

Evolutionary position of a Green turtle with anomalous colouration

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Abstract

One of the most striking sights at the Kosgoda turtle hatchery is a Green turtle showing an unusual white colouration. While anomalous colouration occurs in many tropical vertebrates, they are considered rare in wild populations. Using the classification by Fertl and Rosel (2002), Miller (2005) and Acevedo and Aguayo (2008), the specimen was identified as a Green turtle with leucism. The Green turtle lacked pigmentation in most parts of the body but some pigmentation was seen on the carapace while dark colouration was present in the eyes. The turtle also showed variations in the number and arrangement of the central/vertebral scutes while the other scutes were normal in number and arrangement. Although this turtle was hatched from eggs buried from nesting sea turtles at the Kosgoda beach, it has a relation of sorts to the Green turtle in Oman and Kuwait/ Northwestern Arabian Gulf. The mitochondrial DNA (mtDNA) analysis of a ~420bp control region sequence of the Green turtle with leucism (EF546380) from Sri Lanka showed 100% sequence similarity to a Green turtle from Oman (AJ831526) and Kuwait/ Northwestern Arabian Gulf (EU499302). Molecular level observation of 100% homology of mtDNA D-loop sequence between the mtDNA sequences of Green turtle with leucism from Kosgoda, Sri Lanka and the mtDNA sequence of a Green turtle from Oman and Kuwait/ North Western Arabian Gulf suggests that the specimen could be from the same breeding stock from Oman. It further confirms that the Indian Ocean Green turtle population is distributed right up to the Persian Gulf.

A UPGMA tree was generated after randomly selecting Green turtle (*Chelonia mydas*) sequences from Atlantic Mediterranean as well as Indo-Pacific together with the leucistic Green turtle sequence and two other Green turtle sequences from Sri Lanka to analyze the evolutionary position of the Green turtle with anomalous colouration with respect to the species from Indo Pacific and Atlantic Mediterranean region. The UPGMA tree was consistent with the historical bifurcation of Green turtles into two major clades, the Mediterranean and Atlantic, and Indo Pacific. Further the Green turtle sequences from Sri Lanka fell into a sister clade with the Green turtle sequences from Indian-Pacific supporting the idea of the presence of a major group of *Chelonia mydas*; the Indian-Pacific. Further, the results are consistent with the presence of geographic barriers between the Atlantic and Indian Oceans.

Keywords: Leucism, Green turtle, Mitochondrial DNA.

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Introduction

Studies on mitochondrial DNA (mtDNA) have proven to be particularly effective in detecting the origin of marine turtles. Some of the unique features of mtDNA, such as its maternal mode of inheritance and relatively rapid rate of evolution (Brown *et al.*, 1979) make this molecule particularly appropriate for investigating patterns of gene flow in nesting populations of sea turtles. In the present study, mtDNA polymorphism was used to determine the evolutionary position of the Green turtle with anomalous colouration, which is important to the conservation of this endangered species.

Although anomalous colourations are found in many tropical vertebrates, they are considered rare in wild populations, with few records for the majority of animal taxa. The two factors that determine colour patterns in mammals are the presence and distribution of pigmentation in the skin, hair and eyes (Fertl and Rosel, 2002; Hofreiter and Schöneberg, 2010). Anomalous colour can occur occasionally due to an excess or lack of production of melanin in some regions or throughout the entire body (Acevedo and Aguayo, 2008). According to summers (2009), the terms "partial" and "total" for the two different levels of albinism (hypopigmentation) have become out of date.

According to current zoological studies (Fertl and Rosel, 2002; Miller, 2005; Acevedo and Aguayo, 2008) there are four categories of anomalous colouration; piebaldism, leucism (or partial albinism), albinism and melanism. There is, however, still some discrepancy between authors, mainly because some studies do not take into account the difference between piebaldism and leucism (eg. Geige and Pacheco, 2006; Oliveira, 2009). Piebaldism is lack of pigmentation in some parts of the body, but presenting normal colouration in the eyes (Fertl & Rosel, 2002). Miller (2005) used the term partial albinism to refer to this phenomenon. Leucism is characterized by total or partial absence of pigmentation in the whole body (the individual has a body that is white, whitish or yellowish white colour), but the eyes and/or body extremities still show dark or blue pigmentation (Fertl and Rosel 2002; Miller 2005; Acevedo and Aguayo, 2008) whereas in albinism, individuals present a total absence of pigmentation in the whole body (white, whitish or yellowish white colour) including the eyes which show no traces of pigmentation, but instead red or pink colouration (Fertl and Rosel, 2002; Miler, 2005;

Acevedo and Aguayo, 2008). At the other extreme, overproduction of the pigment melanin results in melanism, or overly dark animals (Visser *et al.*, 2004).

We report here a Green turtle with anomalous colouration from Kosgoda, Sri Lanka. Additionally, as the geographic distribution of mtDNA lineages in Green turtles would provide information relevant to management and conservation of this endangered species, we determined the evolutionary position of this Green turtle with anomalous colouration, using mtDNA control-region-sequence-analysis to provide an insight into the distribution of Green turtles.

Materials and Methods

Morphometric characteristics

The weight of the Green turtle (*Chelonia mydas*) with anomalous colouration, the length of the carapace and the width of the carapace were measured. The colour of the skin, carapace, plastron, and eyes, and presence or absence of deformities in the head, jaw, eyes, nostrils, carapace, plastron and scutes were recorded.

Percentage of Green turtles with anomalous colouration

In the present study percentage of hatchlings that emerged from a single clutch of a female Green turtle and the number of hatchlings with anomalous colouration were recorded.

DNA isolation

Tissue samples were collected from the Green turtle with anomalous colouration (Figs 1-4) and two other Green turtles. The samples were stored in 90% ethanol. The material was ground to a fine powder in 5 ml of a solution containing 2% N-cetyl-*N,N,N*, -trimethyl- ammonium bromide (CTAB), 1.4 M-NaCl, 20 mM-EDTA, 100 mM-Tris-HCl(pH 8). After incubation with 2.5 µl of 20 mg/ml proteinase K at 65⁰C for one hour, the suspension was extracted once with phenol: chloroform: isoamyl alcohol (25:24:1) and then with chloroform: isoamyl alcohol (24:1). Nucleic acids were precipitated by adding 2 ½ volumes of absolute ethanol and 1/10th volume of sodium acetate. Following centrifugation the pellet was washed once with 70% ethanol and air dried. The pellet

was dissolved in 100 l of TE [10 mM-Tris-HCl, 1 mM-EDTA (pH 8.0)] and stored frozen.

PCR amplification and sequence analysis

Amplification was done using mitochondrial control region primers. PCR primers for the ~420bp fragment were, 5' primer, 5'-TTG TAC ATC TAC TTA TTT ACC AC - 3' and 3' primer, 5'-GTA CGT ACA AGT AAA ACT ACC GTA TGC C- 3'. PCR reactions were performed in 10 x PCR buffer, 1.8 µL of 25 mM-MgCl₂, 1 µl of 10 µM primer, and 1 unit of *Taq* polymerase (Promega). 50 ng of template DNA was added to the reaction. The cycling conditions were 2 min at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 54 °C and 40 seconds at 72 °C and a final extension step of 2 minutes at 72 °C. PCR products were visualized on 1.4% agarose gel along with a 1Kb DNA step ladder. PCR products were purified using Ultraclean PCR clean up kit. Single stranded DNA sequencing was carried out by the sequencing facility at the Institute of Biochemistry Molecular Biology and Biotechnology, University of Colombo. Forward and Reverse sequences were aligned and the consensus sequences were created using Bioedit version 7. Homology searches were done with the NCBI Megablast using default conditions. Phylogenetic analyses were conducted in MEGA4.



Fig. 1. Dorsal view of the Green turtle
Cream colour carapace with traces of
pigmentation and vertebral/central
scute abnormalities.

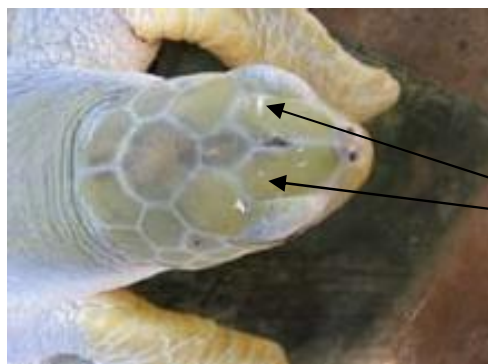


Fig. 2. Ventral view of the Green turtle
Ventral side showing the cream colour
plastron



Fig. 3. Head of the Green turtle

Eye colour of the turtle is normal with no abnormalities in pigmentation.



One pair
of
Prefrontal
scales

Fig. 4. Dorsal view of the head

A pair of prefrontal scales, characteristic of Green turtles

Results

Morphology and Morphometric characteristics

The Green turtle with anomalous colouration had a weight of 12 kg, while the carapace was 49 cm long and 46 cm wide. The animal had silvery white colour and cream coloured carapace and plastron. No deformities were evident in the head, skin, jaws, eyes, nostrils, or plastron. The eyes had dark pigmentation and there were traces of pigmentation on the carapace. Carapacial scute variations were present where there were differences in the arrangement and the number of central scutes. The number of central/vertebral scutes present was eight instead of five. Four costal, five vertebral, one nuchal, eleven marginal and one supracaudal scute were present.

Percentage of Green turtles with anomalous colouration

Of the 180 eggs in the clutch, 165 eggs hatched out. Four hatchlings showed anomalous colouration and of these, only one had survived.

Mitochondrial DNA Control region sequencing

Approximately 420 bp fragment of the D loop of turtle mtDNA control region was generated from three green turtle species including the anomalously coloured green turtle (Fig 5). The sequence of the turtle with anomalous colouration was named GT7 and submitted to the GenBank (Accession number EF546380) along with two other Green turtle sequences (EF587755, EF587754) sequenced.

>gil146428534|gb|EF546380.1| *Chelonia mydas* haplotype GT7 control region, partial sequence; mitochondrial

TGTACATCTACTTATTTACCACTAGCATATGACCAGTAATGTCAACAGTTGATTT
GGCCCTAAACATGAAAATTATTGAATCCACATAAATATTTTGATAGCATGAATAT
TAAGCAGAGAATTAAGAGTGAAATGATATAGGACATAAAATTAACCATTATAC
TCAACCATGAATATCGTCACAGTAATTGGTTATTTCTTAAGTAGCTATTCACGA
GAAATAAGCAACCCTTGTTAGTAAGATACAACATTACCAGTTTCAGGCCCATTT
AGTTTATAGCGTACATAACCTGATCTATTCTGGCCTCTGGTTGTCTTTTCAGGC
ACATACAAATAATAACGTCCATTTCGTTCTCTTTAAAGGCCTTTGGTTGAATG
AGTTCTATACATTAAATTTATAACCTGGCATAACGGTAGTT

Fig. 5. Mitochondrial control region partial sequence ~420bp

Phylogenetic relationship

The sequence of Green turtle with anomalous colouration was subsequently used to search for similar sequences in the GenBank database with Basic Local Alignment and Search Tool (BLAST). BLAST results showed that the sequence of the Green turtle with anomalous colouration (GT7) is identical to the Green turtle from Oman (Accession number AJ831526) with 100% query coverage (Fig. 6). No gaps or missing data were present. Further 100% similarity was found with the Green turtle sequence from Kuwait /Northwestern Arabian Gulf (EU499302) but only 99% query coverage was found.

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>[emb|AJ831526.1| Chelonia mydas mitochondrial D-loop, haplotype CMOM3
Length=435

Score = 776 bits (420), Expect = 0.0
Identities = 420/420 (100%), Gaps = 0/420 (0%)
Strand=Plus/Minus

Query 1 TGTACATCTACTTATTTACCACTAGCATATGACCAGTAATGTCAACAGTTGATTTGGCCC 60
      |||
Sbjct 435 TGTACATCTACTTATTTACCACTAGCATATGACCAGTAATGTCAACAGTTGATTTGGCCC 376

Query 61 TAAACATGAAAATTATTGAATCCACATAAATATTTTGATAGCATGAATATTAAGCAGAGA 120
      |||
Sbjct 375 TAAACATGAAAATTATTGAATCCACATAAATATTTTGATAGCATGAATATTAAGCAGAGA 316

Query 121 ATTAAAAGTGAAATGATATAGGACATAAAATTAACCATTATACTCAACCATGAATATCG 180
      |||
Sbjct 315 ATTAAAAGTGAAATGATATAGGACATAAAATTAACCATTATACTCAACCATGAATATCG 256

Query 181 TCACAGTAATTGGTTATTTCTTAAGTAGCTATTCACGAGAAATAAGCAACCCTTGTTAGT 240
      |||
Sbjct 255 TCACAGTAATTGGTTATTTCTTAAGTAGCTATTCACGAGAAATAAGCAACCCTTGTTAGT 196

Query 241 AAGATACAACATTACCAGTTTCAGGCCCATTTAGTTTATAGCGTACATAACCTGATCTAT 300
      |||
Sbjct 195 AAGATACAACATTACCAGTTTCAGGCCCATTTAGTTTATAGCGTACATAACCTGATCTAT 136

Query 301 TCTGGCCTCTGGTTGTCTTTTCAGGCACATACAAATAATAACGTCATTTCGTTCCCTCTT 360
      |||
Sbjct 135 TCTGGCCTCTGGTTGTCTTTTCAGGCACATACAAATAATAACGTCATTTCGTTCCCTCTT 76

Query 361 AAAAGGCCTTTGGTTGAATGAGTTCTATACATTAAATTTATAACCTGGCATAACGGTAGTT 420
      |||
Sbjct 75 AAAAGGCCTTTGGTTGAATGAGTTCTATACATTAAATTTATAACCTGGCATAACGGTAGTT 16
```

Fig. 6. NCBI -BLAST results for sequence similarity

A UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree was generated after randomly selecting Green turtle (*Chelonia mydas*) sequences from Mediterranean as well as Indian-Pacific together with anomalously coloured Green turtle sequence from Sri Lanka (GT7/EF546380) and two other Green turtle sequences also from Sri Lanka (DQ354570 and EF587754) (Fig. 7.). The UPGMA tree showed that the anomalously coloured Green turtle as well as the other Green turtle sequences from Sri Lanka had a close relationship with those of the Indian-Pacific colonies.

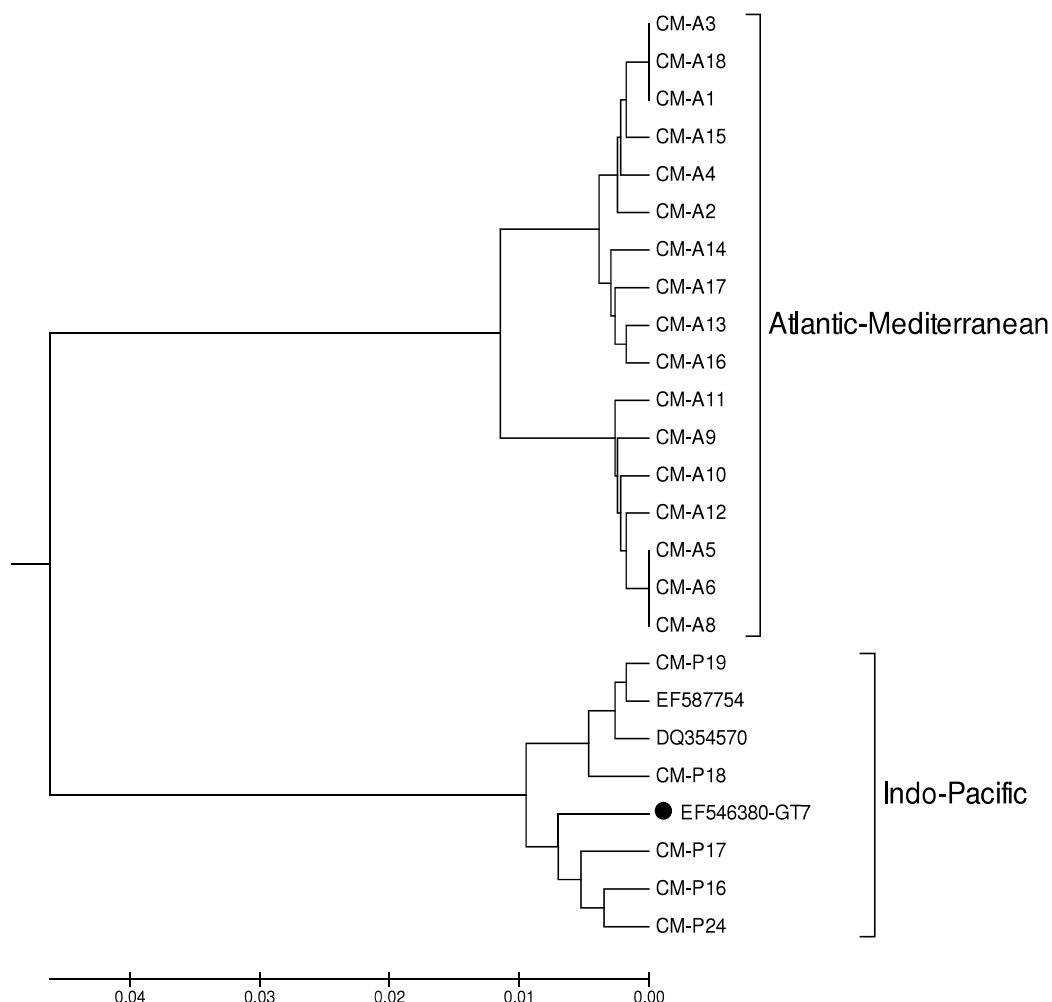


Fig.7. Evolutionary relationships of 25 taxa

The evolutionary history was inferred using the UPGMA method. 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. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

Discussion

Animals that are white instead of their normal colouration quickly capture our attention. Being white doesn't make an animal an albino. The Green turtle at Kosgoda, with anomalous colouration had traces of pigmentation on the carapace and had dark pigmentation in the eye although it showed lack of pigmentation in the body. The distinguishing features were compared with the descriptions of Fertl and Rosel (2002), Miller (2005), Acevedo and Aguayo (2008). Distinguishing characteristics of the skin, plastron, carapace and eye were used to distinguish between piebaldism and albinism. As piebaldism is characterized by patchy body pigmentation and leucism by dark eyed anomalously white animals, the Green turtle with anomalous colouration in the present study was identified as a Green turtle with leucism.

Furthermore, the animal had variations in the number and arrangement of central/vertebral scutes. The turtle had eight central/vertebral scutes instead of five usually found in Green turtles, but the rest of the scutes (nuchal, marginal, costal and supracaudal) were normal in number and arrangement. Newman (1906) argued that the supernumerary scutes are the atavistic appearance of scutes lost during phylogeny (Turkozan *et al.*, 2001). Parker (1901), Hildebrand (1930) and Zangerl (1969) claim that this abnormal situation may take place accidentally or because of a disorder during the process of origin and development of an individual organism from embryo to adult. There are evidences that different kinds of environmental factors play a role in scute variation. Fluctuations in oxygen levels during incubation (Hildebrand 1938), variations in temperature or moisture that cause stress during egg development or incubation, or mutagenic effects of excess salinity during the incubation period (Kazmaier and Robel, 2001) may result in scute anomalies. Deviations from the normal scute formulae of turtles may also result from embryonic mutations, specific causes of which are unknown (Kazmaier and Robel, 2001).

As the phenomenon of anomalous colouration is rare, displays a ghostly beauty, and is likely to have a significant influence on animal survival, the evolutionary position of this animal is of importance to the conservation of this endangered species. Moreover, the

geographic distribution of mtDNA lineages in Green turtles would provide valuable information to the management and conservation of this endangered species.

Recent developments in the application of molecular genetic markers are helpful to identify the evolutionary position of marine turtles. In the present study, a ~420 bp from the control D-loop region of the mtDNA of the Green turtle with anomalous colouration from Sri Lanka was sequenced together with two other Green turtles from Sri Lanka, and were used in subsequent analysis, in order to identify the origin of the turtle with anomalous colouration. NCBI - BLAST results showed a 100% sequence similarity with a Green turtle from Oman (Accession No AJ831526) with 100% query coverage and also with a Green turtle from Kuwait/ Northwestern Arabian Gulf (EU499302) with 99% query coverage. As mtDNA is maternally inherited, the results indicate that this turtle is from the same breeding stock as those from Oman and Kuwait/Northwestern Arabian Gulf area. It further demonstrates that the Indian Ocean Green turtle population is distributed right up to the Persian Gulf. This was also supported by the findings of Shanker (2004) where breeding migration of Green turtles have been seen from the East coast of Africa right up to Sri Lanka and from Bay of Bengal to the Pacific ocean. Tag reports of marine turtles have also indicated the possibility of such movements. The long migration routes of sea turtles span the Gulf area. According to the tag studies, Green turtles tagged in Sri Lanka, have been recovered in Djibouti located in East Africa at the junction of the Red Sea and the Gulf of Aden. It has been found that the flow of the surface current in winter and summer plays a major role in the movement of sea turtles. In winter, with the surface current flowing anti-clockwise in the Arabian Sea and the Northwest parts of the Indian Ocean, the turtles would swim with the surface current to reach Djibouti waters with its path flowing along the coast of India, Pakistan, Oman, Yemen and Somalia (http://www.ioseaturtles.org/feature_deatail.php?id=322). In summer, the surface current will be reversed and would flow clockwise (Shetye *et al.*, 1991; Shetye *et al.*, 1996).

Mitochondrial DNA has proven particularly effective for detecting population structure in marine turtles. Several studies have successfully used mtDNA variants to resolve population boundaries among breeding sea turtles (Bowen *et al.*, 1992; Bowen *et al.*, 1994; Broderick *et al.*, 1994; Norman *et al.*, 1994; Encalada *et al.*, 1996; Bass *et al.*,

1996; Bowen *et al.*, 1998; Dutton *et al.*, 1999). The Green turtles are herbivorous and they inhabit the warm waters of the Mediterranean Sea and the Atlantic, Indian, and Pacific Oceans. The overall degree of mtDNA sequence divergence in Green turtles is said to be low (Bowen *et al.*, 1992) with a historical bifurcation of the *Chelonia mydas* complex in to 1) the Indian and Pacific oceans and 2) the Atlantic Ocean and the Mediterranean Sea (Avise *et al.*, 1987). No evidence of gene flow between Indian and Atlantic Oceans over the last 1.5 million years has been found (Bowen *et al.*, 1992). To see whether the evolutionary relationships inscribed in mtDNA sequences coincide with the said bio-geographic provinces, and to further clarify the origin of the Green turtle with anomalous colouration, a UPGMA tree was generated after randomly selecting Green turtle (*Chelonia mydas*) sequences from Atlantic-Mediterranean as well as Indo-Pacific (Fig. 7.), together with the anomalously coloured Green turtle sequence and two other Green turtle sequences from Sri Lanka. The UPGMA tree was consistent with the historical bifurcation of Green turtles into two major clades, the Atlantic-Mediterranean, and Indo-Pacific, which was consistent with the geographic and climatic boundaries which define Green turtle distributions. Further, the Green turtle sequence with anomalous colouration (EF546380) designated as GT7, together with two other Green turtle sequences from Sri Lanka (DQ354570 & EF587754), fell in to a sister clade with the Green turtle sequences from the Indo-Pacific, supporting the idea of the presence of a major group of *Chelonia mydas* in the Indian-Pacific region. Further, the results are in agreement with the presence of geographic barriers between the Atlantic and Indian Ocean. Previous studies have shown that the Cape of Good Hope was a major bio-geographical barrier between the Atlantic and Indian oceans and has been considered to be an absolute barrier to the mixing of Atlantic and Indo-Pacific populations of Green turtles (Briggs, 1974).

Conclusions

The Green turtle at Kosgoda, with anomalous colouration shows leucism. According to mtDNA control region sequence analysis, the leucistic Green turtle is from the same breeding stock in Oman and Kuwait/Northwestern Arabian Gulf area. It further demonstrates that the Indian Ocean Green turtle population is distributed right up to the Persian Gulf. The Green turtles from Sri Lanka forms a major group of *Chelonia mydas*;

the Indian-Pacific and the result are consistent with the historical bifurcation of Green turtles into two major clades, the Atlantic-Mediterranean, and Indo-Pacific, which defines the presence of geographic barriers between the Atlantic and Indian oceans. The study also demonstrates the need for international cooperation in the general conservation of these Green turtles.

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