



# Examination of Genetic Diversity of Spinner Dolphin (*Stenella Longirostris*) using *cox 1* at the Southern Egyptian Coast of the Red Sea

Mohamed E. Ismail<sup>1</sup>, Mohamed I. Ahmed<sup>2</sup>, Mahmoud H. Hanafy<sup>3</sup>, Fedekar F. Madkour<sup>4</sup>

Department of Marine Science

Faculty of Science, Port Said University, Port Said, Egypt<sup>1,4</sup>

Faculty of Science, Suez Canal University, Ismailia, Egypt<sup>2,3</sup>

## Abstract:

Spinner dolphins *Stenella longirostris* is one of the most important and attractive animals in the southern Egyptian coast of the Red Sea. The knowledge about their genetic diversity in the Egyptian coast is very poor. The current study is focusing on the molecular characterization of *S. longirostris* Egyptian Red Sea which found near their resting area. Samples were collected from a free ranging dolphin during the period between February and April 2017. The collection process was by using biopsy pole system. The samples were analyzed using *cox1* gene to identify their genetic diversity. Aligning samples collected from Egypt with samples of the same species obtained from GeneBank showed a clear divergent between samples in the same species. The analysis indicating the possibility of the population being reproductively isolated from surrounding spinner dolphin population.

## I. INTRODUCTION

Molecular techniques are important indirect approach to study the evolution and population genetics of the marine mammals all over the world which display a fine scale population structure while the extent varies among species (Hoelzel *et al.*, 2002). Recently, molecular markers are useful tool for marine mammals research while they considered a vital tool for research on behavior, ecology and evolution (Zanardo *et al.*, 2016). Molecular genetics characterization is a necessary approach for understanding the population structure of baleen whales (Baker *et al.*, 1994; Bérubé *et al.*, 1998), polar bears (Paetkau *et al.*, 1999), killer whale (Hoelzel and Dover, 1991; Baker *et al.*, 1994; Pilot *et al.*, 2010), harbour porpoise (Rosel *et al.*, 1999; Fontaine *et al.*, 2007), harbor seals (Westlake and O'Corry-Crowe, 2002), dolphins (Natoli *et al.*, 2004; Gowans *et al.*, 2007). *Stenella* is a polytypic genus that considered one of the highly mobile and social marine species and divided into 5 species (Gray, 1866). Three of these species located in the Red Sea. *S. attenuate* is one of the most common dolphin in the Red Sea (Frazier *et al.*, 1987), however, *S. coeruleoalba* has often been retained as being possibly present in the Red Sea (Baldwin *et al.*, 1998, Jefferson *et al.*, 2011). *S. longirostris* have been long known to be frequent in the Red Sea (Leatherwood, 1986). Spinner dolphins *S. longirostris* is a species of small cetaceans which found in the tropical areas overall the world (Perrin, 1998). There are four geographic forms of *S. longirostris*: Costa Rican, northern white belly, southern white belly and eastern (Perrin, 1975). Perrin (1990) hypothesized that the white belly spinner populations comprise a broad zone of hybridization between the eastern form and the spinner dolphins of the Central and South Pacific. Eastern *S. longirostris* form differ morphologically from *S. longirostris* in the rest of the world's oceans: small body and skull size, dark overall coloration obscuring the typical spinner color pattern, and marked sexual dimorphism in shape of body and dorsal fin (Perrin, 1990); breeding season (Barlow, 1984) and also in the ovulation rate, pregnancy rate, age at maturation and testis size (Perrin and Henderson, 1979). No data is

available on the genetic diversity of the Egyptian *S. longirostris* with the other *S. longirostris* from the different areas. Consequently, the current study is focusing on the molecular characterization of *S. longirostris* Egyptian Red Sea which found near their resting area.

## II. MATERIAL AND METHODS

### 2.1. Sample collection

Samples were collected from free ranging Spinner dolphins, *Stenella longirostris*, at their resting areas, Satayah reef, in the southern Egyptian coast Red Sea during the period between February and April 2017. The collection was by using biopsy pole system following the recommendations of Bilgmann *et al.* (2007).

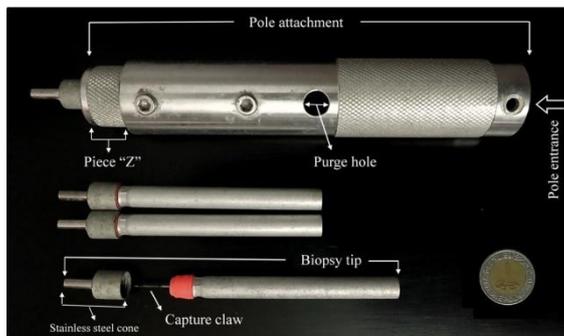
#### 2.1.1. Sampling unit

The used pole is made of two extendable stainless steel sticks with adjusted length range of 1.5-2.5 m. Pole attachment is a piece of stainless steel which used to connect the biopsy tips to the pole. Biopsy tip is inserted in the X opening of the pole attachment and then turned the piece Z (270°) firmly to fix the biopsy tip. Water that flows into the hollow pole throughout the darting purges via a hole called (purge hole) (Fig 1). Biopsy tips have been bought from Pseudart company (product ID: Biopsy RDD Device) (<http://www.pseudart.com>) with a special criteria to fit the targeted dolphin. Biopsy tips are a stainless steel body which designed to remove a sample consisting of both the dermis and hypodermis material attached to the aluminum nose cone that is stainless steel cutter and encompasses a barbed "capture claw" to ensure sample retention (Figure 1).

#### 2.1.2. Sampling process

Collection process was carried out when the dolphins had left the reef and went in the open water to feed. A 6 m inflatable speed boat with twin 115 horse power outboard engine was used for the collection process. Bow-riding dolphins were sampled when they were close to the water surface. Sampling

wasn't attempted on calves, pregnant female or mothers (adult females). The pole was held at an angle of 40° to 60° to the water surface, the speed of the boat was 1-3 knot. Depending on the depth of the dolphin and the speed of the boat, the biopsy pole was either dropped or thrown slightly toward the animal, aiming the lateral side to the base of the dorsal fin. After a successful sampling attempt, the sampler observed the targeted dolphin for the presence of biopsy marks in order to void repeated sampling of the same individuals. Natural marks especially fin notches were also used to prevent resampling. Biopsy samples consisted of cylindrical plugs of skin and blubber about 5 mm in diameter and 5 mm long.



**Figure.1. Components of the pole attachment including biopsy tips.**

Behavioral response of the targeted dolphin was recorded whatever the sampling was successful or unsuccessful. This behavior was documented by following each targeted dolphin for five minutes after the shot was taken. Behavioral responses were characterized following a protocol of Krützen *et al.* (2002) with some modification. Response categories were as follows: (0) no visible reaction, dolphin continued with the previous behavioral, (1) flinch, dolphin moved away but stayed close to the boat, (2) dolphin accelerate and then left the bow, (3) single leap and/or porpoise then dive and (4) individual accelerate then leaves the bow followed by multiple leaps and/or porpoises.

## 2.2. Sample preservation and preparation

All samples were directly preserved in 95% ethyl alcohol in a sterilized labeled 1.5ml Eppendorf tube and then stored at -20 °C. Later, samples were prepared for the DNA extraction step by removing from ethyl alcohol and cut it to small pieces using sterilized disposable surgical blades.

### 2.2.1. DNA extraction

DNA was extracted from tissue samples preserved in 95 % ethanol by using a standard phenol\ chloroform extraction method following Hoelzel (1998) with some modification. Each small slice of Dolphin's tissue was homogenized in 600 µL of 2x CTAB extraction buffer [1.4M NaCl, 0.02M EDTA (pH=7.8), 0.1M Tris-HCl (pH=8.0), 2% CTAB (w/v), and 0.2% β-mercaptoethanol (v/v)] and 10 µL of proteinase K (20 mg/ml). After that the homogenate was incubated at 65°C for 2 hours with frequent shaking each 15 min and in some cases, the homogenate was left in the water path over the night to ensure the digestion of the sample tissue. After incubation, 600 µL of chloroform isoamyl alcohol [24:1 (v/v)] was added to each sample and homogenized using vortex for 3-5 minutes and then the sample was centrifuged for 15 min at 13,000 rpm. After centrifugation the top aqueous layer was transferred to a new labeled 1.5ml Eppendorf tube.

The gDNA in the aqueous layer was further extracted using a 600 µl of phenol chloroform isoamyl alcohol (25:24:1; v/v).

After centrifugation at 13,000 rpm for 15 min, the aqueous layer was transferred to another new labeled Eppendorf tube containing 600 µl chloroform is amyl alcohol and centrifuged at 13,000 rpm for 15 min. Finally, the aqueous layer at this step was transferred to a new labelled Eppendorf tube containing 1 ml of 95% ethanol and incubated overnight at -20°C. The day after, the samples were centrifuged at 13,000 rpm for 30 min. At this step, the ethanol was decanted, while the DNA pellet was kept and rinsed twice with 0.5 ml 70% ethanol to remove phenol and other reagents. Each time the samples were centrifuged at 13,000 rpm for 5 min. Finally, the DNA pellet was dried at room temperature then dissolved in 100 µl double distilled sterilized water and stored at -20°C in the deep freezer.

### 2.2.2. DNA quantity and quality evaluation

Concentrations of the gDNAs (ng/µL) and also the purity (values of 260/280 and 260/ 230 ratios) which have been extracted from the samples were determined spectrophotometrically by using NanoDrop (ND-1000) after calibration with sterilized double distilled water as a blank. DNA integrity was assessed by using gel electrophoresis techniques (*see section 4.2.6*)

### 2.2.3. Polymerase chain reaction (PCR)

The mitochondrial DNA *cox 1* was amplified either with universal primers FishF1(5'- TCAACC AACCACAAA GACATTGGCAC) and FishR1 (5'- TAGACTTCT GGGTG GCCAAAGAATCA) after Lakra *et al.*, 2011. The PCR reaction conditions were followed as: 100 µM dNTPs, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris- HCl (pH=8.4), 50 mM KCl, 200 nM of each forward and reverse primer, 0.02 U/ µL *Taq* polymerase and approximately 20-40 ng/ µL of gDNA. The PCR cycling profile was started at 95°C for 2 min followed by 35 cycles of 94°C for 30 seconds, 1 min at 54 °C and 10 min at 72°C, lastly, 8 min at 72°C and hold at 4°C.

### 2.2.4. Gel electrophoresis

gDNA integrity and PCR products were visualized on 1.5% (w/v) agarose gel by dissolving 1.125g of agarose in 75 ml of 1x sodium borate buffer (SB). The working 1x SB buffer was prepared by diluting 20x stock solution [0.20M NaOH and 0.76M H<sub>3</sub>BO<sub>3</sub>; pH=8.0]. For gel staining, ethidium bromide (0.1 µg/ml) was added to 1x SB buffer. To dissolve agarose, 10-15 minutes of heating with continuous swirling were applied until there is no agarose lenses present in the working buffer. After solidification in 15x10 cm tray of SafeBlue Electrophoresis System (01D), gel was covered with 1x SB buffer and the samples to be analysed were loaded with 1x loading-EZ dye. Samples were allowed to run along with 3 kb molecular ladders. Loaded samples were left to run under 150v and 95 mA for 60 min. bands had been visualized and photographed in UV photo-documentation unit. Sizes of PCR products were determined using Gel-Pro Analyzer V4 software.

### 2.2.5. mtDNA *cox 1* sequencing

Sequencing of mtDNA *cox 1* for all samples were done in Biotechnology Research Centre at Suez Canal University using forward primer only. For sequence editing, chromatogram of each sample was revised using MEGA version 6 software. After editing, Basic Local Alignment Search Tool (BLAST) search was used to verify existing similarities with deposited sequences in the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Corresponding genome of spinner

dolphin's sequences from NCBI GenBank was downloaded in FASTA format.

### 2.2.6. Phylogenetic analysis

For construction of phylogenetic trees, mtDNA *cox 1* sequences in the current study and that downloaded from NCBI GenBank were initially aligned together using ClustalW. A best-fit substitution model was selected according to lowest Bayesian Information Criterion (BIC) for Maximum Likelihood method (Kimura, 1980). Reconstruction of phylogenetic trees was based on 1000 bootstrap replication value. All phylogenetic analyses were conducted using MEGA v6.0 (Tamura *et al.*, 2013).

## III. RESULTS

### 3.1. DNA quality and quantity

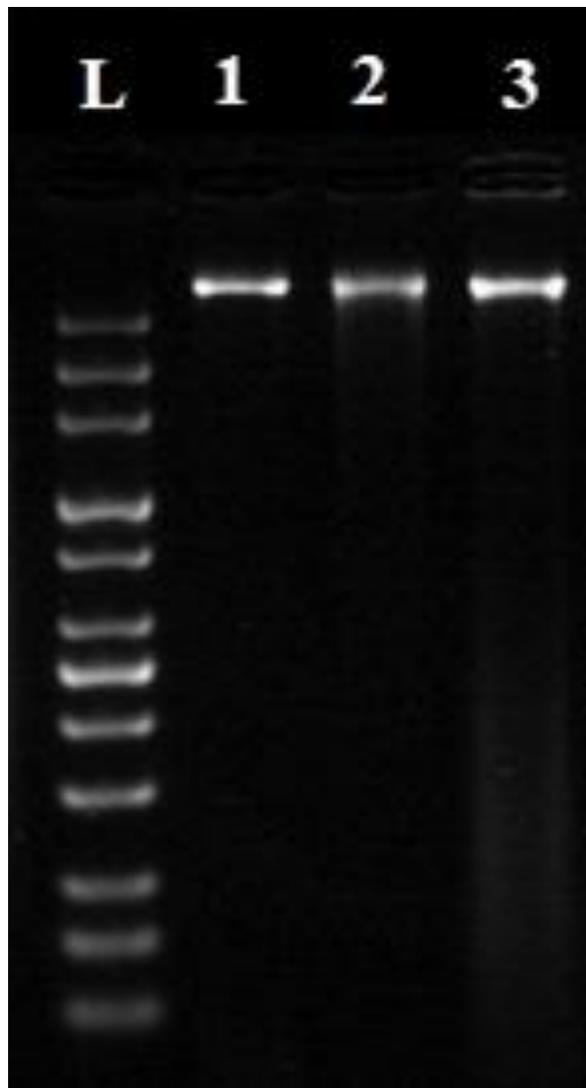
DNA samples from 17 samples of *S.longirostris* at the southern Egyptian coast of the Red Sea, Satayah, were primarily assessed based on both agarose gel electrophoresis and NanoDrop readings. First, agarose gel electrophoresis indicated high integrity of DNA extracted from skin tissue preserved in absolute ethanol using phenol-chloroform extraction methodology. However, electrophoresis analysis displayed different concentrations of DNA. About 70.5 % of the extracted samples were higher than 100 ng/ $\mu$ L as indicated by NanoDrop readings (Table 1). Nevertheless, according to concentration and purity parameters of DNA, only 5 sequences were successfully worked PCR analysis.

**Table.1. NanoDrop readings of DNA concentration and purity extracted from skin tissue of *S. longirostris*.**

Sample number	concentration	Purity
01 X	42.3	2.01
02 X	36.6	1.47
03 X	172.6	1.94
04 X	190.0	2.15
05 X	220.4	1.94
06 X	1.2	0.21
07 X	275.4	2.12
08 X	136.8	2.09
09 X	25.8	5.2
10 X	164.0	2.11
11 X	342.4	2.01
12 X	161.5	2.1
13 X	324.7	2.04
14 X	267.1	2.04
15 X	52.7	2.55
16 X	141.7	2.13
17 X	285.0	2.06

### 3.2. COI molecular identification

For molecular characterization of *S. longirostris* samples (n=3), *cox 1* gene was successfully amplified with molecular size of about 700 bp (Figure 2). These PCR amplicons, however, were eventually sequenced to produce COI gene sequences. On the other hand, edited sequences of COI gene were archived at NCBI GenBank (<https://www.ncbi.nlm.nih.gov/>).



**Figure.2. 1.5% agarose gel electrophoresis of gDNA after had been successfully amplified by PCR**

### 3.3. Phylogenetic relationships

17 samples have been collected from the populations of spinner dolphins *S.longirostris* in their resting area Satayah reef. The samples were analyzed using *cox1* gene to identify the genetic diversity in Satayah reef. Aligning samples with samples obtained from Genebank showed a clear divergence between *Stenella longirostris* and the other marine mammals (Figure 3). On the other hand, COI sequences from Red Sea samples (current study) showed distinct phylogenetic divergence when compared with NCBI GenBank COI data. The neighbor joining phylogenetic analysis also showed that Eastern Tropical Pacific (ETP) population is significantly subdivided into two subgroups. In contrast, the North Western Atlantic (NWA) and Gulf of Mexico (GOM) populations were significantly divergent (Figure. 4).

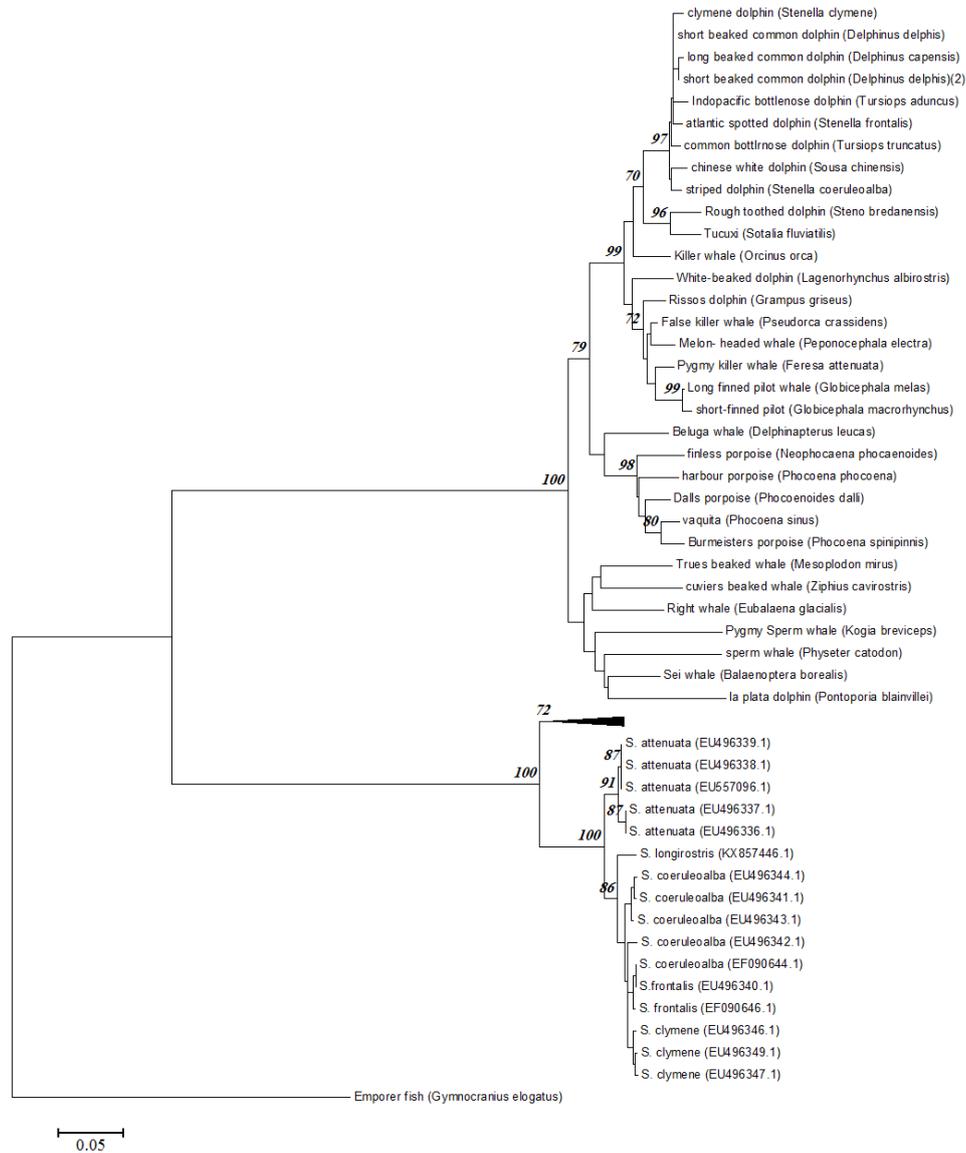


Figure.3. Phylogenetic tree of COI sequences of *S. longirostris* and other marine mammal's species based on Neighbour joining method with bootstrap value of 1000.

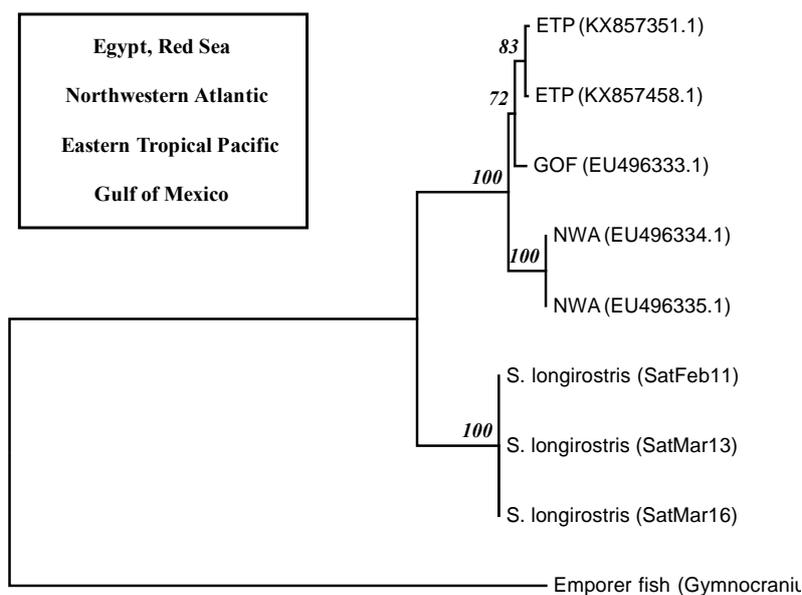


Figure.4. Neighbor joining phylogenetic tree of *S. longirostris*, inferred from COI sequences from the current study (Denoted by circles) and that available at the NCBI GenBank, with bootstrap value of 1000. Note: North West Atlantic (NWA), Eastern Tropical Pacific (ETP), Gulf of Mexico (GOM) and Egyptian Red Sea (EGRS).

#### IV. DISCUSSIONS

Identifying dolphins using the morphological techniques are vital but these techniques not capable for identifying the higher level of taxonomy including cryptic, hybrid or a new species (Hoelzel *et al.*, 2002). Molecular techniques are important indirect approach to study the evolution and population genetics of the marine mammals all over the world which display a fine scale population structure while the extent varies among species (Hoelzel *et al.*, 2002). Recently, molecular markers are useful tool for marine mammals research while they considered a vital tool for research on behaviour, ecology and evolution (Zanardo *et al.*, 2016).

Molecular genetics characterization is a necessary approach for understanding the population structure of baleen whales (Bérubé *et al.*, 1998; Baker *et al.*, 1994), polar bears (Paetkau *et al.*, 1999), killer whale (Hoelzel and Dover, 1991; Baker *et al.*, 1994; Pilot *et al.*, 2010), harbour porpoise (Rosel *et al.*, 1999; Fontaine *et al.*, 2007), harbor seals (Westlake and O'Corry-Crowe, 2002), dolphins (Gowans *et al.*, 2007 and Natoli *et al.*, 2004). DNA barcoding aims to develop a universal DNA-based species diagnosis technique in which a single reference gene is used to classify a specimen to species through DNA sequence comparisons (Hebert *et al.*, 2003a). The mitochondrial cytochrome oxidase I (*cox1*) gene has been most heavily promoted for this purpose (Hebert *et al.*, 2003b). Not only capable of identify genetic diversity between species but also within species additionally capable of identify the cryptic species. In Egypt, 4 samples have been collected from the populations of spinner dolphins *Stenella longirostris* in their resting area Satayah reef.

The samples were analyzed using *cox1* gene to identify the genetic diversity in Satayah reef. Aligning samples with samples obtained from Genebank showed a clear divergence between *Stenella longirostris* and the other marine mammals. In addition, the samples from *S. longirostris* were aligned with other *Stenella* species, *S. attenuate*, *S. frontalis*, *S. coeruleoalba* and *S. clymene*. Aligning samples collected from Egypt with samples of the same species obtained from GeneBank showed a clear divergent between samples in the same species. Egyptian samples which we recollected from Satayah reef are clearly divergent forming a separate clade with bootstrap value of 100 indicating that Egyptian spinner dolphin are genetically differ than spinner dolphins from Eastern Tropical Pacific Ocean, Gulf of Mexico, North Western Atlantic.

The levels of genetic differences need more examination in order to determine whether it is a cryptic species complex or the separation of a new species. Analysis on the samples that collected from the Red Sea indicates the formation of a separate gene pool with all samples mostly genetically identical indicating the possibility of the population being reproductively isolated from surrounding spinner dolphin population. Our results need further examination In order to verify the level of the reproductive isolation within spinner dolphin population. As a conclusion, molecular examination indicates that Egyptian population of spinner dolphins is genetically isolated from other spinner dolphin's population worldwide. The present study recommends using other barcoding genes in order to identify whether spinner dolphin population in the Red Sea form a cryptic species complex or a new species. Further examination is required to examine

genetic diversity of Spinner dolphin population in different localities within the Red Sea (Samadai reef, Shaab alam).

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