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DNA Barcoding and Intra Species Analysis of the Ember Parrot Fish Scarus Rubroviolaceus using mtCO1

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ABSTRACT

In this study S.rubroviolaceus form very ancient family group Scorpaenidae with structurally diverse group of subphylum Vertebrata was collected form from parangipettai (MAB06)for species identification with inter specific variation among barcoding region sequenceing cytochrome oxidase subunit I (COI) gene comparing with Australia (EF609452), Tonga (FJ584082), South Africa (GU805008), Iran (HQ149928). The intraspecific variation analysis in NCBI screend for COI sequence form different geographical regions results 99% to 100% identity. BOLD's identification engine shows 99.68% to 100% similar of 20 specimens with conformation of COI species database tree that S.rubroviolaceus belongs to order Carangidae. The molar concentration of nucleotides in the COI region profiling barcoding valuates that high in thymine content are similar in all the samples, Cytosine as second predominant and guanine as least molar concentration. A helical wheel is ploted to illustrate the properties of alpha helices in proteins. Multiple sequence alignments for DNA sequences similarities query were analysed with Clustal W from EMBL tools.

Keywords: Interspecific variation, barcode, cytochrome oxidase, *Scarus rubroviolaceus*

Introduction

As mostly biological research carrying species diagnoses, taxonomic expertise is confusing. The way was made by the construction of barcodes as taxon validating based on DNA[1]. An extricating method for species identification, and can consistently proclaim the unknown species to known, also denoting potential mystrious species and genetically distant populations[2]. It was constructed under mitochondrial gene cytochrome *c* oxidase I (COI) as kernel part for animals global bioidentification system[1] brought this breakthrough identification with mitochondrial genome and introduce the name DNA barcoding. Reasons for choosing mitochondrial (mtDNA) over nuclear DNA include uniparental inheritance (in a majority of animal phyla), high evolutionary rate, lack of introns, large copy numbers in every cell, and limited recombination. As mitochondrial DNA has uniparental inheritance (mainly in animal phyla), evolution rate, lack of introns, large copy numbers in each cells and limited recombination[3]. The term "DNA barcode" was projected to imply that the characteristics of nucleotide sequences can be used to indicate a species is similarly as like the 11- digit Universal Product Codes in labeling retail products[4]. The DNA barcode project was initially conceived as a standard system for fast and accurate identification of animal species. Its scope is now that of all eukaryotic species[5]. The DNA barcode itself consists of a 648 bp region 58–705 from the 50-end of the cytochrome c oxidase 1 (COI) gene using the mouse mitochondrial genome as a reference[6]. It is based on the postulate that every species will most likely have a unique DNA barcode (indeed there are 4650 possible ATGC-combinations compared to an estimated 10 million species remaining to be discovered[7] and that genetic variation between species exceeds variation within species[1] . The efficiency of species identification by molecular methods is judged by the levels of intraspecific homogeneity and interspecific heterogenenity displayed by the intended method[8,9] . Mitochondrial COI gene, as an attractive "species barcode", its high efficiency in species identification has been reported in Australia marine fishes[10] , Canadian freshwater fishes[11] and ornamental fishes in the market of North America[12].

Redlip Parrotfish is the common name of *S.rubroviolaceus* which occurs in seaward reefs. Usually over rocky or coral substrates, at boulder-strewn slopes at the base of high-island cliffs where it may occur in large schools. *S.rubroviolaceus* comes under subfamily Scarinae. The Scarini was under the family Labridae[13]. The Chlororus and Scarus are different monophyletic lineages[14]. While comparing the species in pacific and Indian ocean there is no absolute conformation for morphological differences but has genetic breaks between them[15]. *Scarus rubroviolaceus* and *S. psittaccus* is high in central Indo-Pacific population and has genetic divergence found at the periphery of each species range[16,17]. Only few studies are made in this fish merely based on its prominence in recreational, artisanal and commercial fisheries

throughout its distribution[18,,19,20,21,22]. The association between diversification and evolutionary innovations has been well documented and tested in studies of taxonomic richness but the impact that such innovations have on the diversity of form and functions are not well understood[23].

Basically Parrotfishes consume algaes associated in the coral reef and those fishes are bioeroders that makes useful for healty reef ecosystem in the tropics[24,25,26]. *S.rubroviolaceus* as coral associated fishes, for fisheries and tourisum these fishes are economically valuble[27,19,20]. All parrotfishes are sequential protogynous hermaphrodites which exposes two colour phases[28]. Fish with essentially females having small male parts is Initial phase (IP) and only male without female is the terminal phase (TP)[28]. In Hawaii areas Spawning in parrotfishes contineouses for whole year and peaks up in summer months form May to July[30,29] and species may migrates to certain places for spawning[30]. 50% of *S.rubroviolaceus* are immature upto 13.5 inches fork length growth In Hawwaii islands[31].

This species is fished in some parts of its range where destructive fishing practices and habitat destruction are prevalent. It is heavily fished in the northern Solomon Islands with evidence of a decline in mean size and numbers of this species in the markets from 2004-2006. It is the second or third most important species in the parrotfish fishery in 2004, but by 2006 the importance of this species in the fishery had declined[15]. It is a component of subsistence fisheries and is heavily fished in parts of its range. There have been indications of localized population declines in Indonesia, Philippines and the Solomon Islands. It is found in a number of marine reserves and in remote localities. It is therefore listed as Least Concern. Although there are numerous marine reserves in areas where this species is heavily fished (Coral Triangle Region), most reserves are not very well managed. However, in well-managed reserves parrotfishes tend to recover comparatively quickly and therefore increased management in protected areas and potentially fishery protection might offset the overexploitation of this species.

Materials and methods

Wet lab methodologies

Sample 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 were excised from the lateral side of *Scarus rubroviolaceus* and cut into small pieces (< 5-7 mm) to permit adequate fluid penetration and preserved in fresh 95% ethanol using 1.5 ml labeled tubes. The ethanol was poured off after few days of collection and replaced with fresh 95% ethanol to optimize DNA preservation. The tubes were stored under refrigerated condition.

DNA extraction

Salting out procedure was adapted to extract DNA from *S.rubroviolaceus* 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 were transferred in to 1.5 ml tube and grounded in micro pestle with 500µl of solution 1 (500mM Tris-HCL, 20mM EDTA and 2% SDS).

After homogenizing the tissues were added with 5µl of Proteinase K (20mg/ml). The tubes were incubated at 55ºC in water bath for 2 hours with occasional mixing by 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. 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 H_2O.

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

OD_{260} of 1= 50µg/ml DNA

7Dilution factor =100

$$\text{Concentration of DNA in a given solution} = \frac{50 \times OD \times \text{Dilution factor } \mu g/\mu l}{1 \times 1000}$$

PCR amplification

Primers

The primer set MAB Fw and MAB Rw designed in the conserved region was used for the amplification of the COI region of the test organisms and the primer sequences are;

MAB Fw3: 5'- TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC -3'

MAB Rw3: 5'- CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA -3'

PCR Set up

A 1.0µl of Sample DNA (approximately 100 ng/µl) was added to PCR Mixture containing 100mM Tris HCl (pH 8.3), 500mM KCl (pH 8.3), 2.5µl $MgCl_2$ (25mM), 2.0µl dNTP's (2.5mM), 1.0µl Primer Forward & Reverse (each of 10pm/µl) and 1u /µl of Taq Polymerase (Bioserve Make) & the final volume made to 25 µl with nuclease free water.

DNA sequencing

QIAGEN QIAquick™ kit was used for sequencing reaction. The sequencing PCR was done to amplify one strand of barcode gene employing the primer FISH F1only under standard PCR conditions. The samples were precipitated and suspended in 40µl of loading solution provided with the kit. Sequencing was done with MegaBace sequencer- Bioserve India, Hyderabad.

Dry lab methodologies

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 a taxonomic assignment 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. Further validation with independent genetic markers will be desirable in some forensic applications. 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 of *Scarus rubroviolaceus*

The molecular weight of the single stranded barcode DNA was calculated as the sum of the monophosphate forms of each deoxyribonucleotide minus 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. Any DNA sequence has only A, G, C and T and these were represented by four bars on the graph.

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 *Scarus rubroviolaceus.* 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.MShowing translation alignment was optional, and aminoacids were displaied as a 1-letter aminoacids code.

1-letter aminoacid codes

A alanine
B aspartate or asparagine
C cysteine
D aspartate
E glutamate
F phenylalanine
G glycine
H histidine
I isoleucine
K lysine
L leucine
M methionine
N asparagine
P proline
Q glutamine
R arginine
S serine
T threonine
U selenocysteine
V valine
W tryptophan
Y tyrosine
Z glutamate or glutamine
X any

Amino acid composition summaries and plots were obtained by choosing "Amino Acid Composition" from the "Protein" submenu of the "Sequence" menu. Bar plots showed the Molar percent of each residue in the sequence (Fig 10). Amino Acid plots and summaries were similar, though residues 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 of alpha 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 11).

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

Neighborhood 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[32]. 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 separate pair wise alignments.

MEGA (Molecular Evolutionary Genetic Analysis) version 5

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[34].

Bootstrapping

One of the most commonly used tests of the reliability of an inferred tree is[35] bootstrap test which is evaluated using[36] 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 *n* nucleotides were randomly chosen with replacements, giving rise to *m* rows of *n* columns 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 bootstrap tree 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 for phylogram construction. Bootstrap test for phylogeny was preferred to detect the reliability of each branch in phylogram. As a general rule if the bootstrap value for a given interior branch is 95% or higher than the topology of that branch then the value is considered "correct"[32].

Results

Quantitation of DNA by electrophoresis

A thick band was seen above the 300kb band of λHind III marker (marker not shown here). This indicates high molecular nature of genomic DNA (Fig1). In the electropherogram the bands of the size

~700bp (for sample MAB06) was observed against 100bp DNA ladder (Fig 2). There was no overlapping of the bands 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 99% to 94%. The maximum score ranged from 1153 to 991. The query coverage was found to be as 100%. The summary of the results is depicted in Fig 3. The distance tree comparison of the study organism showed similar evolutionary similarity with *Scarus rubroviolaceus* (Fig 4).

BOLD's search

Identification summary (Fig 5) showed the probability of placement (99.8%) along with taxonomic level and taxon assignment. The distance summary is illustrated in Fig 6. A species level match has been made. The bolds search showed top 20 specimen similarity with 96.11% to 99.84%. (Fig 7). The COI species database tree confirmed that the study organism belongs to the order Perciformes which was very closely related with *Scarus rubroviolaceus* (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 (JX120608) for which the accession numbers were cross checked from the database. The accession numbers were EF609452 (Australia) FJ584082 (Tonga), GU805008 (South Africa), HQ149928 (Iran).

Profiling the barcode region of *Scarus rubroviolaceus*

Nucleotide composition summaries were obtained and shown in Table 1. The table represents the Molar concentration of DNA nucleotides in the COI region of *Scarus rubroviolaceus* sample (MAB06) from Parangipettai waters versus closely related organisms. Upon comparison the results showed that the thymine and cytosin content was high and While comparing with all the samples they are different . The least molar concentration was observed in guanine in all the samples.

Barcode protein profiling

DNA to Protein

The protein's secondary structures of figure 11 explains the alpha helices in proteins showing that hydrophobic aminoacids of Alanine (A), Isoleucine (I), Leucine (L), Phenylalanine (F), Valine (V), Glycine (G) where concentrated as groups, and only Serine (S), Threonine (T) and Tyrosine (Y) interfered between those hydrophobic aminoacids in the first circle.

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 Clustal W from the tools option of EMBL. The results page displays the similarities between the sequences. The similarities in sequences of the study animal with intra species is shown in fig 9.

Evolutionary relationships of taxa by UPGMA Method

From fig 12 we are resulted that the evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 8.67922062 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 642 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Discussion

This study clearly revealed that COI (mitochondrial cytochrome c oxidase subunit I) could be a barcode sequence distinguishing *Scarus rubroviolaceus* to its species level both through the phylogram and by search result of barcode of life database in fig 5. Minimum evolutionary distance of 0.02 in the scale was observed (Fig 12). While comparing the Phylogeographical signals and the arrangement of *Scarus rubroviolaceus* (MAB06) sample from parangipettai with Australia (EF609452), Tonga (FJ584082), South Africa (GU805008), Iran (HQ149928). The parangipettai sample is closely relationship with Iran sample with same distance of about 0.0210 (Fig 12)which may indicates that the species have evolved from Bushehr, Iran "27.40 N 52.60 E" to India ("11 ^{0} 29' N, Long 79 ^{0} 46'E") or vise versa. Surprisingly here the result also denotes that Australian ("11.62 S 143.57 E") species (EF609452) and South African ("30.333 S 30.75 E") species have same evolution relationship of 0.0201(Fig 12) as the distance between the two region is too long. The Tonga species (FJ584082) was the most isolated and genetically diversed while comparing to those other four samples of about 0.0322(Fig 12) as may due to the enterly different region in the world map and notably worlds most divergent species living biggest island Australia is also distanced to this Tonga region.

It was observed that other one *Scarus rubroviolaceus* from Iran Sea shows 99.84% by which it conforms the species *Scarus rubroviolaceus*.. The profiling study on the barcode regions of *Scarus rubroviolaceus* revealed that barcode region was rich in thaimin and cytosine consisting equal value of about 29.3% and 18.5% as least of guanine content. The GC content of *Scarus rubroviolaceus* from various waters was found to be about 47.8%. The protein's secondary structures of figure 11 exposing hydrophobic aminoacids concentrated as groups which reveals that one face of the helix is oriented towards the hydrophobic core and other face is oriented towards the solvent exposed surface of the alpha helical globular proteins. This will makes the further steps for characterizing the protein folds and its docking motifs.

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FIGURES AND TABLES

Table 1 Nucleotide composition of Test organism & closely related sequences

Name of species	Accession ID	Base pair length	G+C content (%)	A+T content (%)	Nucleotide Number and Mol%			
					A	T	G	C
MAB06	**JX120608**	642	47.8%	52.2%	147	188	119	188
					22.9%	29.3%	18.5%	29.3%
	EF609452	655	48.5%	51.5%	149	188	120	198
					22.7%	28.7%	18.3%	30.2%
	FJ584082	652	47.4%	52.6%	153	190	117	192
					23.5%	29.1%	17.9%	29.4%
	GU805008	652	48.0%	52.0%	149	190	119	194
					22.9%	29.1%	18.3%	29.8%
	HQ149928	648	48.6%	51.4%	148	185	121	194
					22.8%	28.5%	18.7%	29.9%

Figure 1 Genomic DNA (sample MAB02) were shown 300kb band

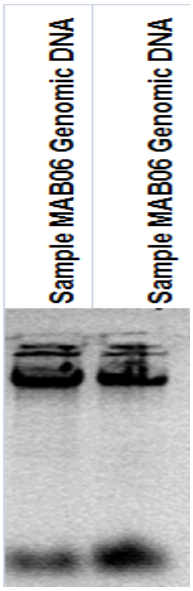


Figure 2 Electrophoresing the amplicons size were shown ~700bp.

Figure 3 Top 10 Sequences Producing Significant Alignments from NCBI

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU805008.1	Scarus rubroviolaceus voucher ADC09_221.15#7 cytochrome oxidase	1153	1153	100%	0.0	99%
FJ227899.1	Scarus rubroviolaceus mitochondrion, complete genome	1122	1122	100%	0.0	99%
HQ149928.1	Scarus ghobban voucher NPPF1123 cytochrome oxidase subunit 1 (C	1041	1041	100%	0.0	96%
EF609452.1	Scarus ghobban voucher BW-A1264 cytochrome oxidase subunit 1 (	1041	1041	100%	0.0	96%
FJ449707.1	Scarus ghobban mitochondrion, complete genome	1036	1036	100%	0.0	96%
HQ149930.1	Scarus ghobban voucher NPPF1097 cytochrome oxidase subunit 1 (C	1032	1032	100%	0.0	96%
FJ237699.1	Chlorurus gibbus voucher MBCSC:HN SY08421 cytochrome oxidase s	1023	1023	98%	0.0	96%
FJ584082.1	Scarus iseri voucher HLC-12101 cytochrome oxidase subunit 1 (COI)	996	996	100%	0.0	94%
GU225430.1	Scarus iseri voucher MX103 cytochrome oxidase subunit 1 (COI) gen	991	991	100%	0.0	94%
GU225429.1	Scarus iseri voucher MX104 cytochrome oxidase subunit 1 (COI) gen	991	991	100%	0.0	94%

Figure 4 The Distance Tree of the Results from NCBI

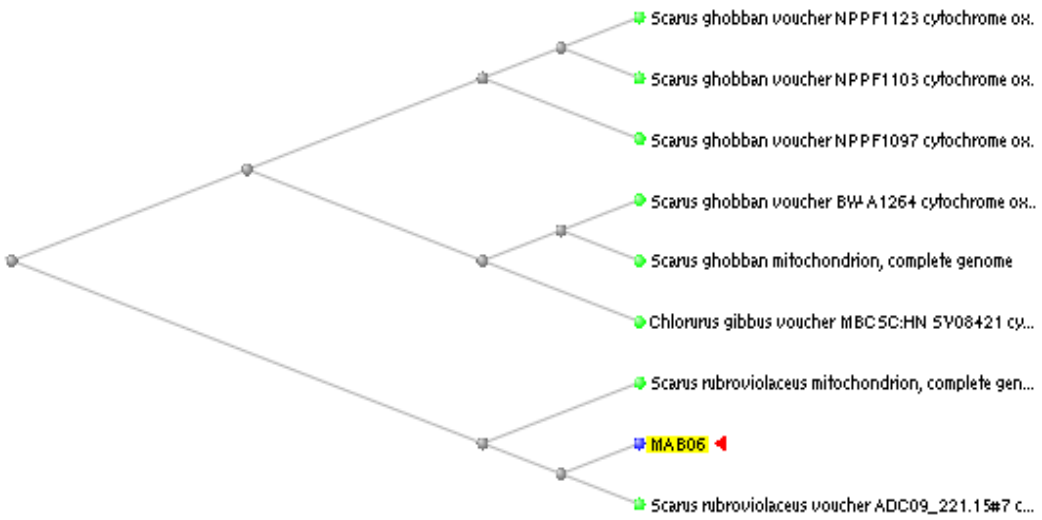


Figure 5 BOLD's search Result

Search Request:

Type : COI SPECIES DATABASE

Search Result:

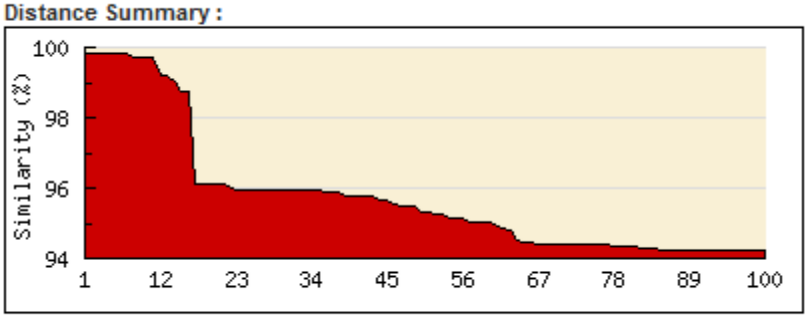
Identification Summary :

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
phylum	Chordata	100
class	Actinopterygii	100
order	Perciformes	100
family	Scaridae	100
genus	Scarus	100
species	Scarus rubroviolaceus	99.8

A species level match has been made. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

Figure 6 Result of the distance summary

Distance Summary :



Similarity scores of the top 100 matches

Figure 7 Results of top 20 specimen similarity with 99.68% to 100%.

TOP 20 Matches :

Phylum	Class	Order	Family	Genus	Species	Specimen Similarity (%)
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.84
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.84
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.84
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.84
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.84
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.84
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.84
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.69
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.69
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.69
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.69
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.22
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.22
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	99.05
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	98.75
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*rubroviolaceus*	98.75
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*ghobban*	96.11
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*ghobban*	96.11
Chordata	Actinopterygii	Perciformes	Scaridae	Scarus	*ghobban*	96.11
Chordata	Actinopterygii	Perciformes	Scaridae	Chlorurus	*gibbus*	96.11

Figure 8 COI SPECIES DATABASE Tree

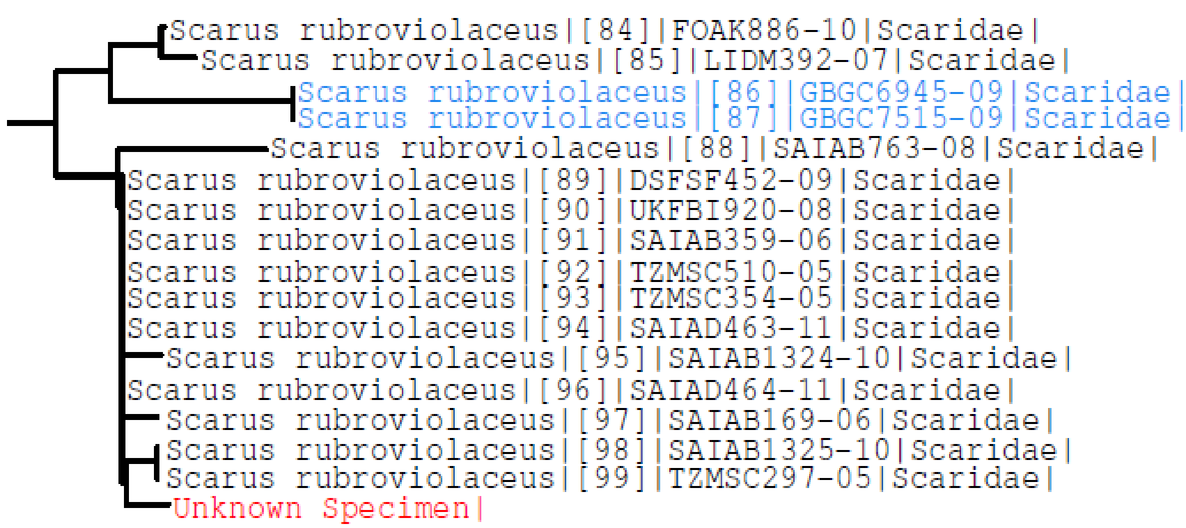
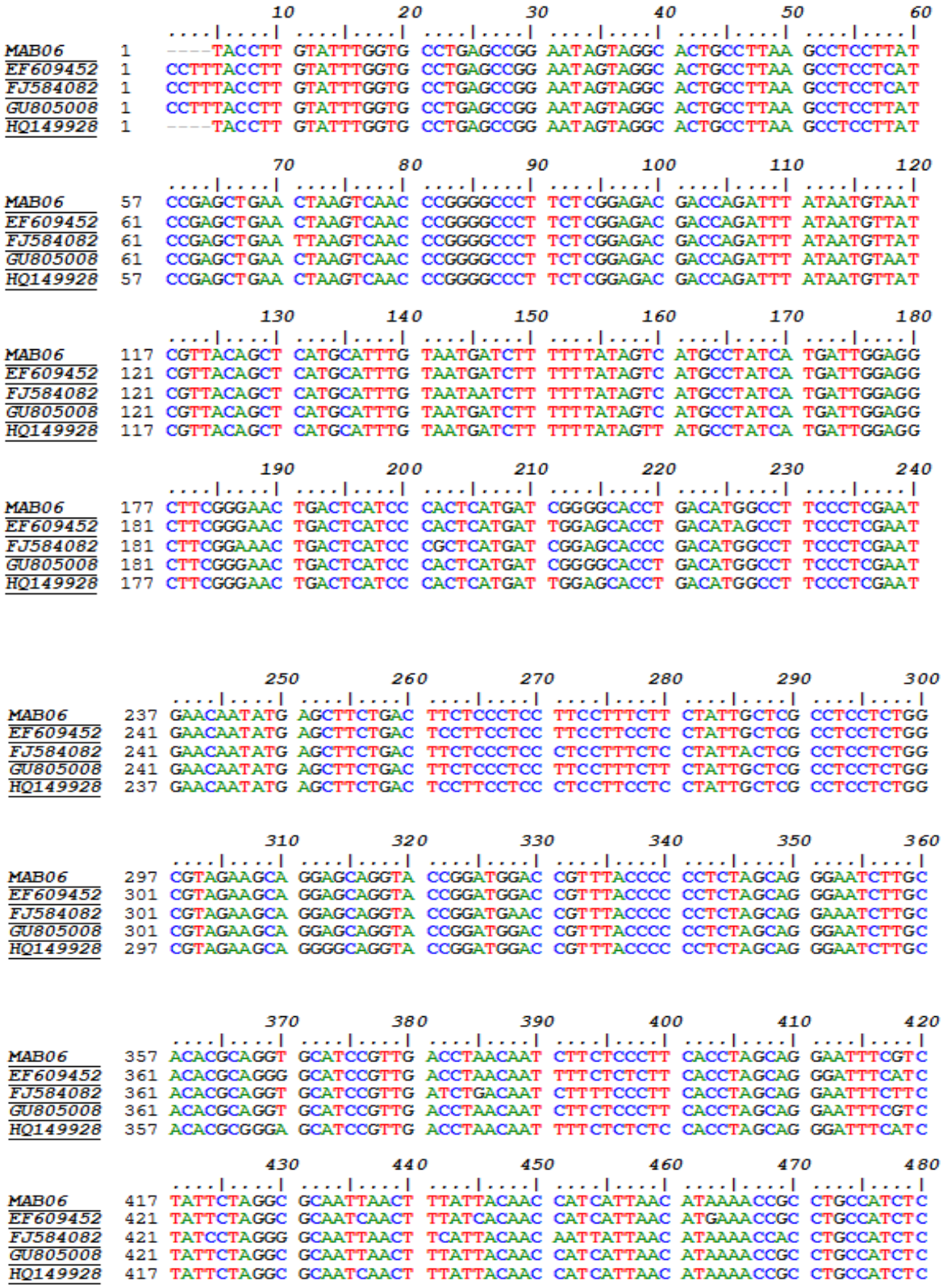


Figure 9 CLUSTAL 2.0.10 Multiple Sequence Alignments



```
                          490         500         510         520         530         540
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
MAB06        477 CCAGTACCAA ACACCCCTCT TCGTATGAGC TGTCTTAATT ACTGCCGTGC TTCTTCTCCT
EF609452     481 CCAATACCAA ACGCCCCTAT TCGTATGAGC TGTTTTAATT ACTGCCGTGC TTCTTCTCCT
FJ584082     481 CCAGTACCAA ACCCCACTGT TCGTATGGGC TGTTTTAATT ACTGCCGTAC TCCTTCTCCT
GU805008     481 CCAGTACCAA ACACCCCTCT TCGTATGAGC TGTCTTAATT ACTGCCGTGC TTCTTCTCCT
HQ149928     477 CCAATACCAA ACGCCCCTGT TCGTATGAGC TGTTTTAATT ACTGCCGTGC TTCTTCTCCT

                          550         560         570         580         590         600
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
MAB06        537 CTCGCTTCCT GTCCTTGCTG CAGGAATCAC AATGCTTCTT ACAGATCGAA ATCTAAACAC
EF609452     541 CTCGCTCCCT GTCCTTGCTG CAGGAATCAC AATGCTTCTC ACAGATCGAA ATCTAAACAC
FJ584082     541 CTCGCTTCCT GTCCTCGCTG CAGGAATTAC AATGCTTCTT ACAGATCGAA ATCTAAACAC
GU805008     541 CTCGCTTCCT GTCCTTGCTG CAGGAATCAC AATGCTTCTT ACAGATCGAA ATCTAAACAC
HQ149928     537 CTCGCTTCCT GTCCTTGCTG CAGGAATCAC AATGCTTCTC ACAGATCGAA ATCTAAACAC

                          610         620         630         640         650
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|
MAB06        597 TACCTTCTTT GACCCTGCAG GCGGAGGAGT CCCAATTCTT TATCAA----- -----
EF609452     601 TACCTTCTTT GACCCTGCAG GCGGAGGAGA CCCGATTCTC TATCAACACC TCTTC
FJ584082     601 TACTTTCTTT GACCCTGCAG GCGGAGGAGA CCCGATTCTT TACCAACACC TG---
GU805008     601 TACCTTCTTT GACCCTGCAG GCGGAGGAGA CCCAATTCTT TATCAACACC TC---
HQ149928     597 TACCTTCTTC GACCCTGCAG GCGGAGGAGA CCCAATTCTC TATCAACACC TC---
```

Figure 10 Amino acid composition of *S. rubroviolaceus* sample MAB06 of Graph showing hypothetical barcode profiling of *S. rubroviolaceus* of Parangipettai waters.

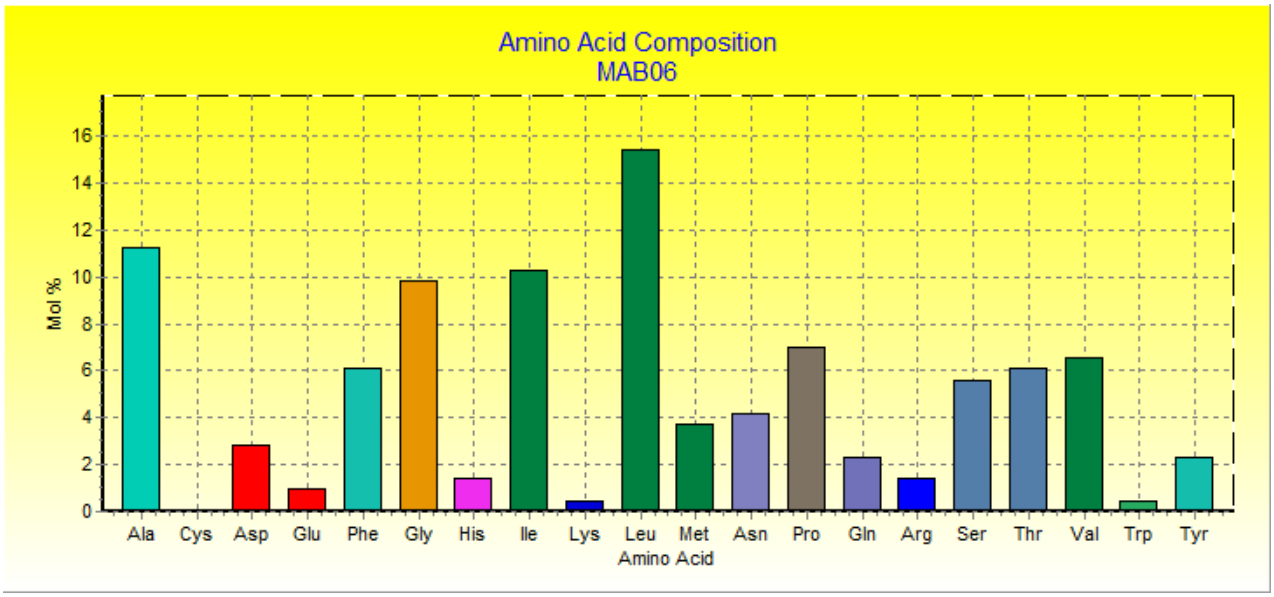


Figure 11 Helical wheel Diagram for Hypothetical Protein of sample MAB06

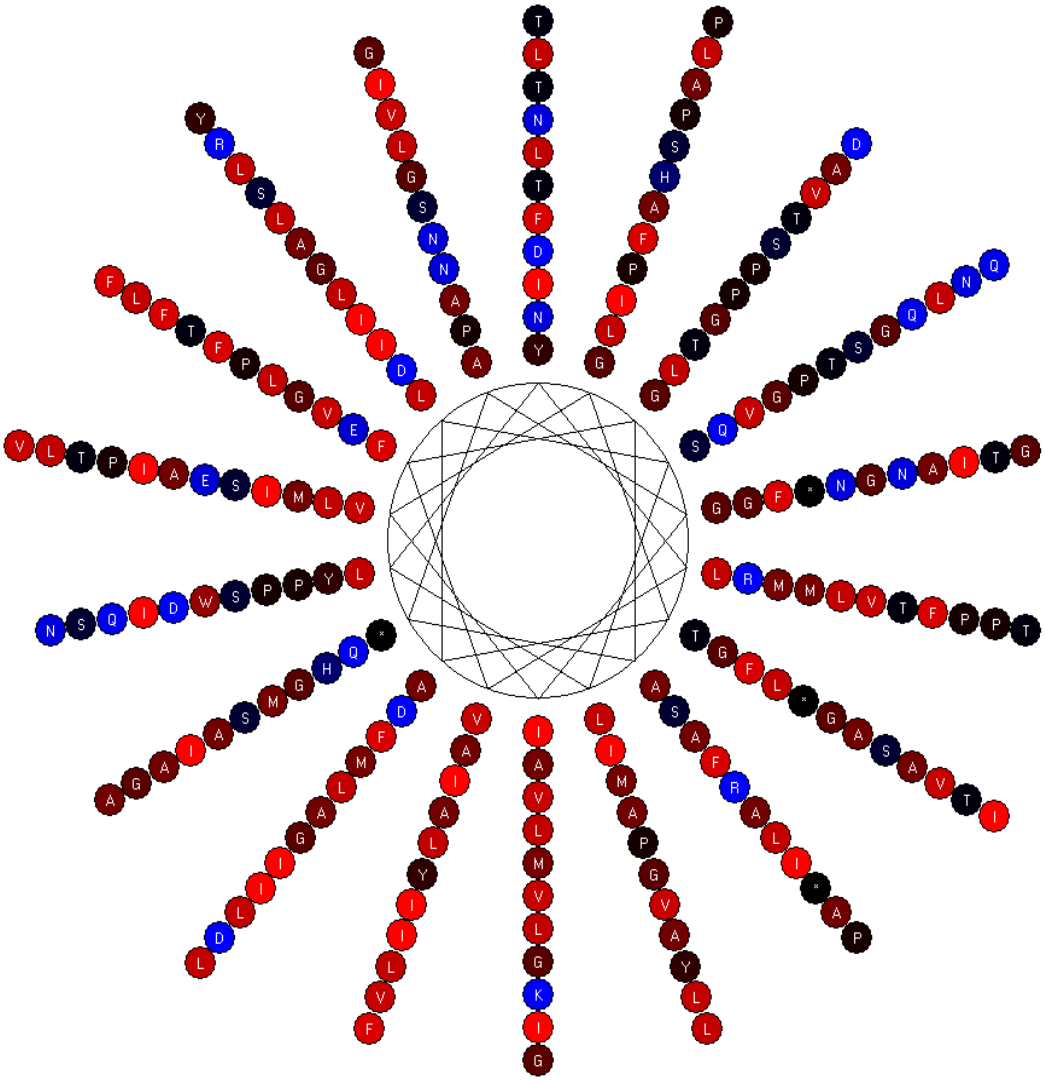


Figure 12 Evolutionary relationships of taxa by UPGMA Method