actinopterygii	Beloniformes	Exocoetidae	Cheilopogon	abei	95.31	Private

Figure 6: Results of top 20 specimen similarity with 96.05% to 100%

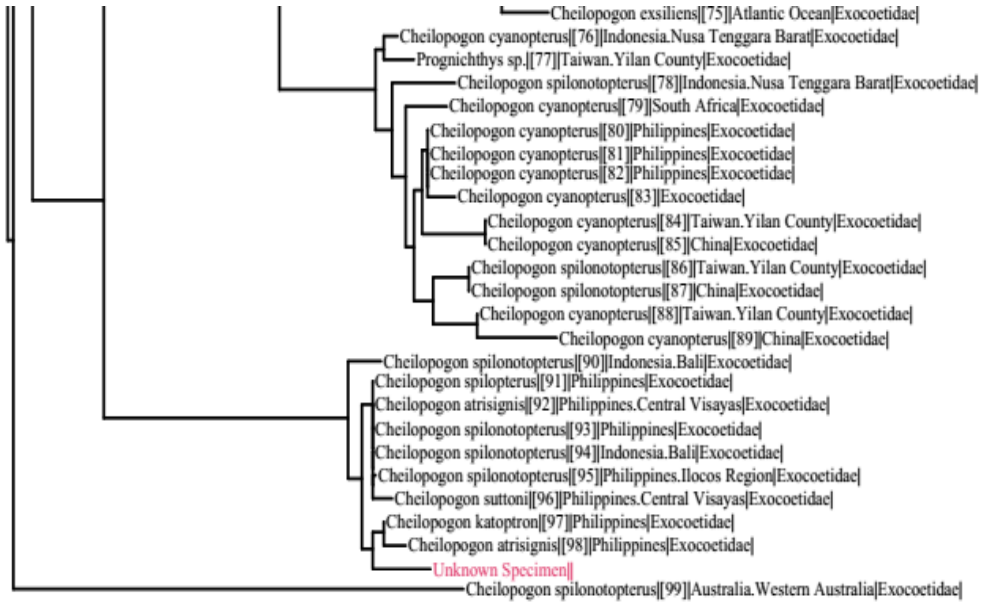

Figure 7: COI species database tree

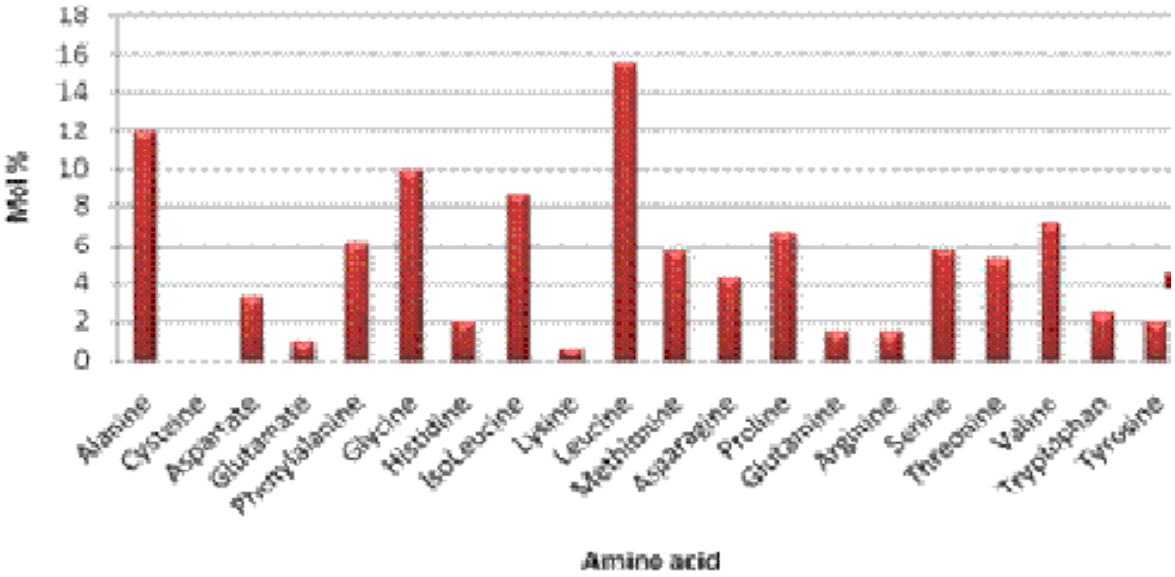

Figure 8: Graph showing hypothetical barcode profiling Cheilopogon spilonotopterus **of Parangipettai waters**

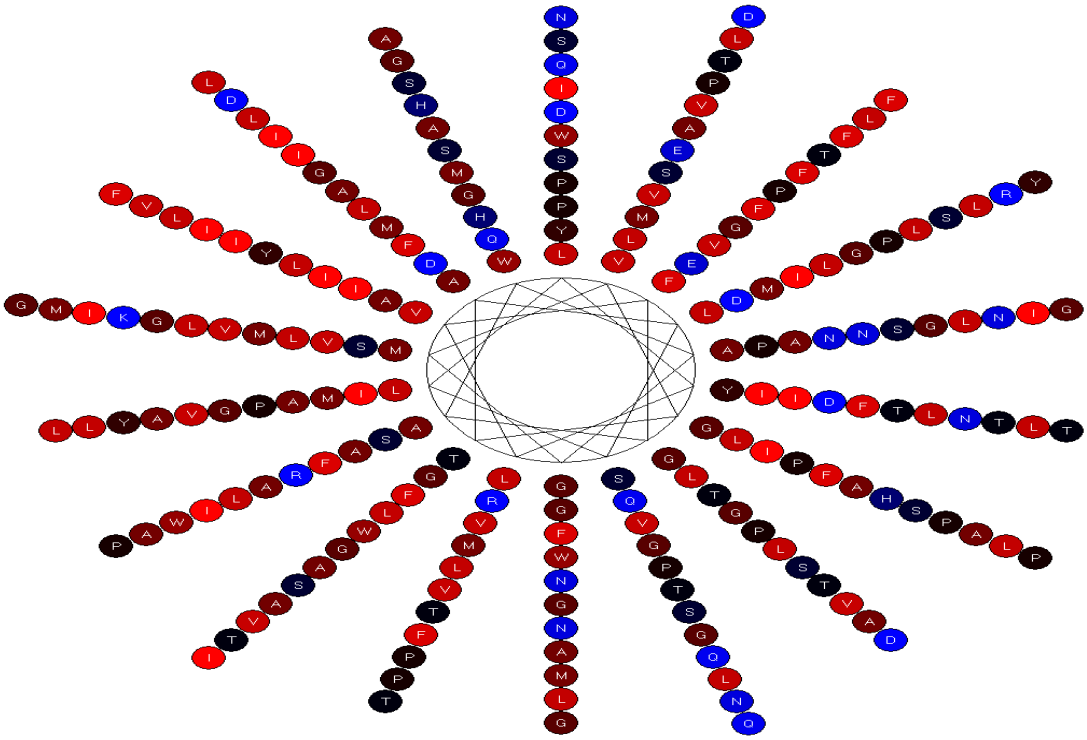

Figure 9: Protein's secondary structure is plotted in a rotating manner where the angle of rotation between consecutive amino acids is 100°.

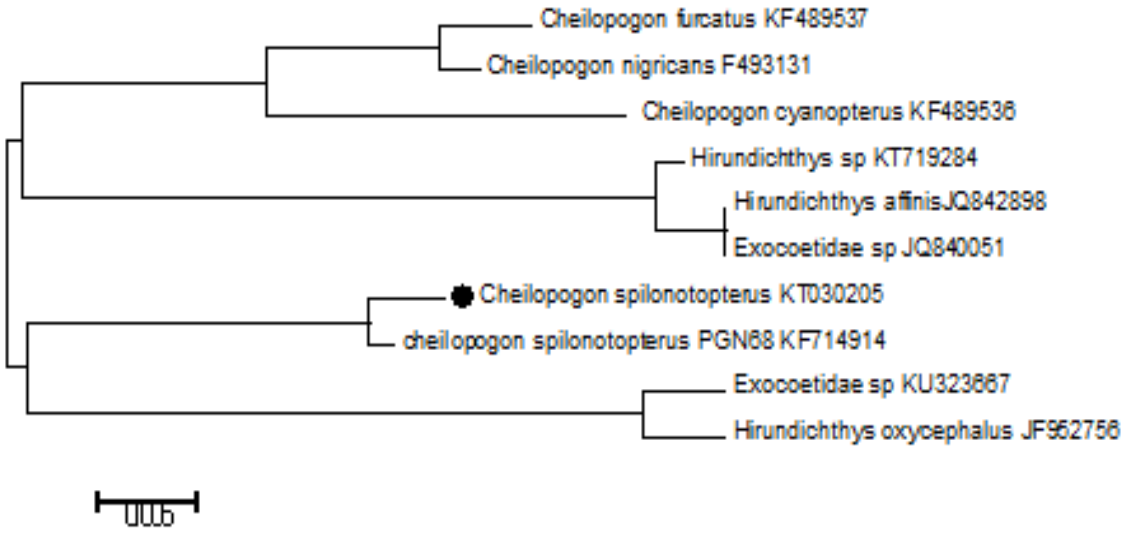

Figure 10: Evolutionary relationships of taxa by UPGMA Method

Profiling the barcode region of *Cheilopogon Spilonotopterus:*

Nucleotide compositionsummaries were obtained and shown in Table 1. The table represents the Molar concentration of DNA nucleotides in the COI region of *C.Spilonotopterus* sample from Parangipettai waters versus closely related organisms. Upon comparison the results showed that the Thymine content was high and similar in all the samples. The least molar concentration was observed in Guanine in all the samples. Cytosine was the second predominant in molar concentration next to thymine which is followed by adenine.

Name of species	**Accession ID**	**Base pair length**	**G+C content (%)**	**A+T content (%)**	**Nucleotide Number and Mol%**			
					A	T	G	C
Cheilopogonspilonotopterus	**KT030205.1**	622	44.2%	55.8%	26.4%	29.4%	17.2%	27.0%
	KF714914.1	695	43.9%	56.1%	26.2%	29.9%	17.7%	26.2%
	KF489537.1	652	44.3%	55.7%	26.1%	29.6%	17.6%	26.7%
	JF493131.1	652	43.7%	56.3%	26.4%	29.9%	17.3%	26.4%
	KF489536.1	652	43.7%	56.3%	26.4%	29.9%	17.5%	26.2%

Table1: The Molar concentration of DNA nucleotides in the COI region of C.spilonotopterussample from Parangipettai waters versus closely related specimens

Barcode protein profiling:

DNA to Protein:

The translation alignment was optional, and amino acids were displayed as a 1-letter amino acids code. Aminoacid composition summaries and plots were obtained bychoosing "Amino Acid Composition"

from the "Protein" submenu of the "Sequence" menu. Bar plots showed the Molar present of each residue in the sequence (Fig 9). Amino Acid plots and summaries were similar, thoughresidues other than the standard 20 amino acids were ignored. A helical wheel is a type of plot or visual representation used to illustrate the properties ofalpha helices in proteins. The sequence of amino acids that make up a helical region of the protein's secondary structure are plotted in a rotating manner where the angle of rotation between consecutive amino acids is 100° , so that the final representation looks down the helical axis. The plot reveals whether hydrophobic amino acids are concentrated on one side of the helix, usually with polar or hydrophilic amino acids on the other (Fig 8).

CLUSTAL W:

The similarities between two or more DNA sequences were compared using multiple sequence alignments. The query sequences were posted on the query box in ClustalW from the tools option of EMBL. The results page displays the similarities between the sequences.

DISCUSSION

As a part of this initiative we have selected C.Spilonotopterusa commercially exploited species as a nutritive sea food for DNA barcoding to solve the ambiguity in its specieslevel identification. The BOLD database was found efficient in identifying the queried barcode sequence as *C.spilonotopterus* as the distance summary was cent percent and the phylogram constructed by BOLD revealed the same. barcode profiling studies clearly revealed that thebarcode region of *C.Spilonotopterus* from different waters had high cytosine content whereas guanine content was found least. Interesting results were obtained in case of hypothetical barcode protein profiling, as the percentage of leucine was found higher in barcode proteins of *C.Spilonotopterus* from dissimilar region of Uttar Pradesh 1 – 4 (India - accession no. KF 714914.1, KF 489537.1, JF 493131.1 and KT 719284.1) waters whereas in Parangipettaiwaters percentage of serine was found at the highest level. However multiple sampling approaches had to be adopted to justify the statement. In the phylogram drawn with the aid of Bioeditsoftware tool with Neighbor-joining methodology, two clades were evident one with the out group species other clade consisting of *C.Spilonotopterus* from international waters (Fig 12). Though phylogeographical signals were witnessed, interestingly two of Uttar Pradesh 2 and 3 barcode sequences clustered with the Parangipettai sequences, where as the Uttar Pradesh 1sequences were kept outseparately in the Uttar Pradesh 4 sequence clade ofthe constructed phylogram. The two main ambitions of DNA barcoding are to (i) assign unknown specimens to species and (ii) enhance the discovery of new species and facilitate identification, particularly in cryptic, microscopic and other organisms with complex or inaccessible morphology (Hebert et al., 2003). This study clearly revealed that COI could be abarcode sequence distinguishing *C.Spilonotopterus* to its species level both through the phylogram and by search result of barcode of life

database. The profiling study on the barcode regions of *C.Spilonotopterus* revealed that barcode region was rich in cytosine and least in guanine content. The GC content of *C.Spilonotopterus* was found to be about 44.2%. Both phylography and phylogeographic signals were evident from the phylogram constructed with *C.spilonopterus* as the same group. Hence this sort of study can affirm that COI could be a potential barcode gene for accurate species level identification of *C.Spilonotopterus*.

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