



Divergent domain D11 of 28S rRNA gene as a molecular marker in the phylogenetic relationship study of some parrotfish species from the Egyptian Red Sea

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ABSTRACT

Parrotfish is a ubiquitous and very varied group connected to coral reef ecosystems. A comparative genetic investigation was achieved using divergent domain D11 of 28S rRNA sequences in six parrotfish (*Scarus ferrugineus*, *Scarus psittacus*, *Scarus frenatus*, *Chlorurus sordidus*, *Scarus ghobban* and *Cetoscarus bicolor*) to manifest a generic visibility about the phylogenetic linkages of those species. The sequenced regions of divergent domain D11 of 28S rRNA in six parrotfish species submitted in the GenBank/NCBI under accession numbers (MW144763 - MW144768). Length of nucleotide sequence, based on divergent domain D11 of 28S rRNA, ranged from 612 bp. to 616 bp. The average nucleotide frequencies of adenine (A), thymine (T), cytosine (C) and guanine (G) were 21.9%, 23.8%, 23.1% and 31.2% respectively. The CG- contents were higher than that of AT- contents in all species. The average content of C+G was 54.3%. Genetic distance among parrotfish species ranged from 0.000% to 0.006%. Overall, the distance value among all fishes was 0.007%. Due to the limited data on phylogenetic inferences in parrotfish based on divergent domain D11 of 28S rRNA, the present study could be helpful in the detection of genetic relationships for parrotfish species using 28S rRNA gene marker.

INTRODUCTION

Numerous vital biological affairs need to be resolved, among which is a necessity to safeguard the genetic biodiversity, maintain species and avert pandemics. It is very important to study and analyze the diversity of species or genera to provide the required information to determine the belonging of species or genus (Dayrat, 2005; Templonuevo *et al.*, 2018).

New molecular genetic techniques have enabled scientists to identify different species or strains. Studies based on ribosomal RNA (rRNA) genes have been used in many animals to examine the evolutionary linkages and description of genome structure

(Verma & Serajuddin, 2012). The 28S ribosomal RNA gene consists of many highly conserved cores, interrupted by divergent domains that evolve rapidly with substitution rates, which are at least two orders of magnitude higher than those of core regions that create a possibility for variations in these fast-evolving divergent domains. These evolving domains are efficient to analyze the phylogenetic linkages between closely related species (Olsen & Woese, 1993; Awasthi *et al.*, 2016).

Parrotfish is a ubiquitous and very varied group connected to coral reef ecosystems. They have the unique ability to modify the benthic structure of coral reefs by either scraping surfaces, digging up the calcareous structure (including live coral (Bellwood & Choat, 1990) or redistributing sediments within habitats (Bellwood, 1995). There are about 10 parrotfish genera comprised approximately 90 species, for instance, genus *Scarus* contained more than half of all species (Parenti & Randall, 2000; Streebman *et al.*, 2002). Parrotfish feed on algae related to the coral reef and those fishes are bioeroders that are useful for healthy reef ecosystem in the tropics (Done, 1992; Williams *et al.*, 2001; Mumby, 2006). Labrid fish represent wide morphological diversity in aspects of cranial morphology and function (Wainwright *et al.*, 2004; Westneat *et al.*, 2005). Parrotfish is considered as an unusual group within the family Labridae and provided with a modified skull, fused jaws for scraping algae off the substrate, in addition to hypertrophied pharyngeal jaws to grind their food (Smith *et al.*, 2008).

The present study was performed to estimate the nucleotide differences in the divergent domain D11 of 28S rRNA and construct the phylogenetic linkages among six parrotfish species from the Red Sea in Egypt.

MATERIALS AND METHODS

Samples collection

Six parrotfish species (*Scarus ferrugineus*, *Scarus psittacus*, *Scarus frenatus*, *Chlorurus sordidus*, *Scarus ghobban* and *Cetoscarus bicolor*) were collected from the Red Sea, Hurghada, Egypt. The morphological identification of all samples was according to Randall (1982). We separated muscle tissues from each sample and stored at -20 °C until further usage.

DNA Extraction and PCR amplification

Total genomic DNA was extracted from the preserved muscle tissues using the DNA extraction method of QIAamp DNA Mini kit (Qiagen, Hidden, Germany) and following the manufacturer's guidelines.

The divergent domain (D11 of 28S rRNA) was amplified in Six parrotfish species (*S. ferrugineus*, *S. psittacus*, *S. frenatus*, *Chlorurus sordidus*, *S. ghobban* and *Cetoscarus bicolor*) using primers following the methods of **Zardoya and Meyer (1996)**, **Verma et al. (2011)** and **Awasthi et al. (2016)**. The PCR reactions comprised of 10 pmoles of each forward and reverse primer, 25 µL PCR master mixed with 100 ng of genomic DNA and in a final reaction volume of 50 µL. The PCR cycling conditions were performed with an initial denaturation for 3 min at 94 °C, followed by 30 cycles of denaturation for 30s at 94°C, annealing for 30s at 50°C, and an extension at 72 °C for 60 sec with post cycling extension at 72 °C for 5 min. The PCR products were separated on 1.5% agarose gel with ethidium bromide (0.5µg/ml).

The Sequencing of PCR Products and phylogenetic tree construction

All DNA sequencing was performed by Macrogen (South Korea). The sequenced regions of the divergent domain D11 of 28S rRNA were submitted to GenBank/NCBI to obtain accession numbers (Table 1). Sequence alignment was performed using MUSCLE (**Edgar, 2004**) with default settings.

Molecular evolutionary genetic analysis (MEGA) version 7.0 18 (**Kumar et al., 2016**) was applied to perform the phylogenetic trees analyses using three phylogenetic methods of trees construction; Maximum Likelihood (ML), Neighbour Joining (NJ) and Minimum Evolution (ME) and using 1000 bootstrap iterations (**Felsenstein, 1985**). Calculation of sequence divergences was occurred by utilizing Kimura two-parameter distances.

RESULTS

The present study was performed to estimate the phylogenetic relationship among six parrotfish species (*S. ferrugineus*, *S. psittacus*, *S. frenatus*, *Chlorurus sordidus*, *S.*

ghobban and *Cetoscarus bicolor*) from the Red Sea in Egypt using the divergent domain D11 of 28S rRNA.

The sequenced regions were submitted to the GenBank under accession numbers (MW144763-MW144768). The divergent domain D11 of 28S rRNA was rooted with the outgroup sequences from *Iracundus signifer* (GenBank accession number AY539086.1), *Pontinus longispinis* (GenBank accession number AY539087.1) and *Scorpaena brasiliensis* (GenBank accession number AY539088.1) (Table 1).

Table 1 The understudying parrotfish species with outgroup from the GenBank/ NCBI based on divergent domain D11 of 28S rRNA gene sequence.

No.	Species	Accession number
1	<i>S. ferrugineus</i>	MW144763
2	<i>S. psittacus</i>	MW144764
3	<i>S. frenatus</i>	MW144765
4	<i>Chlorurus sordidus</i>	MW144766
5	<i>S. ghobban</i>	MW144767
6	<i>Cetoscarus bicolor</i>	MW144768
Out group	<i>Iracundus signifer</i>	AY539086.1
	<i>Pontinus longispinis</i>	AY539087.1
	<i>Scorpaena brasiliensis</i>	AY539088.1

The length of nucleotide sequence, based on divergent domain D11 of 28S rRNA in six parrotfish species, ranged from 612 bp to 616 bp. The results revealed that *S. ferrugineus* has the longest nucleotide sequences (616 bp.), while *Cetoscarus bicolor* have the shortest nucleotide sequences (612 bp). The average nucleotide frequencies of adenine (A), thymine (T), cytosine (C) and guanine (G) were 21.9%, 23.8%, 23.1% and 31.2% respectively. The base pair length, nucleotide frequencies%, the average content of A+T and C+G were summarized in Table (2).

Table 2 Accession numbers, Nucleotide frequencies%, A+T contents%, C+G contents% and their averages of divergent domain D11 of 28S rRNA sequence in six parrotfish species.

No.	Species	Accession number	Base pair length	Nucleotide frequencies %				A+T Content (%)	C+G Content (%)
				A	T	C	G		
1	<i>Scarus ferrugineus</i>	MW144763	616	21.8	23.7	23.3	31.2	45.5	54.5
2	<i>Scarus psittacus</i>	MW144764	614	21.8	23.9	23.2	31.1	45.7	54.3
3	<i>Scarus frenatus</i>	MW144765	615	22.0	23.7	23.1	31.2	45.7	54.3
4	<i>Chlorurus sordidus</i>	MW144766	614	21.8	23.8	23.1	31.3	45.6	54.4
5	<i>Scarus ghobban</i>	MW144767	615	22.0	23.7	23.2	31.1	45.7	54.3
6	<i>Cetoscarus bicolor</i>	MW144768	612	21.9	23.9	23.0	31.2	45.8	54.2
	Average %	-	614	21.9	23.8	23.1	31.2	45.7	54.3

Pairwise genetic distances were estimated by MEGA software version 7.0 18 (Kumar *et al.*, 2016) using the Kimura 2- parameter method with gamma correction. Overall, the distance value among all fishes was 0.007%. The P-distances among all samples ranged from 0.000% to 0.006% (Table 3). Among understudied parrotfish, the highest P-distance (0.004) was found between *S. psittacus* and *S. ghobban*. While the lowest value (0.000) was found between *S. ferrugineus* and *S. frenatus*, likewise between *Chlorurus sordidus* and *Cetoscarus bicolor* (Table 3). The final alignments consisted of 617 bp, among which 612 were conserved sites (Fig. 1).

Table 3 Pairwise distances based on divergent domain D11 of 28S rRNA in six parrotfish species in addition to outgroup using Kimura 2- parameter.

No.	Species	1	2	3	4	5	6	7	8	9
1	<i>Scarus ferrugineus</i>		0.002	0.000	0.002	0.003	0.002	0.004	0.005	0.005
2	<i>Scarus psittacus</i>	0.002		0.002	0.003	0.004	0.003	0.005	0.006	0.006
3	<i>Scarus frenatus</i>	0.000	0.002		0.002	0.003	0.002	0.004	0.005	0.005
4	<i>Chlorurus sordidus</i>	0.002	0.005	0.002		0.002	0.000	0.004	0.004	0.004
5	<i>Scarus ghobban</i>	0.005	0.007	0.005	0.002		0.002	0.004	0.005	0.005
6	<i>Cetoscarus bicolor</i>	0.002	0.005	0.002	0.000	0.002		0.004	0.004	0.004
7	<i>Scorpaena brasiliensis</i>	0.009	0.012	0.009	0.007	0.009	0.007		0.002	0.002
8	<i>Pontinus longispinis</i>	0.012	0.014	0.012	0.009	0.012	0.009	0.002		0.000
9	<i>Iracundus signifer</i>	0.012	0.014	0.012	0.009	0.012	0.009	0.002	0.000	

To estimate the phylogenetic linkage among six parrotfish species by using the sequences of divergent domain D11 of 28S rRNA, we used three phylogenetic methods: Maximum Likelihood (ML), Neighbour Joining (NJ) and Minimum Evolution (ME). The divergent domain D11 of 28S rRNA was rooted with outgroup sequences from *Iracundus signifer* (GenBank accession number AY539086.1), *Pontinus longispinis* (GenBank accession number AY539087.1) and *Scorpaena brasiliensis* (GenBank accession number AY539088.1) (Table 1).

All the methods showed the same relations among parrotfish with some differences in support values and revealed 5 main features: (1) species of the outgroup formed a separate cluster, (2) *S. psittacus* and *S. frenatus* found as a sister clad, (3) each of *S. ferrugineus*, *Chlorurus sordidus*, *S. ghobban* and *Cetoscarus bicolor* formed a separate cluster, (4) *Chlorurus sordidus* found between *S. ferrugineus* and *S. ghobban*, and (5) *Cetoscarus bicolor* formed a basal clade with the rest of understudied species (Figs. 2-4).

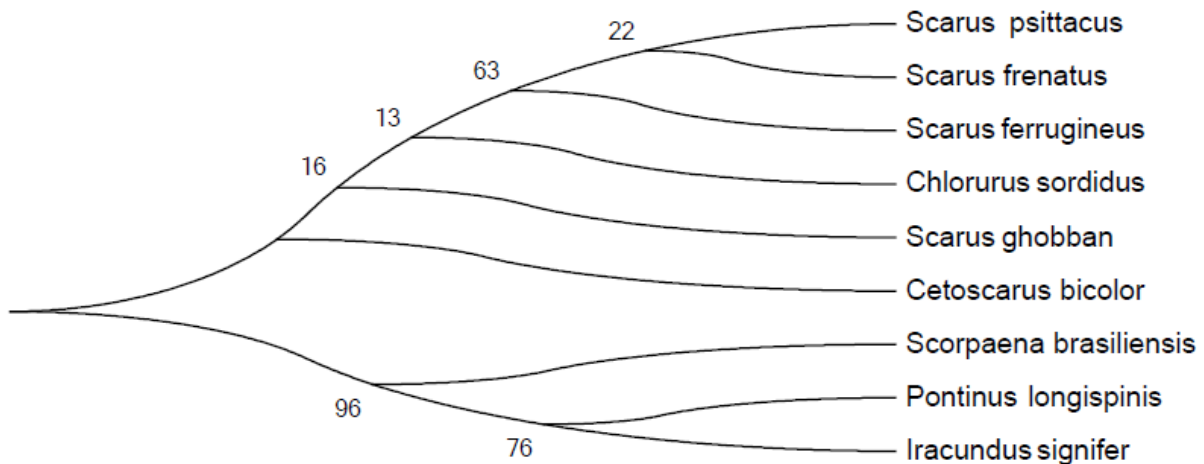


Fig.2. Phylogenetic tree using the Maximum likelihood method among understudying parrotfish species in addition to outgroup species based on divergent domain D11 of 28S rRNA gene sequences

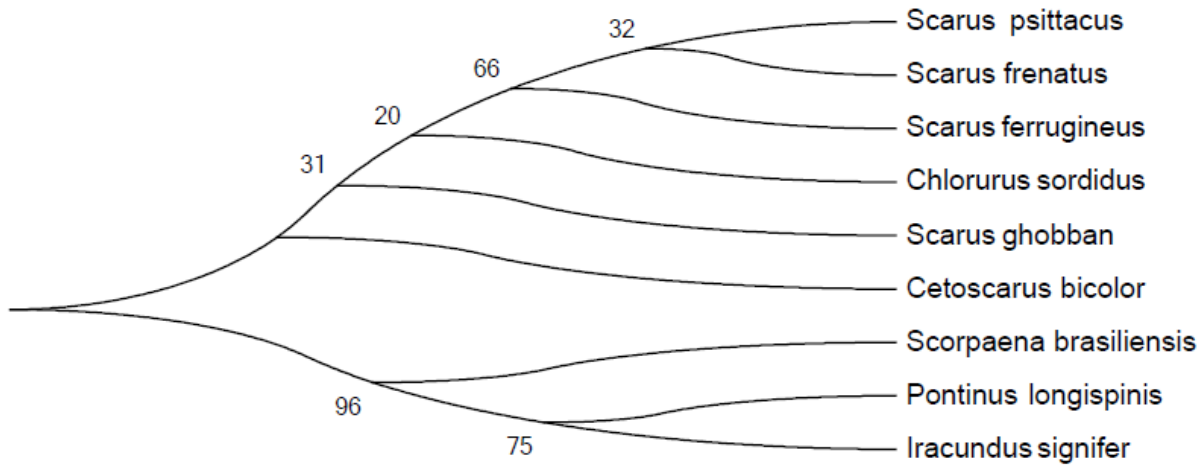


Fig.3. Phylogenetic tree using the Neighbour Joining method among understudying parrotfish species in addition to outgroup species based on divergent domain D11 of 28S rRNA gene sequences

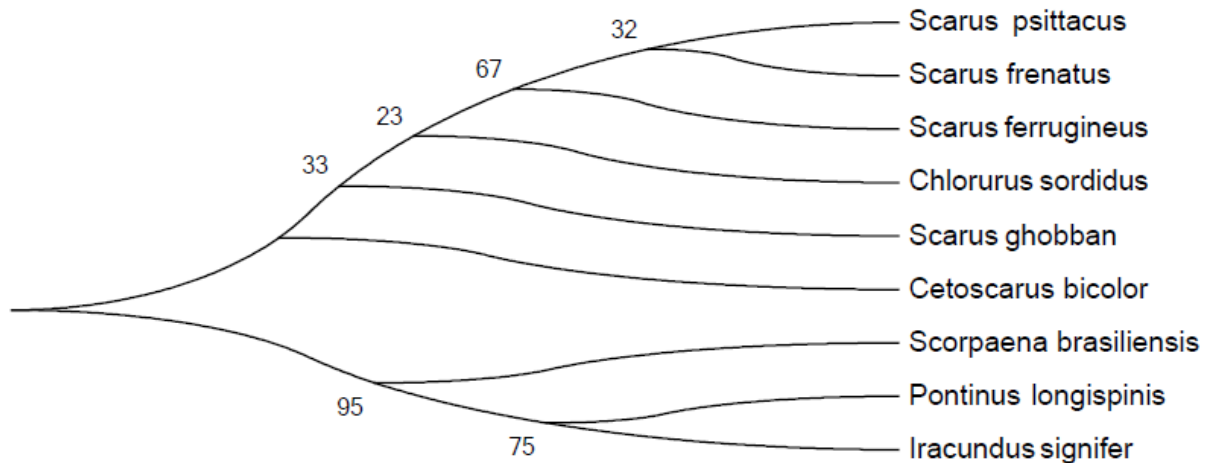


Fig.4. Phylogenetic tree using the Minimum Evolution method among understudying parrotfish species in addition to outgroup species based on divergent domain D11 of 28S rRNA gene sequences

DISCUSSION

Divergent domains are continuously employed in a large scale to analyze linkages among species using a phylogenetic structure method in several organisms (Vidigal *et al.*, 2000, 2004; He *et al.*, 2005; Verma & Serajuddin, 2012). According to Torres and

Santos (2020) the identification of species is a very important step in the observation and monitoring biodiversity.

Identification and evolution of parrotfish species using genetic studies and analysis are not fully maximized; therefore, it is very important to investigate the genetic diversity and structure of parrotfish because of their economic genetic resources by using an easy, highly polymorphic and effective molecular technique. Moreover, assessment of the genetic variation based on molecular markers should supply the necessary information to help in the kindly management of parrotfish wild stocks, where the careless management of these fishes in the Red Sea will cause loss of these important fishes (**Saad *et al.*, 2012; Saad *et al.*, 2013 a,b**). Parrotfish represent a very varied and ubiquitous group common to coral reef ecosystems, as well as it was considered as one of the most prevalent groups of non-cryptic fishes throughout coral reefs worldwide (**Johnson *et al.*, 2019**).

In the present work, the sequencing of divergent domain D11 of 28S rRNA in six parrotfish species revealed nucleotide length ranging from 612 bp to 616 bp. This concurs with the findings of **Awasthi *et al.* (2016)** who proclaimed that the nucleotide sequences of divergent domain D11 in four Gouramis fishes ranged from 585 to 613 bp. The C+G content was higher than that of A+T content in all species. The average content of C+G was 54.3%. This coincides with **Zardoya and Meyer (1996)**, **Verma *et al.* (2011)** and **Awasthi *et al.* (2016)** who detected a high G+C content in D11 domain of fish. The final alignments consisted of 617 bp, out of which 612 were conserved sites. The high conserved sites of divergent domain D11 of 28S rRNA in the understudied fish are in agreement with the results of **Jansen *et al.* (2006)** who reported that, within a genome, the genes coding for 18S, 5.8S and 28S rRNA are highly conserved due to concerted evolution of intra- and inter-chromosomal loci.

Phylogenetic analysis showed that *Chlorurus sordidus* formed a separate clade found between *S. ferrugineus* and *S. ghobban*, which expressed closely genetic relation between *Chlorurus sordidus* and genus *Scarus*. This result agrees with the molecular analysis of **Streelman *et al.* (2002)**. Likewise, **Smith *et al.* (2008)** supported the same result and concluded that the genera *Chlorurus* and *Scarus* were found to be monophyletic assister group. Furthermore, **Saad *et al.* (2019a)** indicated closed

relationship between *Chlorurus* and *Scarus*. All phylogenetic methods; Maximum Likelihood, Neighbour Joining and Minimum Evolution revealed that *Cetoscarus bicolor* formed a basal clade with the rest of understudied species, this correlates with the results of **Streelman *et al.* (2002)** and **Smith *et al.* (2008)**, who found that *Cetoscarus bicolor* formed a basal clade. The present findings showed that *S. psittacus* and *S. frenatus* found as a sister clade. This is supported by **Smith *et al.* (2008)** who reported that *S. psittacus* is the sister to the rest of the *Scarus* clade in their trees.

Developing of molecular markers through an accurate identification system would provide a reliable solution for removing the collapses in fish species identification and evolution. The molecular identification system should be potentially supported by an understanding of evolution through accurate phylogenetic relationships among fish species (**Saad *et al.*, 2019b**).

The 28S rRNA domains have been used as successful genetic markers for estimating the phylogenetic relationship both at lower and higher taxonomic levels (**Al-Banna *et al.*, 1997, 2004; Duncan *et al.*, 1999; Subbotin *et al.*, 2005, 2007, 2008; Vovlas *et al.*, 2008**). **Zardoya and Meyer (1996)** used 28S rRNA genes to determine the relationships of lungfishes, coelacanth, rainbow trout, eel, sturgeon and tetrapods and declared the divergent domains of 28S rRNA genes are beneficial in phylogenetic studies (**Awasthi *et al.*, 2016**).

Few investigations have been interested in studying the level of genetic variations of parrotfish and mainly focused on their taxonomy and evolution (**Almeida *et al.*, 2017**). Parrotfish identification is knotted because of color changes that occur during development. Until now, the genetic diversity and evolution of parrotfish in the Red Sea area have been examined at little scale. The phylogenetic linkages among parrotfish are ancient troubles in ichthyology that require wide genetic analysis to be resolved (**Saad *et al.*, 2019a**). The characterization of parrotfish species in the Red Sea was investigated with few studies due to the difficulties in morphological identification, thus, the use of molecular barcoding is significant. These genetic markers provide an effective and a reliable tool in parrotfish identification (**Hassan *et al.*, 2020**).

CONCLUSION

This study was established to assay the phylogenetic relationships among six parrotfish fishes by using the sequences of divergent domain D11 of 28S rRNA gene sequence. Phylogenetic analysis among six parrotfish based on divergent domain D11 of 28S rRNA revealed a close genetic distance between *Chlorurus* and *Scarus*. The presented study could be salutary in assessing the genetic variation among parrotfish, and would allow an accurate taxonomy to species level, revealing the potential of D11 of 28S rRNA gene as a genetic marker in the DNA barcoding among fishes, especially parrotfish.

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