Original Research

Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/42/1/MRN-1526

The efficacy of *COI* barcoding and ISSR markers in molecular identification of diverse bird *Sternula saundersi* populations along the Red Sea coast, Kingdom of Saudi Arabia

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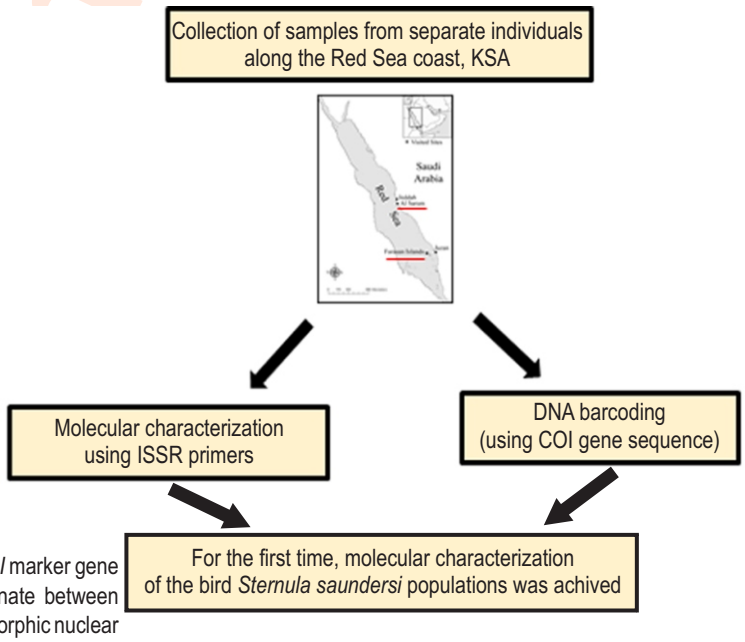
Received: 20.05.2020 *Revised*: 13.08.2020 *Accepted*: 31.08.2020

Abstract

Aim: To demonstrate the first DNA barcode and molecular diversity of bird *Sternula saundersi* (Saunders's tern) populations collected from the Red Sea coast at the Kingdom of Saudi Arabia.

Methodology: The mitochondrial cytochrome c oxidase subunit I (COI) gene was isolated and sequenced from 26 different individuals of *S. saundersi,* whose blood samples were collected from two different areas at the Red Sea coast of the Kingdom of Saudi Arabia. Also, the genetic diversity of 26 individuals were investigated using highly polymorphic nuclear markers, *i.e.,* inter simple sequence repeat [ISSR] technique.

Results: The *COI* gene sequences of all 26 individuals were documented and submitted to the GenBank database. Five unique single nucleotide polymorphisms were found in all individuals of *S. saundersi* that were not observed in other species of the genus Sternula. Consequently, these five nucleotides can be used as a distinctive DNA fingerprint of *S. saundersi*. Additionally, it was possible to identify molecular diversity within different populations from two different areas using eight different ISSR markers.



Interpretation: The present data demonstrate the power of *COI* marker gene in identifying *S. saundersi, i.e.,* it can be utilized to discriminate between different species of the genus *Sternula.* In addition, highly polymorphic nuclear markers (ISSR) were demonstrated to have significant power to accurately distinguish between individuals collected from different widely spaced areas.

Key words: *COI* gene, DNA barcode, ISSR markers, *S. saundersi*

How to cite : Almalki, M., M. Ismail and A. Gaber: The efficacy of *COI* barcoding and ISSR markers in molecular identification of diverse bird *Sternula saundersi* populations along the Red Sea coast, Kingdom of Saudi Arabia. *J. Environ. Biol.*, **42**, 24-32 (2021).

Introduction

Seabirds have a significant role to play within marine and earthly biological systems (Parsons *et al.,* 2008). They are beneficial to communities in numerous ways: as pointers of fish schools for human fishers (Einoder, 2009), as nourishment for indigenous gatherers (Klein *et al.,* 2010), as guano for plant manure (Schnug *et al.,* 2018), and as an attraction for eco-tourists (Wilson and Tisdell, 2002). The extinction of a specie is irreversible and represents a serious threat to the well-being of the planet (Costello *et al.,* 2013). Seabirds reportedly account for approximately 25% of all marine deaths and are the most threatened bird group (Dulvy *et al.,* 2003).

Sternula saundersi (Saunders's tern) is a seabird of family *Laridae*. It is a little-known specie and is more widely recognized as the black-shafted tern (BirdLife International, 2018). It is a small bird, weighs 40-45 g, and it is similar to *S. albifrons* (little tern); they are easily mistaken for each other (BirdLife International, 2018). Originally, *S. saundersi* was classified as a subspecies of *S. albifrons* (Harrison, 1983); however, ten years later, it has been recognised as a separate species (Sibley and Monroe, 1990). *S. saundersi* is pale grey above and white below. It is characterised by a black cap and a white triangular patch on its forehead (Porter and Aspinall, 2010). The global distribution of *S. saundersi* is confined to the Northern Indian Ocean. Breeding occurs along the Red Sea coast and the Arabian Gulf, extending to north-west India, Sri Lanka and Maldives (Bird Life International 2018; Sibley and Monroe, 1990). Numerous nesting species are located in the Kingdom of Saudi Arabia along the Red Sea and coasts and islands of the Arabian Gulf (Shobraka and Aloufib, 2014).

Species identification is a crucial step in all biological studies (Kesler and Haig, 2007). Without proper species identification, appropriate action regarding biological issues is not be possible (Hebert *et al.,* 2004). Traditional taxonomy has sometimes been demonstrated to be ineffective for species identification, or qualified personnel to perform proper identification is lacking. Researchers are, therefore, seeking new and precise methods of species characterisation that are not dependent on taxonomic expertise. Molecular identification provides more precise and correct means of species documentation than morphology-based methods (Rajpoot *et al.,* 2016). To date, DNA barcoding has performed well in unravelling a wealth of new information, and it is expected to continue to provide invaluable insights (Aliabadian *et al.,* 2013; Abdul Aziz *et al.,* 2016; Gaber *et al.,* 2020). DNA barcoding in animal kingdom facilitates the ability to make evidence-based distinctions between species and assess biodiversity by utilising the whole nucleotide sequence of mitochondrial genes (Aliabadian *et al.,* 2013; Gaber *et al.,* 2020).

Since mitochondrial proteins have fundamental metabolic capacity, their DNA nucleotide sequences are extremely conservative (Amer *et al.,* 2013). Thus, the characterisation of mitochondrial genes makes them suitable for molecular identification of species (Amer *et al.,* 2013; Gaber *et al.,* 2020). Furthermore, a barcode from the mitochondrial genome, rather than a nuclear genome, represents the most effective single gene marker due to its smaller population size, thereby increasing general concordance in the gene tree and fundamental species tree (Amer *et al.,* 2013). Most DNA barcoding studies achieve this by utilising the gene-encoding cytochrome oxidase subunit I (COI). This gene sequence marker can be evaluated against a database of known sequences in GenBank to perform species molecular identification (Aliabadian *et al.,* 2013; Gaber *et al.,* 2020). The main purpose of this research was to document, for the first time, the molecular identification of *S. saundersi* using *COI* marker gene. Therefore, *COI* was isolated and sequenced from 26 individuals collected from two different areas at Red Sea coast of the Kingdom of Saudi Arabia. Additionally, the inter simple sequence repeat (ISSR) technique as a nuclear genetic marker was used to determine the effect of biogeographical divergence on genetic diversity between individual birds.

Materials and Methods

Collection of samples: Field work was conducted during the breeding season of *S. saundersi* from first week of March to first week of June 2013 in Al Sarum city and Farasan islands in the Kingdom of Saudi Arabia. Ethical committee of Taif University had approved the study. Two different strategies were utilized to find nests; either a vehicle was driven at a moderate speed to flush out hatching birds or parents returning to their nests from the sea were recognised. Adult *S. saundersi* was captured followers the methods previously reported (Bub, 1991). The captured birds were marked with a ring on the right leg using an unmistakable 26 numbers supplied by the Saudi Wildlife Authority. About 50 µl of blood sample was assembled from individuals for DNA extraction. The birds were then freed at the sites of capture.

Amplification of cytochrome oxidase subunit I gene: Isolation of total genomic DNA obtained from the blood of each sample was achieved using DNeasy® Blood and Tissue Kits (Qiagen, Germany) as described by the supplier. The target region of the COI gene (about 900 bp in length) was amplified using specific designated primers as documented previously (Amer *et al.,* 2013). The reaction mix and thermocycler protocol were performed as described recently (Hebert *et al*., 2004). Specific products of the COI gene from the individuals were purified and sequenced using same primers in both the forward and reverse directions, and the procedure was repeated to confirm that the results were reproducible. The results of crude sequencing were collected and analyzed by Seqscape software version 2.7 (ABI Applied Biosystems).

Sequence analysis: The *COI* gene sequences from 26 individuals, GenBank accession numbers MT857928 to MT857953, together with other *COI* gene sequences obtained from GenBank, were adjusted via ClustalW® and MEGA® (version 7.0) (Kumar *et al.,* 2016). To avoid sequence

mismatching and provide a uniform length for all *COI* genes (about 700 bp), 100 bp at both ends of each gene product was excluded. Maximum likelihood (ML) estimation trees were constructed in MEGA® program (version 7.0) following the Kimura two parameter model of evolution (Kimura, 1980). One thousand bootstrap replicates were used to evaluate clade reliability (Felsenstein, 1985). Bayesian inference was performed using BEAST® (version 1.8.0) and XML input files were generated in BEAUti® (version 1.8.0) (Drummond *et al.,* 2012). Almost 10% of the results were discarded prior to the analysis as burn-in period. A maximum clade credibility tree was obtained with TreeAnnotator® (version 1.8.0). The trees were modified using FigTree® (version 1.4.4) according to a procedure that was described previously (Rambaut, 2018).

ISSR experiments: PCR reaction mixture and protocol were achieved using eight ISSR primers to amplify the inter-repeat regions of the genomic DNA from all 26 individuals as recently reported (Buhroo *et al.*, 2018). The amplified fragments were examined in 2% agarose gel and visualized using UV light (Bio-Rad® Gel Doc 2000, Germany).

Gene flow between populations: Prior to gene flow assessment, 26 genotypes of *S. saundersi* were grouped into two populations according to collection locations (350 km apart). The level of population differentiation (ΦPT, Nei 1978) as an indication of gene flow was then estimated by hierarchical analysis of molecular variance (AMOVA) as implemented in GenAlex 6.5 (Peakall and Smouse, 2012), its significance was determined using 1000 permutations. The number of migrants (N_m) between population was determined according to [N_m = 0.5 (1-ΦPT)/ ΦPT] (McDermott and McDonald, 1993). It is worth mentioning that due to elevated mitochondrial *COI* sequence similarity among the collected samples, it was not possible to compute the gene flow using *COI*.

Statistical analyses: The ISSR-PCR DNA products were counted either as '0' for absent loci, '1' for existing loci and '9' for infrequent unamplified data. A similarity matrix was constructed using the Unweighted Pair Group Method with Arithmetic Mean, and a dendrogram was built using NTSYS-PC® (version 2.01) (Rohlf, 2000).

Results and Discussion

The consortium for the Barcode of Life initiative was created in 2004 with a view to encourage and establish a worldwide DNA barcoding project. Thereafter, the All Birds Barcoding Initiative project was initiated with the aim of barcoding at least five individuals per species of bird (Ratnasingham and Hebert, 2007). As part of this initiative, the aim of the present research was to report, for the first time, on the efficacy of mtDNA *COI* gene in molecular documentation of *S. saundersi.* The topologies generated from the examination of mtDNA genes appeared to be more noteworthy in terms of investigation than those inferred from nuclear genes. Thus, it was anticipated that those obtained from the examination of mtDNA genes would provide an extremely high rate of sequence evolution in animal rather than nuclear DNA (Brown *et al.,* 1985). The first DNA barcoding study of birds was conducted in 2004, and approximately 40% of North American bird species were inspected (Hebert *et al.,* 2004). This study reported that a *COI* gene of 650 bp in length facilitated the identification of bird's species (Hebert *et al.,* 2004). The *COI* gene was successfully amplified and sequenced from all individuals collected from two different areas.

The *COI* DNA sequences of 26 individuals were completely identical. The gene sequences of all 26 individuals were documented and submitted to the GenBank database under the accession numbers MT857928 to Mt857953. The base composition of sequenced *COI* gene was A = 179, T = 197, G = 114 and C = 200. BLAST search analysis between *COI* gene of *S. saundersi* and other *COI* reference genes in GenBank was performed. Most of the obtained DNA sequences (95-98%) matched the sequences of the genus *Sternula* (data not shown). The *COI* gene sequences revealed five unique single nucleotides variations in *S. saundersi* compared to other species of the genus Sternula, specifically *S. albifrons, S. superciliaris* and *S. antillarum* (GenBank Accession numbers KT350612.1, EU525525.1 and EU525523.1, respectively) (Fig. 1). These five variations in *S. saundersi* were G, T, G, G and A at positions 184, 406, 472, 487 and 535, respectively (Fig. 2). These five polymorphisms did not affect the amino acid sequences of the *COI* protein because all the variations were silent mutations. These data demonstrate the power of *COI*

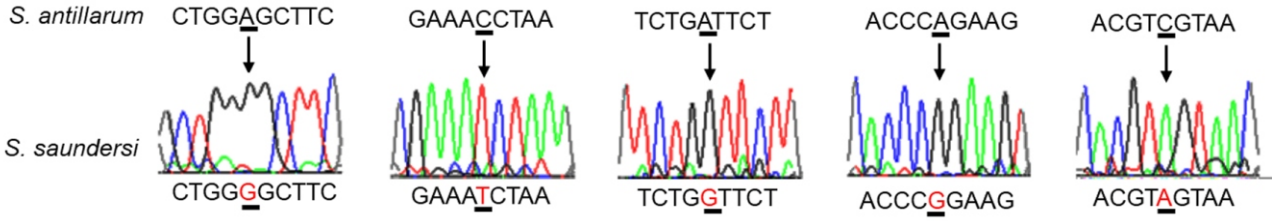


Fig. 1: Recognition of five unique single nucleotide polymorphism in *COI* gene of *Sternula saundersi* comparing with the sequence of *Sternula antillarum* (upper line).

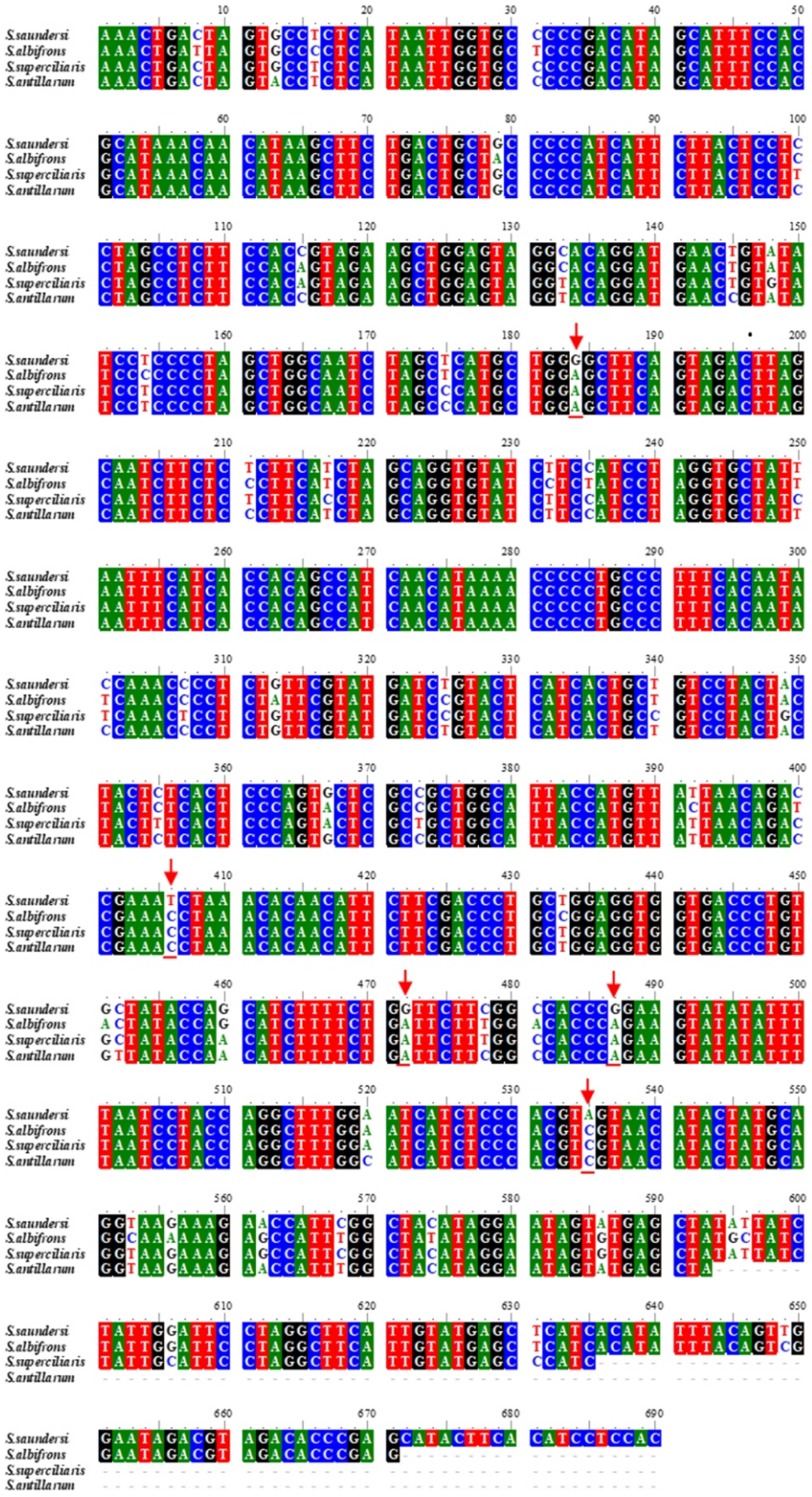


Fig. 2: Nucleotide sequence alignment of COI gene (690 bp) of *Sternula saundersi* with other species of the genus *Sternula* listed in the GenBank.

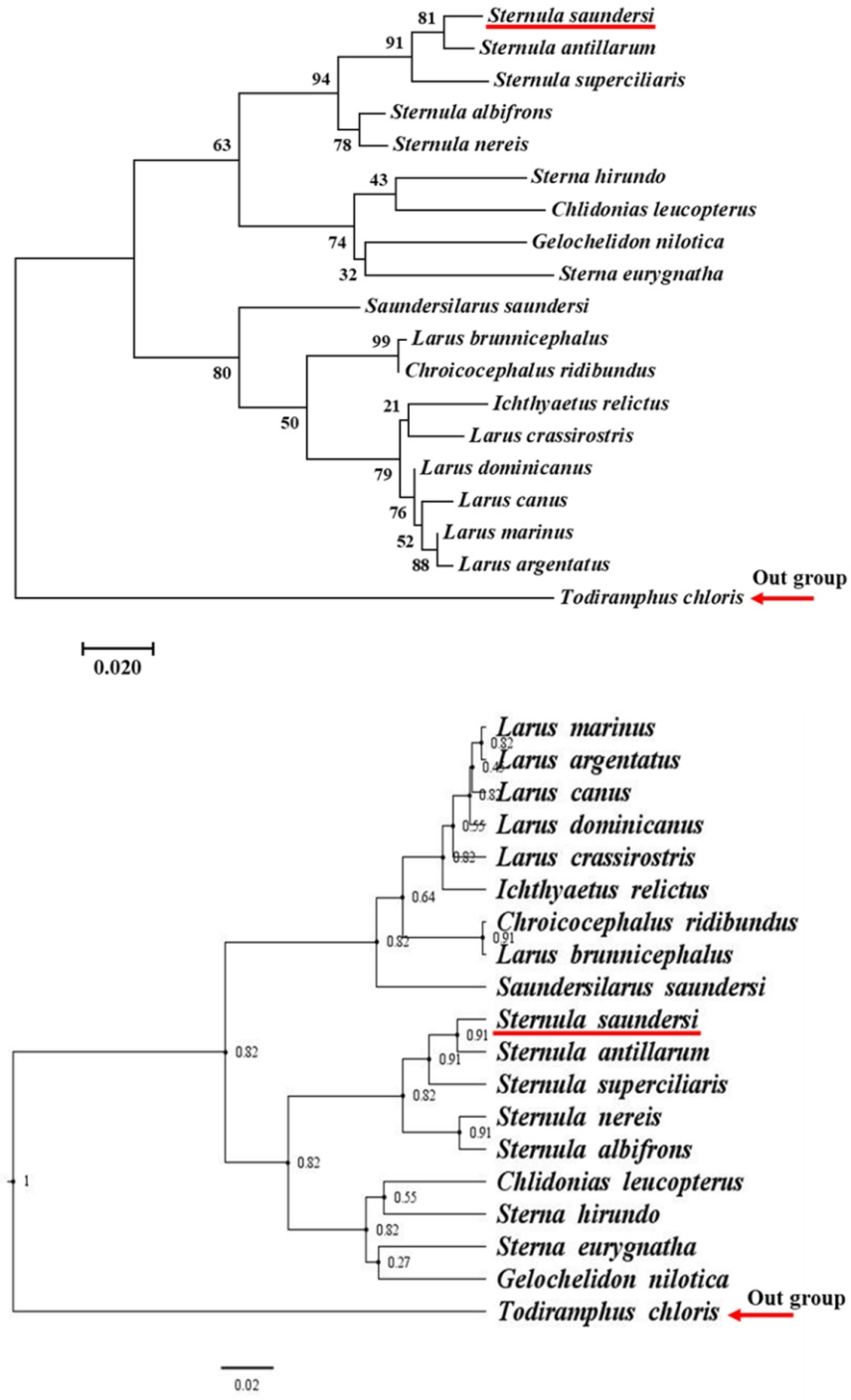


Fig. 3: Depiction of molecular phylogenetic tree by Maximum Likelihood method (upper)or Bayesian analysis (lower) of *Sternula saundersi* and different genera of *Laridae* family using *COI* gene. *Todiramphus chloris* was utilized as out-group taxa.

marker gene in identifying *S. saundersi* and this means that it can be utilized to discriminate between different species of the genus *Sternula* (Fig. 2). Two different approaches to sequence analysis, ML estimation and Bayesian inference, were used to compare sequence similarities and reconstruct the phylogenetic trees between different *Laridae* family genera based on the *COI* gene sequences (Fig. 3). Interestingly, both methods revealed that the 26 individuals were located in the same monophyletic

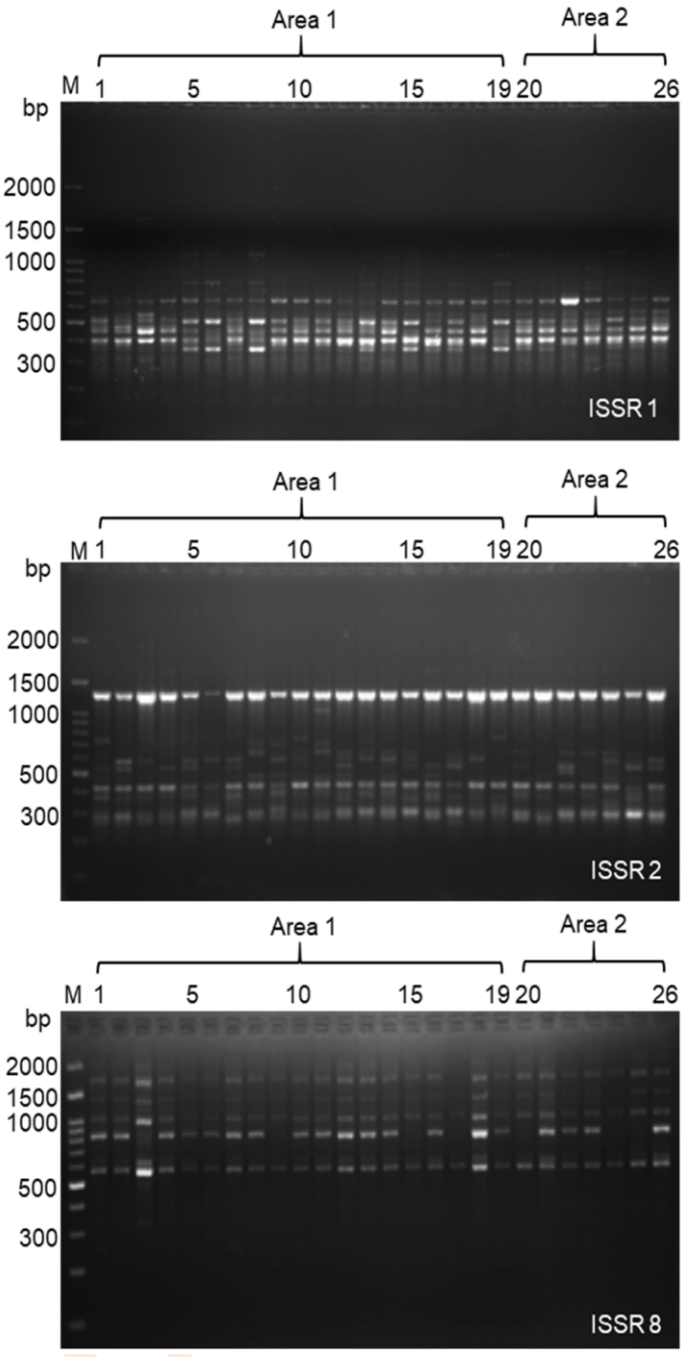


Fig. 4: Amplification profile of DNA loci distinguished using ISSR primers in 26 *Sternula saundersi* individuals. Full details about area 1 and 2 represented in ''Materials and Methods section''.

clade with *S. antillarum* using high bootstrap values. Until now, it is difficult to distinguish between *S. saundersi* and *S. albifrons* due to high degree of similarity between them (Bird Life International, 2018). But, as a result of *COI* sequence analysis, it has been proved that genetically *S. saundersi* is closely related to *S. antillarum* rather than *S. albifrons* (Fig. 3). By using two phylogenetic analyses methods, two groups were generated, and the first group was divided into two clades. Four species of the genus *Sternula* were clustered in the same clade while *Sterna*, *Chlidonias* and *Gelochelidon* were clustered in the second clade (Fig. 3).

These data clearly indicate that either ML estimation or Bayesian Inference can efficiently distinguish between *S.*

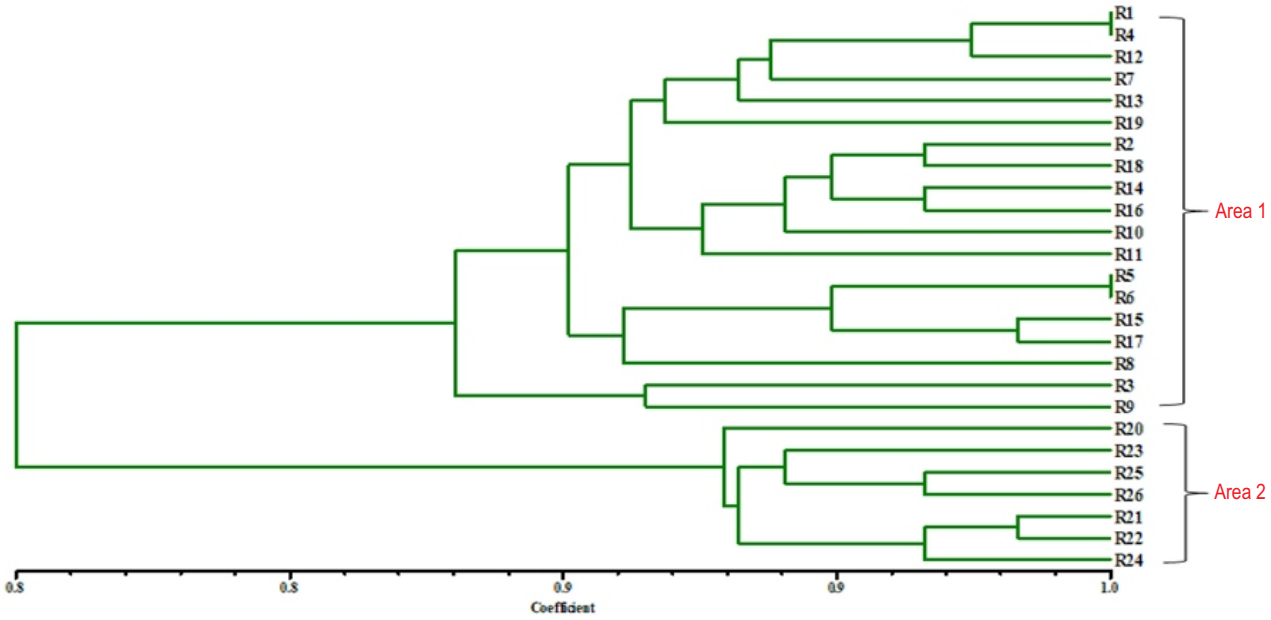


Fig. 5: Dendrogram analysis of 26 *Sternula saundersi* individuals using UPGMA clustering method resulting from eight ISSR markers.

Table 1: Primer names, sequences, level of polymorphism and range of amplified loci for 26 *Sternula saundersi* individuals analysed using eight ISSR primers

Primers	Sequence (5'-3')	Loci	ML	PL	% PL	Range of amplified loci (bp)
ISSR-1	(AG) 8 TG	9	2	7	77.70	300–1100
ISSR-2	(GA) 8 TT	14	3	11	78.60	280–1400
ISSR-3	(GA) 8	11	7	4	36.40	250–1100
ISSR-4	(AG) 8 T	13	4	9	69.20	310–1800
ISSR-5	(AG) 8 C	11	5	6	54.50	220–1400
ISSR-6	(TC) 8 C	10	6	4	40.00	690–1700
ISSR-7	(AC) 8 C	10	9	1	10.00	450–1700
ISSR-8	(AG) 8 TT	11	4	7	63.60	350–1900
Average		11	5	6	53.75	
Total		89	40	49		

ML: Monomorphic loci; PL: polymorphic loci

Table 2: Analyses of molecular variance (AMOVA) for ISSR data of *Sternula saundersi*

Source of variation	df	SS	MS	Est. Var.	%	ΦPT
Among populations	1	21.662	21.662	1.745	31	0.314*
Within populations	24	91.338	3.806	3.806	69	
Total	25	113.000	25.467	5.551		

Df: Degrees of Freedom; SS: Sum of Squares; MS: Mean Squares; Est. Var: Estimated Variance; %: proportion of genetic variability, *P< 0.05

saundersi and closely related species. ML analysis of phylogenetic trees was the standard method used in the BOLD project (Ratnasingham and Hebert, 2007), and it has been broadly utilised in DNA barcoding reports on birds (Hebert *et al.,* 2004; Gaber *et al.,* 2020). Thus, it was employed in the current study to facilitate a comparison between *S. saundersi* and other species, even though it was acknowledged that the nucleotide frequencies may not have been equal.

ISSR is considered as an effective DNA nuclear marker in genotyping research, genome evolution and investigating for molecular characterization and diversity (Buhroo *et al.,* 2018; Gaber *et al.,* 2020). Recently, ISSR has proved to be a powerful molecular identification marker to investigate genetic dissimilarity between different individuals of *Todiramphus chloris* subspecies collected from different areas in the Arabian Peninsula (Gaber *et al.,* 2020). Therefore, in the present study, eight different ISSR primers were used to amplify the nuclear DNA fragments of all 26 individuals; three of them are represented in Fig. 4. As reported previously, ISSR primers are attached in repeat nucleotide regions in the genome, therefore; they comprise 2-5 nucleotides with a haphazard attaching sequence of 1-3 nucleotides (Lin *et al.,* 2012; Sesli *et al.,* 2020). In the current study, 90% of the eight primers were constructed as dinucleotides (Table 1). Previous studies have documented that repeat dinucleotide ISSR markers are more effective in identifying molecular diversity between the individuals of same species than other nucleotide repeats (Lin *et al.,* 2012; Gaber *et al.,* 2020; Sesli *et al.,* 2020). The number of amplified fragments scored for each ISSR primer was recorded (Table 1). A total of 89 loci were identified by eight ISSR primers. These loci were used to explore genetic similarities and dissimilarities and consequently, to construct the phylogenetic tree (Table 1; Fig. 4). The total number of polymorphic amplicons was 49.

The number of amplified loci varied from 14 bands (ISSR-2) to 9 bands (ISSR-1). Based on the genetic diversity and similarity identified by eight ISSR markers, the samples of 26 individuals were categorized into two main groups (Fig.5). The first group pertained to samples collected from area 1 (Farasan Islands; samples numbers 1-19). The second group pertained to clustered samples collected from area 2 (the Al Sarum; samples numbers 20-26) (Fig. 5). Analysis of molecular variance indicted that most of genetic variation occurred within, rather than among populations (Φ_{PT}= 0.314, P< 0.05, Table 2). Using ΦPT, we were able to estimate another genetic parameter (N_m) which was calculated to N_m = 1.092. Since $N_m \approx 1$, it indicated that the two populations experienced limited gene flow (Crow and Aoki, 1984), which is also supported by moderate population differentiation (Φ_{PT}= 0.314). Based on the present data, it can be concluded that *COI* gene marker has the ability to identify *S. saundersi* at the molecular level since there are five unique single nucleotide polymorphism recognized in *S. saundersi* only. In addition, the use of an ISSR-nuclear marker was highly effective in accurately differentiating between *S. saundersi* individuals collected from two different areas.

Acknowledgment

The authors acknowledge the support of Taif University Researchers Supporting Project number (TURSP-2020/39), Taif University, Taif, Saudi Arabi.

Add-on Information

Authors' contribution: M. Almalki: Conceptualization, data curation, data validation and draft preparation; **M. Ismail:** Formal analysis, software and data visualization; **A. Gaber:** Supervision, visualization, methodology, writing original draft and editing and funding acquisition.

Research content: The research contents is original and has not been published elsewhere

Ethical approval: The authors have followed and complied with the National Guidelines for the use of plants/animals/humans involved in the experimental study. The blood samples were assembled from bird individuals for DNA extraction and the birds were then freed at the sites of capture.

Conflict of interest: The author declares that there is no conflict of interest.

Data from other sources: Not Applicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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