Original article

Molecular identification of *Todiramphus chloris* subspecies on the Arabian Peninsula using three mitochondrial barcoding genes and ISSR markers

Ahmed Gaber [a,b], Mohamed M. Hassan [a,c], Christopher Boland [d], Abdullah Alsuhaibany [h], Jem Babbington [e], John Pereira [f], Jane Budd [f], Mohammed Shobrak [a,g,*]

[a] Department of Biology, Faculty of Science, Taif University, Saudi Arabia
[b] Department of Genetics, Faculty of Agriculture, Cairo University, Egypt
[c] Department of Genetics, Faculty of Agriculture, Menoufia University, Egypt
[d] Saudi Aramco, Environmental Protection Department, Dhahran, Saudi Arabia
[e] Saudi Aramco, EXPEC Advance Research Center, Dhahran, Saudi Arabia
[f] Environment & Protected Areas Authority, Sharjah, United Arab Emirates
[g] Saudi Wildlife Authority, Prince Saud Al Faisal Research Center, Taif, Saudi Arabia
[h] Ministry of Environment, Water, and Agriculture, National Wildlife Center, Riyadh, Saudi Arabia

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ABSTRACT

The Collared Kingfisher *(Todiramphus chloris)* is widely distributed across the Indian and western Pacific Oceans and consists of about 50 subspecies. Two different subspecies of *T. chloris* occur in the Arabian Peninsula: *T. c. abyssinicus* from the Red Sea coast and *T. c. kalbaensis* from the Arabian Sea coast in the United Arab Emirates and Oman. The aim of this study was to determine the molecular relationship between the two Arabian subspecies and to establish the first DNA barcodes from the Arabian Peninsula for this species. Three different mitochondrial genes were used: (i) cytochrome *c* oxidase subunit I (*COI*), (ii) 12S rRNA (*12S*) and (iii) NADH dehydrogenase-1 (*ND1*). The *COI* gene sequences of the two subspecies were 100% identical, while the *12S* and *ND1* gene sequences revealed a unique single nucleotide variation between the two subspecies. Thus, this single nucleotide variation can be used as a DNA barcode to discriminate between two subspecies. Furthermore, the genetic profile or fingerprint for both subspecies were compared using ten primers of the highly polymorphic nuclear markers (Inter Simple Sequence Repeat, ISSR). As expected, the DNA analysis of the ISSR markers was able to distinguish between the specimens of the two subspecies. These results suggest that *T. c. abyssinicus* and *T. c. kalbaensis* are not identical and thus belong to different subspecies. Besides, the sequences of the *COI* gene for *T. c. abyssinicus* and *T. c. kalbaensis* differs by only 1.28% from *T. sanctus* suggesting that the Arabian subspecies are closely related to the Sacred Kingfisher (*T. sanctus*).

1. Introduction

The Collared kingfisher (*Todiramphus chloris*) belongs to the family of Alcedinidae, subfamily Daceloninae, occasionally under an alternative family of Halcyonidae (Moyle, 2006). The genus *Todiramphus* is composed of 24 species (Woodall, 2018a). *Todiramphus chloris* is a pervasive species occurring through the Red Sea coast of Ethiopia as well as Saudi Arabia, through United Arab Emirates (UAE), Oman, India, Southeast Asia, northern Australia and the western Pacific Islands to Tonga, with 50 subspecies currently recognized (Woodall, 2018b; Gill and Donsker, 2019). The spreading of *T. sanctus*, only migratory *Todiramphus,* comprehensively covers with numerous populations in *T. chloris* complex. All sympatric *Todiramphus* populations display environmental, morphological and behavioral differences, together with detachment by habitat favorite, recommending reproductive isolation between each population (Woodall, 2018a). Two subspecies of *T. chloris*

* Corresponding author at: Department of Biology, Faculty of Science, Taif University, Al-Haweiah, P.O. Box 888, Taif, Saudi Arabia.
E-mail address: shobrak@saudibirds.org (M. Shobrak).
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occur in the Arabian Peninsula (Jennings, 2010; Woodall, 2018b). *T. c. abyssinicus* is spreading along Saudi Arabia's southern Red Sea coast, whereas *T. c. kalbaensis* occurs at Khor Kalba in the UAE and Khor Liwa and Khor Shinas in Oman (Dickinson, 2003; Eriksen et al., 2013; Porter et al., 1996). The distance between the two subspecies is about 1900 km. *Todiramphus c. kalbaensis* is classified as Critically Endangered and consumes a population of less than 50 pairs (Aspinall, 2007; Symes et al., 2015). Cowles (1981) reported for the first time that *T. c. kalbaensis* is a new subspecies of kingfisher was documented several morphological differences between the two subspecies. The *T. c. kalbaensis* differs

in holding a distinct and unmistakable white superciliary line spreading from the edges of the brow to overhead and passing to the eye. Additionally, a white superciliary is covered with blue-sloped feathers occurs over the ear coverts. This area become a marked area with bright blue-green and white color. In addition to that, the bill is smaller in *T. c. kalbaensis* than *T. c. abyssinicus*. Finally, the color of the upper tail cover and rump are increasingly in blue-green. (Cowles, 1981).

Species and subspecies identification are important in biological studies and for prioritizing conservation actions, especially for isolated species (Kesler and Haig, 2007). However, traditional
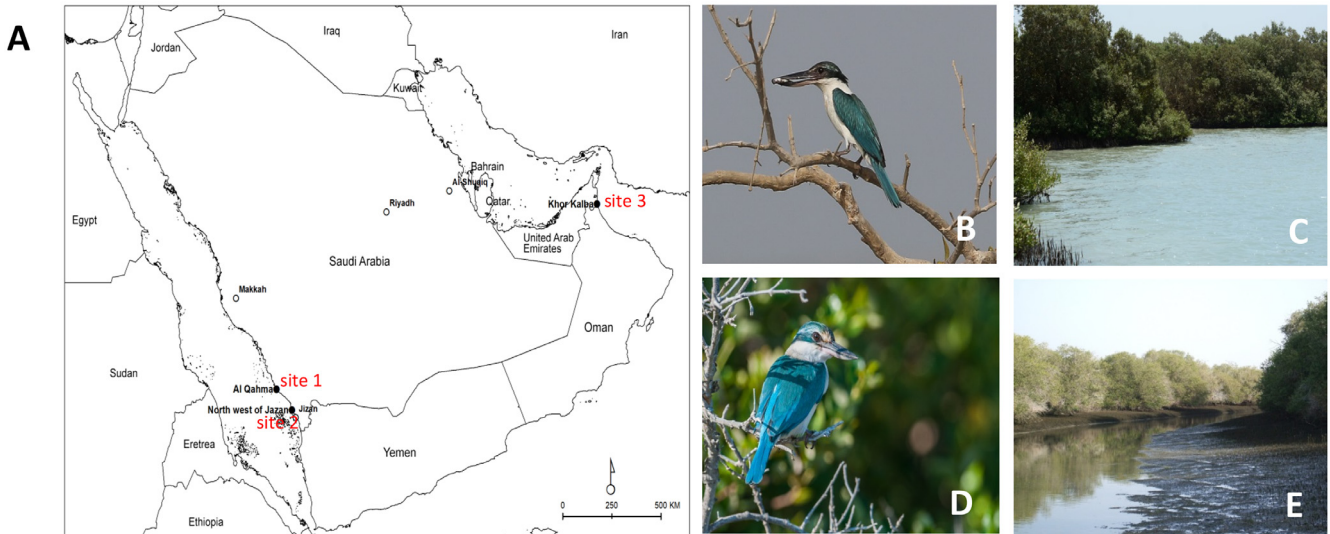
Fig. 1. (A) Map demonstrating the sampling of *Todiramphus chloris* subspecies, used in this study. Black circles represent the three sites of sampling collecting points for the *T. chloris*. Sampling GPS site localities are described in Methods section. (B and C) Representative image of mangrove habitats of *T. chloris abyssinicus*, while, (D and E) Representative image of mangrove habitats of *T. Chloris kalbaensis*. Image B was taken by Jem Babbington and image C was taken by Mohammed Shobrak, whereas, images D and E were taken by Ahmed H. Al-Ali.
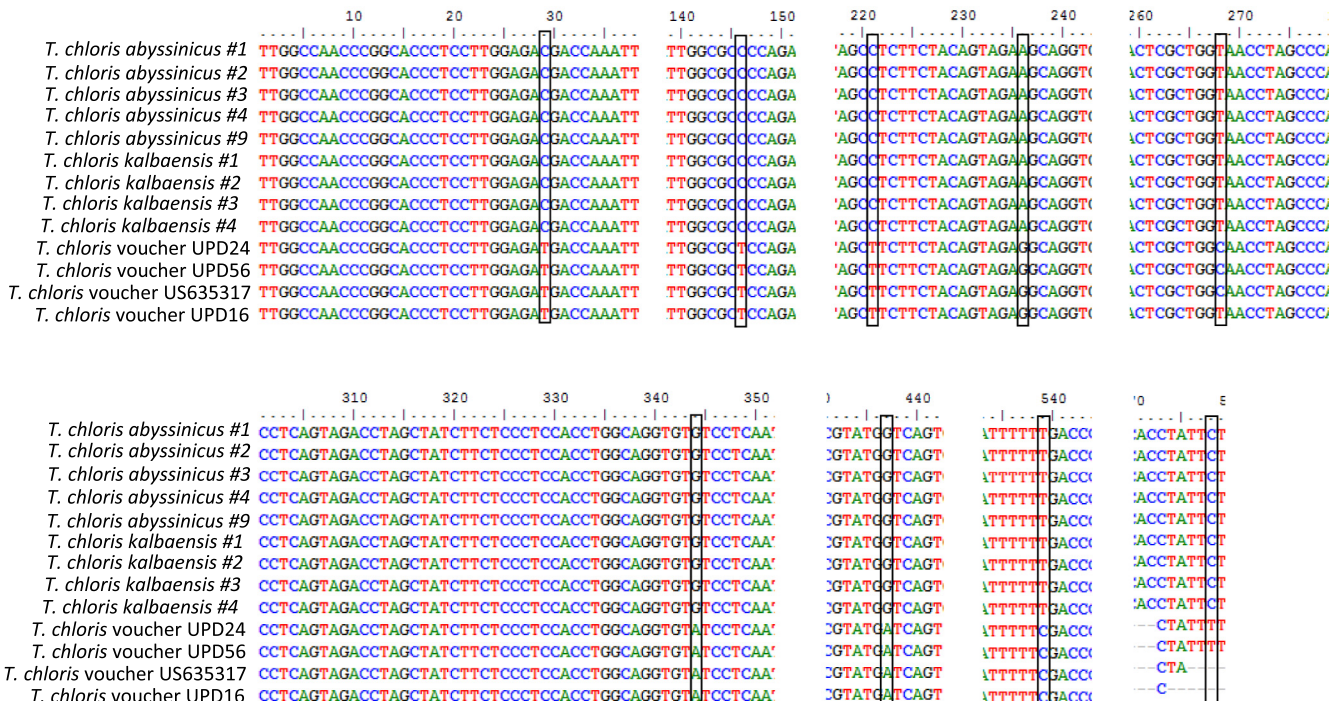
Fig. 2. The alignment of cytochrome oxidase subunit-I nucleotide sequences of selected samples of *T. c. abyssinicus* (5 samples) and *T. c. kalbaensis* (4 samples) with other *T. Chloris* DNA samples from the Gene Bank.

taxonomy may sometimes fail to accurately discriminate between species or subspecies. Therefore, it is important to develop a further rapid and objective method of species identification. Molecular techniques provide a fair more accurate and quantitative method of species designation, allowing identification of all life stages as well as fragments and products of organisms (Hebert et al., 2003). Accordingly, DNA barcoding is used in nucleotide sequences from the mitochondrial genome, has already unraveled a whole wealth of new taxonomic information (e.g. Hebert et al., 2004; Amer et al., 2013; Andersen et al., 2013). We performed this study to molecularly differentiate between two closely related subspecies of Collared Kingfisher, *T. c. abyssinicus* and *T. c. kalbaensis*.

Most DNA barcoding studies accomplish specific tasks through *COI* gene; oftenly considered as the gold standard in animal DNA barcoding. However, earlier studies have documented both *12S* and *ND1* genes which provide robust evidence for species differentiation (Balitzki-Korte et al., 2005; Humphries and Winker, 2011; Cawthorn et al., 2012; Siddappa et al., 2013). Thus, in the present research, we opted three mitochondrial DNA (mtDNA) barcoding genes: the cytochrome *c* oxidase subunit I (*COI*), 12S rRNA (*12S*) and NADH dehydrogenase-2 (*ND1*). Additionally, we carried out a genome fingerprinting analysis using the inter simple sequence repeat-PCR (ISSR-PCR) technique to further explore the genetic differentiation between the two subspecies.

2. Materials and methods

2.1. Sampling

We extracted DNA from 13 individual birds between June 2015 to 2017, consisting of nine samples of *T. c. abyssinicus* from Saudi Arabia Red Sea coast, and four samples of *T. c. kalbaensis* from Kalba

Table 1
Primer sequence, length of the amplified bands and levels of polymorphism among the two populations using ten ISSR primers.

Primer code	Sequence 5′ to 3′	Length of amplified bands	Total amplified Bands	Number of polymorphic Bands	Percentage of polymorphic Bands (%)
ISSR-1	AGAGAGAGAGAGAGAGAGTG	400–700	6	1	16.7
ISSR-2	GAGAGAGAGAGAGAGAGATT	290–750	11	2	18.2
ISSR-3	GAGAGAGAGAGAGAGA	350–2000	14	4	28.6
ISSR-4	AGAGAGAGAGAGAGAGAGT	290–1200	13	3	23.1
ISSR-5	AGAGAGAGAGAGAGAGAGC	230–1100	16	5	21.3
ISSR-6	TCTCTCTCTCTCTCTCC	250–1500	10	1	10.0
ISSR-7	ACACACACACACACACC	300–2000	14	2	14.3
ISSR-8	AGAGAGAGAGAGAGAGTT	350–650	11	4	36.4
ISSR-9	GTGGTGGTGGTGGTGGTG	600–1300	10	1	10.0
ISSR-10	ACACACACACACACACT	390–1100	13	4	30.8
Total			118	27	22.89
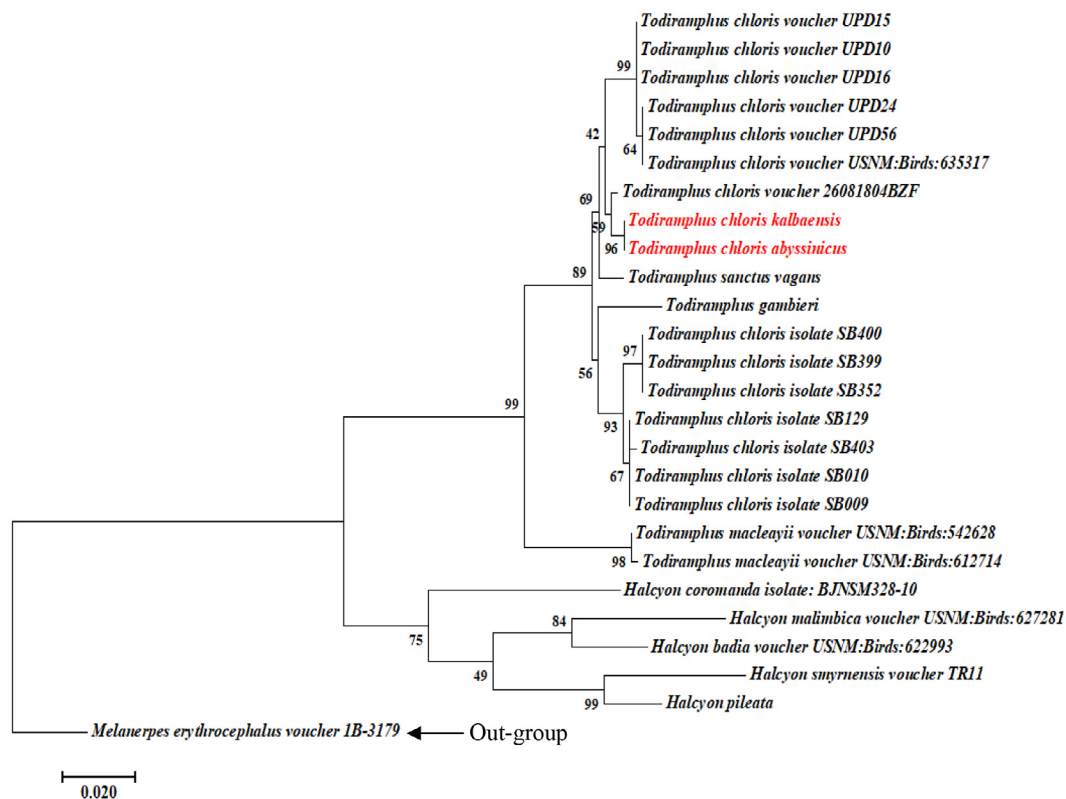
Fig. 3. Molecular phylogenetic tree of the *T. c. abyssinicus* and *T. c. kalbaensis* with different species of the Alcedinidae family based on 800 nucleotides of the *COI* gene using the Maximum Likelihood method (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Numbers by nodes indicate Maximum Likelihood bootstrap. *Melanerpes erthrocephalus* (Picidae) were used as out-group taxa. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). See methods for further details.

Mangroves in the Arabian Sea coast of UAE (Fig. 1). Saudi Arabian samples were collected from two sites along with the Red Sea coast in Jazan and Asir Provinces in southwestern Saudi Arabia in 2015 and 2016. Both sites consisted primarily of mature Gray Mangroves- *Avicennia marina*- with a supreme altitude of 5 m. There are two trapped area, first one (17.9671°N, 41.6770°E) is located nearby Al Qahma area, about 155 km northwest of Jazan. At this site, we collected eight birds (1–8) which trapped on 29 June 2015 from site 1. The bird number 9 was trapped on July 2, 2016 from the area number 2 (17.17648°N, 42.3791°E) which is situated close to Either area, about 25 kms from northwest of Jazan (Fig. 1A). The nine birds were captured by Abdullah Alsuhaibany and Jem Babbington using mist-nets that fixed among mangrove tree, especially wherever new mangrove growth was exist (Fig. 1B and C). Nets were erected over land that inundated only during extreme high tides, and a maximum of 75 m from the water's edge. Throughout all catching times, day by day temperatures were in increased to 40 °C and moisture over 90%. Consequently, mist-nets were fixed before dawn (05:30) and ringing proceeded until late morning when temperatures turned out to be excessively hot and the wind grabbed, making ringing impossible. For the UAE samples, the sample area (24.9888°N 56.3695°E)

was selected as it annually supports the highest density of Arabian Collared Kingfisher nesting sites in Khor Kalba (Fig. 1A, site 3). Khor Kalba is the only mangrove-lined creek on the east coast of the United Arab Emirates. The site supports some of the oldest mangrove trees in the country and has been formally protected under Sharjah's Environment and Protected Areas Authority since 2012 (Fig. 1D and E). Approximately, eight pairs of nests, each breeding season in an area of less than 0.04 square kilometres. Seven revisited nesting cavities occur in the similar area. Old growth mangrove stands with a canopy height of 5–7 m characterizes the sample area. Reduced mangrove density occurs under the old growth canopies. Sampled nest cavities occur exclusively in large older mangrove trees. Kalba is one of the western Indian Ocean's northernmost mangrove habitats. The mangrove vegetation is mature; dense and supports abundant and diverse fauna.

2.2. DNA amplification and sequencing

Total genomic DNA from individual samples was isolated from blood using Qiagen DNase kit (Germany) as described by the producer's directions (Khan et al., 2019). Target regions of the *COI* (950 bp), *12S* (984 bp) and *ND1* (800 bp) genes were amplified
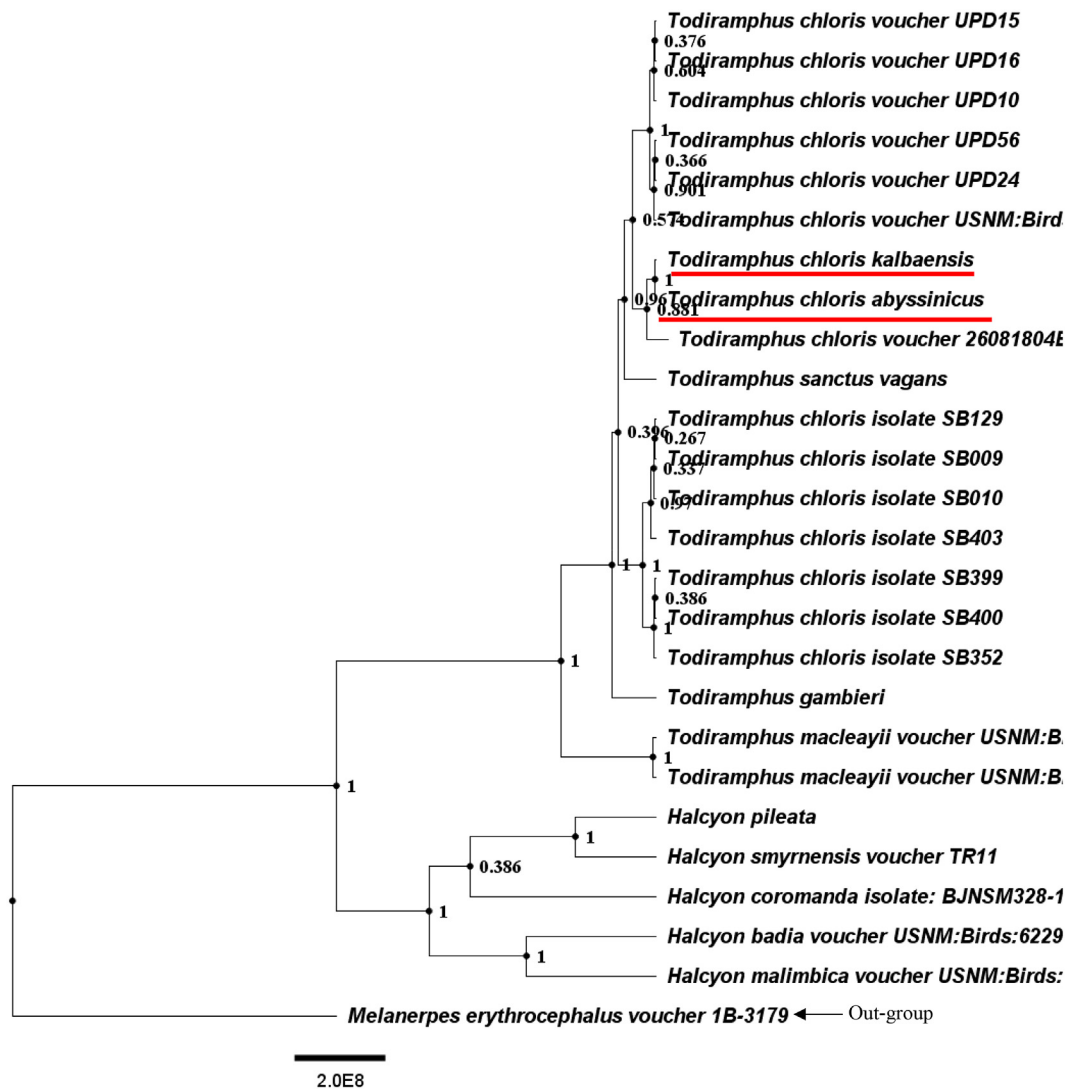

Fig. 4. Bayesian phylogenetic tree analysis of the *T. c. abyssinicus* and *T. c. kalbaensis* with different species of the Alcedinidae based on 800 nucleotides of the *COI*. *Melanerpes erthrocephalus* (Picidae) were used as out-group taxa. Black circles and numbers on nodes represent Bayesian posterior. See methods for the complete details.

predominantly using our designed primers as previously reported (Amer et al., 2013). The final 25-μL PCR reaction mix consists of 12.5 μl GoTaq buffer master mix from Promega (USA), 25 ng of template DNA, 0.2 μM of each amplification primers and up to a final desired volume with deionized distilled water. The PCR thermocycler protocol was achieved as reported previously (Hebert et al., 2004; Luczon et al., 2010; Cawthorn et al., 2012). The specific bands relating to desired genes were excised and cleansed from the gel by Promega kit (Wizard SV Gel and PCR clean up) as designated by the maker's procedure. The cleaned fragments were sequenced on both directions with the same primers and repeated at least three times to ensure reproducible results. Sequencing was achieved using Applied Biosystems 3130 genetic analyzer device (Applied BioSystems, Boston, MA) and BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems) as described by the protocols supplied from the manufacturers. The raw sequencing data were assembled via ABI software data collection version 3.1 (ABI, Applied Biosystems) and the data were analyzed by ABI Seqscape software version 2.7 which was used for base-calling and single nucleotide polymorphism detection. To avoid sequence mismatching, 75 base pair from both ends of each genes were excluded. Therefore, a uniform length of about 800 base pairs for *COI* and *12S* genes or 650 base pairs of *ND1* were aligned by ClustalW in MEGA 7.0 program (Tamura and Nei, 1993; Kumar et al., 2016). Nearest-Neighbor-Interchange heuristic tree was performed using MEGA 7.0 (Kumar et al., 2016). Statistical support for this topology was achieved by running 1000 bootstrap replicates to assess clade credibility (Felsenstein, 1985) in MEGA 7.0. Distance calculations were performed, and the Maximum Likelihood trees were inferred with the Kimura two parameter (K2P) evolutionary model that permits for diverse rates of transition and transversion, but assumes equal nucleotide rates (Kimura, 1980). The Maximum Likelihood method is the typical model used for analyses achieved by the Barcode of Life Data System (BoLD) management system (Ratnasingham and Hebert, 2007; e.g., Hebert et al., 2004; Yoo et al., 2006; Kerr et al., 2007, 2009; Johnsen et al., 2010).

Bayesian phylogenetic tree analysis was accomplished using BEAST v1.8.0 with XML input files made by BEAUti v1.8.0 (Drummond et al., 2012). All Bayesian analyses used four Markov chains with the temperature profile at the default setting of 0.2. The data were partitioned by codon position for the three mtDNA genes. Five independent MCMC runs of one million generations were achieved with a sample taken every 1000th generation. Each file was then handled using BEAST v1.8.0 (Drummond et al., 2012). Nearly, 10% of the samples were omitted before analysis as a burn-in. A maximum clade of credibility tree was made for each analysis using TreeAnnotator v1.8.0 to make a consensus phylogram and posterior probabilities for individual branches. The phylogenetic trees with the best topology were amended with FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. ISSR experiment

A total of 10 ISSR primers (Table 1) were chosen as previously described (Haig et al., 2003; Gómez-Díaz and González-Solis, 2007). These primers were utilized to intensify the inter-repeat regions in the genomic DNA of the 13 individual bird as stated previously (Haig et al., 2003). The amplified DNA were tested in 1.5% agarose gels. The 100 bp DNA Ladder RTU (GeneDireX, Germany) was used as a standard marker to analyse the base pairs for the
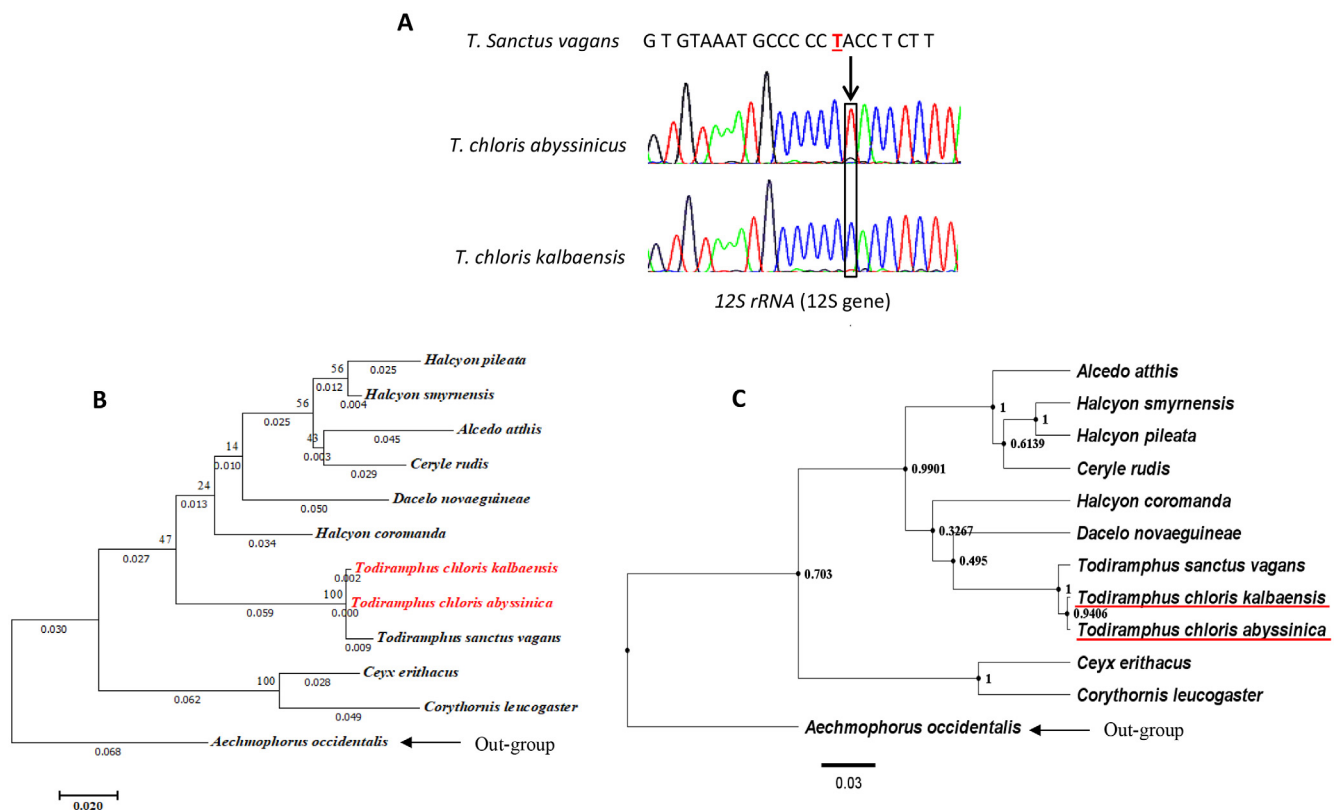
Fig. 5. (A) Detection of the single nucleotide polymorphism by direct sequencing between *T. c. abyssinicus* and *T. c. abyssinicus* based on 800 nucleotides of the *12S* gene. (B) Molecular phylogenetic tree of the *T. c. abyssinicus* and *T. c. kalbaensis* with different species of the Alcedinidae family based on 800 nucleotides of *12S* gene using the Maximum Likelihood method (Tamura and Nei, 1993). Numbers by nodes indicate Maximum Likelihood bootstrap. (C) Bayesian phylogenetic tree analysis of the *T. c. abyssinicus* and *T. c. kalbaensis* with different species of the Alcedinidae based on 800 nucleotides of the *12S gene*. *Aechmophorus occidentalis* (Podicipedidae) were used as out-group taxa. Black circles and numbers on nodes represent Bayesian posterior. See methods for details.

loaded samples. Gels were stained and captured using Gel Doc 2000 (Bio-Rad, Germany).

2.4. ISSR data analysis

Allele size ranges were assessed optically by comparing the length of the bands with a standard 100-bp DNA marker. The resulting product of ISSR-PCR was scored either 0 for absent or 1 for present. The irregular or absent data was scored by 9. Genetic distances and phylogenetic relationship were calculated via Jaccard's similarity coefficient using NTSYS-PC version 2.01 (Rohlf, 2000).

3. Results and discussion

3.1. Sequence attributes and phylogenetic relationships using three mtDNA barcode genes

This study has used three mtDNA barcode genes for molecularly differentiate between two closely related subspecies of Collared Kingfisher, *T. c. abyssinicus* and *T. c. kalbaensis*. An earlier molecular study of *T. chloris* focused on the phylogeny of the species using mtDNA marker (Luczon et al., 2010). It is known that the topologies derived from the results of mtDNA analysis yielded greater resolution than those resulting from the analysis of nuclear introns, therefore, the sequence evolution recovered from mtDNA give a higher rate compared to nuclear DNA (Brown, 1985). Alignment lengths were 800 bp for *COI* and *12S* genes, while 650 bp for *ND1*. Individual gene trees were highly concordant (Figs. 3–6). These alignment lengths should be sufficient for species identification as a seminal avian barcoding analysis of ~40% of

North American species demonstrated that a 648-bp part of the 5′ end of the *COI* was extremely powerful for identification of species (Hebert et al., 2004). The sequence analysis and phylogenetic tree results revealed as *COI* genes of the two subspecies were 100% identical (Figs. 3 and 4). On other hand, *COI* gene barcoding of the two subspecies revealed nine nucleotide variations compared to other *T. chloris* subspecies, specifically, *T. chloris* voucher UPD56, UPD16, UPD24 and USNM: Birds:635317 (GenBank Accession numbers HM622577.1, HM622579.1, HM622578.1 and JQ176503.2, respectively) (Fig. 2). These variations did not disrupt the reading frame because they are all silent mutations.

Due to the lack of sequence differentiation and DNA barcode divergence between *T. c. abyssinicus* and *T. c. kalbaensis* by using *COI* gene, we have applied *12S* and *ND1* genes for possible molecular differentiation between the two subspecies. Numerous studies demonstrate that *12S* and *ND1* are useful DNA barcoding genes for species identification (Balitzki-Korte et al., 2005; Humphries and Winker, 2011; Cawthorn et al., 2012; Siddappa et al., 2013). Interestingly, we found a single-nucleotide polymorphism (SNP) in both genes only in all four individuals of *T. c. kalbaensis*. (Fig. 5A and 6A). Both SNPs can be used to differentiate between the two subspecies (Figs. 5 and 6). However, our results show low genetic variability between the two subspecies, therefore, there is a possibility that the low subspecies are diverged very recently. On other hand, several studies confirmed the efficacy of mtDNA genes in the differentiating between subspecies within evolutionary lineages. The results of the current study exhibited some subspecies were differentiated through SNP (Arias and Sheppard, 1996; Ilyasov et al., 2016; Aliabadian et al., 2013; Bilgin et al., 2016).

DNA barcoding could be used for delineating species by using a species threshold concept in which sequences are regarded as
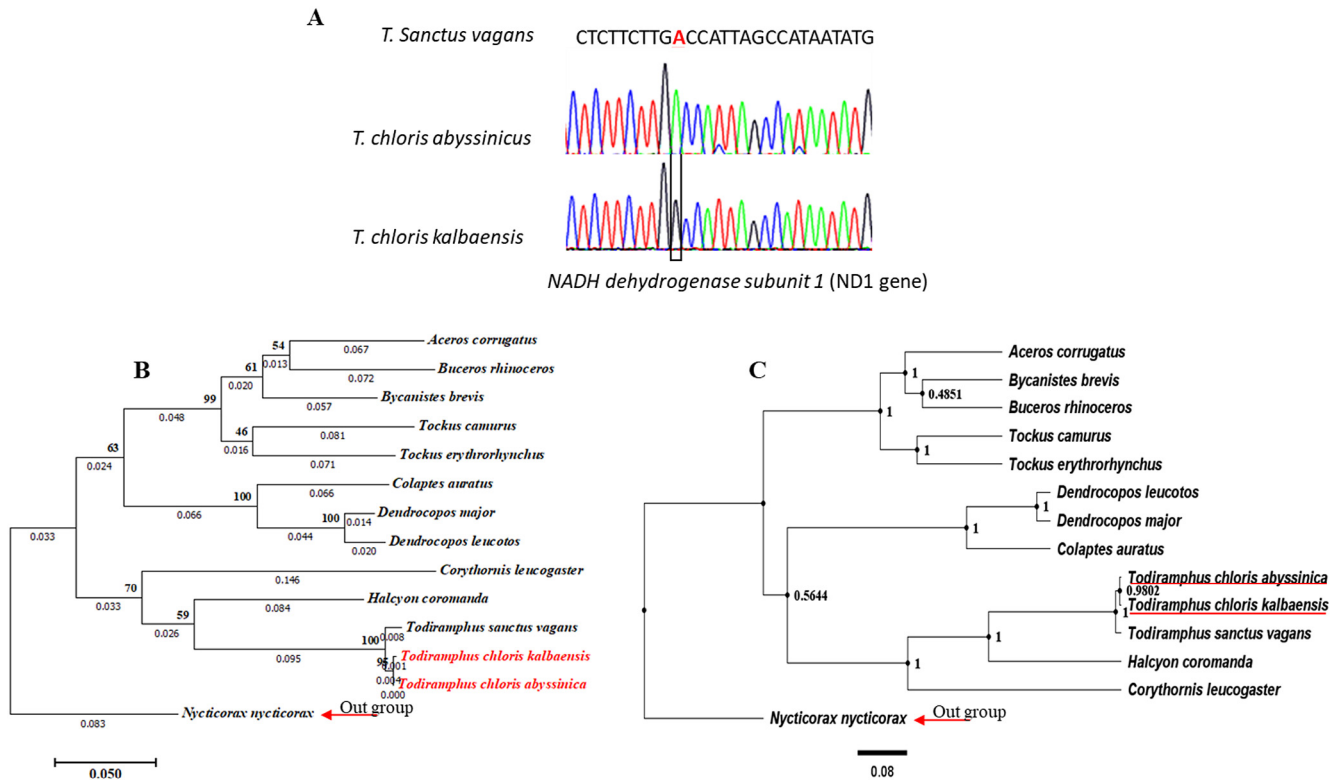
Fig. 6. (A) Detection of the single nucleotide polymorphism by direct sequencing between *T. c. abyssinicus* and *T. c. abyssinicus* based on 650 nucleotides of the *ND1* gene. (B) Molecular phylogenetic tree of the *T. c. abyssinicus* and *T. c. kalbaensis* with different families, including Alcedinidae based on 650 nucleotides of *ND1* gene using Maximum Likelihood method (Tamura and Nei, 1993). Numbers by nodes indicate Maximum Likelihood bootstrap. (C) Bayesian phylogenetic tree analysis of the *T. c. abyssinicus* and *T. c. kalbaensis* with different species of the Alcedinidae based on 650 nucleotides of the *ND1* gene. *Nycticorax nycticorax* (Ardeidae) were used as out-group taxa. Black circles and numbers on nodes represent Bayesian posterior. See methods for details.

belonging to distinct species if they differ either (i) by more than ten times the minimum intraspecific variation or (ii) beyond an empirically determined divergence limit (i.e., 2.7%) (Hebert et al., 2004). Significantly, the sequences of the *COI* gene for *T. c. abyssinicus* and *T. c. kalbaensis* differ by only 1.28% from *T. sanctus,* which is less than the half of empirical divergence limit of 2.7%. This suggests that *T. c. abyssinicus* and *T. c. kalbaensis* might belong to the similar species as *T. sanctus.* On contrary to Hebert et al. (2004) concept, we believe that DNA barcoding is a species identification method, but not a species delimitation method. The DNA barcoding

sequences was used to have a first idea of the level of genetic differentiation of the populations. Species delimitation is a much more complex process that, if done using molecular data, it has to incorporate multi-locus coalescent analyses including mitochondrial and nuclear genes.

As shown between the Figs. 3–6, the Maximum Likelihood and Bayesian analysis phylogenetic trees of the Alcedinidae family were based on 800 nucleotides of *COI* and *12S* genes or 650 of *ND1* gene found that *T. chloris* and *T. sanctus* are most likely conspecific and should be flagged for taxonomic reevaluation, thus
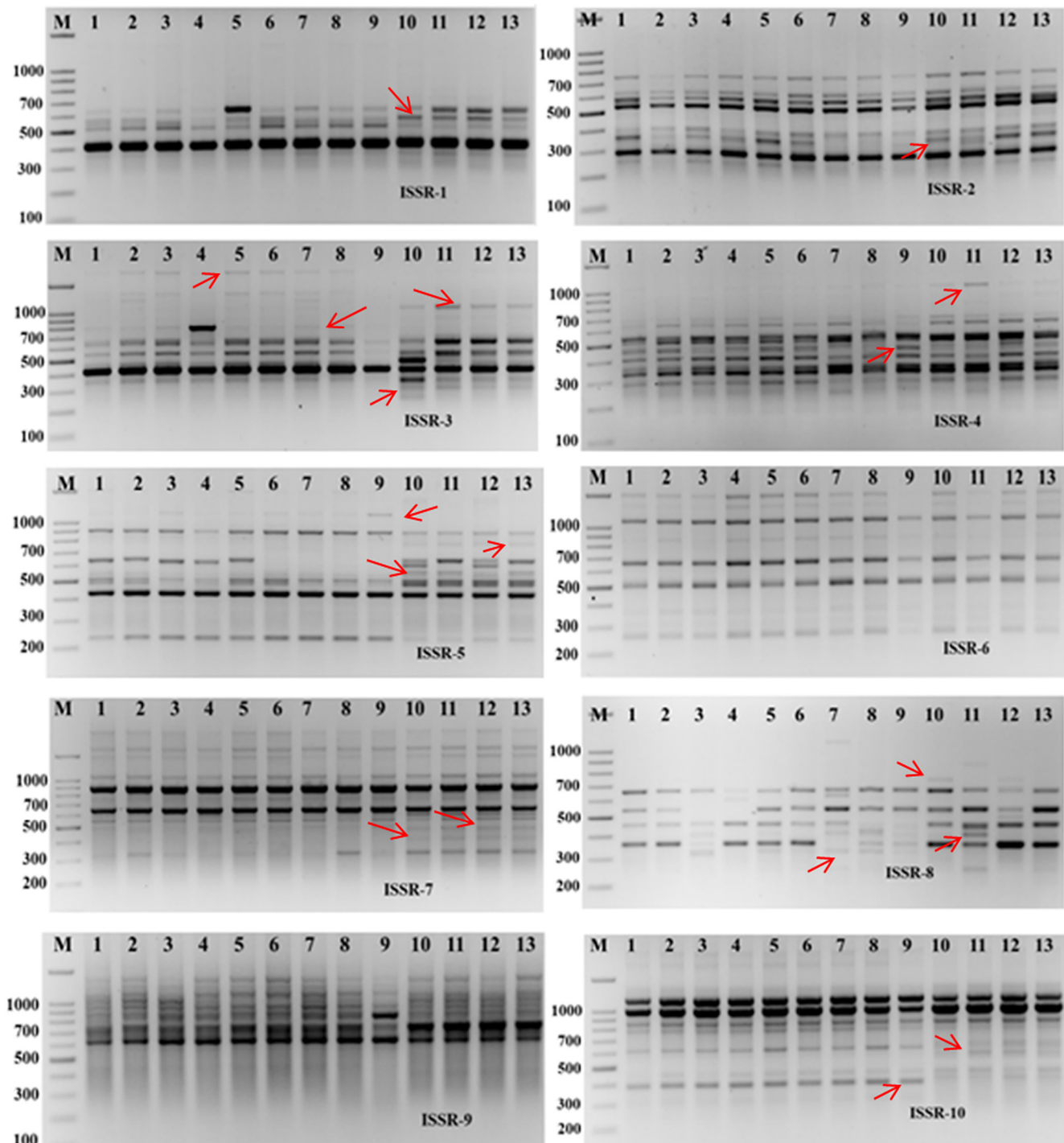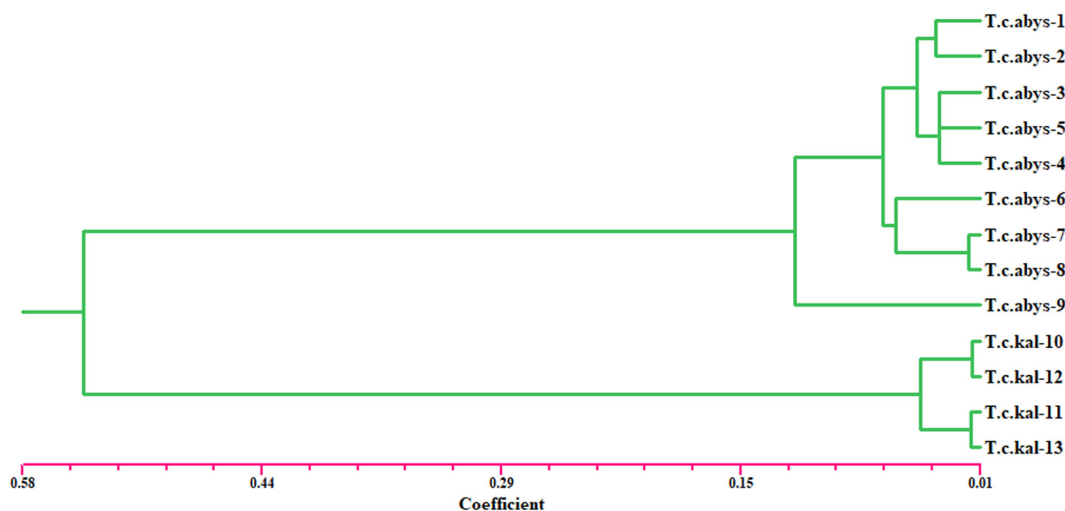

Fig. 7. Amplification profile of DNA detecting ISSR primers fragments in 13 *T. chloris* samples (from 1 to 9 for *T. c. abyssinicus* and from 10 to 13 for *T. c. kalbaensis*). The red arrows indicate a unique positive or negative band. Lane M is a 100 bp DNA ladder.

Fig. 8. Dendrogram analysis of the 13 individual samples (1 to 9 for *T. c. abyssinicus* and 10 to 13 for *T. c. kalbaensis*) from cluster analysis (UPGMA) based on genetic similarity estimated from the ten ISSR markers.

supporting the similar results of Andersen et al. (2015), which have documented the *T. chloris* complex is paraphyletic comprising as many as 10 distinct species. To settle the real taxonomy of these two subspecies and to unravel their genetic relationships, future genomic studies using thousands of loci should be used.

3.2. ISSR-PCR markers and genetic variation

ISSR is a powerful DNA molecular marker for phylogenetic studies, genome evolution and diversity analyses (Arif and Khan, 2009; Labastida et al., 2015; Buhroo et al., 2018). This research represents the first usage of ISSR genotyping technique to measure the genetic variation of two different populations of *T. chloris* subspecies from the Arabian Peninsula. From the recent literature, ISSR primers were recognized as a repeat nucleotide motif between two-five nucleotides with a random attaching sequence of one to three nucleotides (Askari et al., 2011; Lin et al., 2012). Here, 90% of the primers were based on di-nucleotides (Table 1). Several reports stated that di-nucleotide repeats are more agreeable to ISSR investigation than other nucleotide repeats (Askari et al., 2011; Moresco et al., 2013). The selected ISSR primers gave 118 bp amplified bands (Fig. 7). The average number of amplified bands was 11.8/primer. Averagely 22.9% of the bands were polymorphic, while 77.1% was monomorphic. Table 1 illustrated the total number of amplified bands that counted for each ISSR. All amplified bands were used for documentation of genetic similarities and thus constructing the phylogenetic tree for the two subspecies (Fig. 8). ISSR primers numbers 3, 8 and 10 produced the maximum polymorphic markers within the 13 samples. Primer ISSR 5 produced 16 bands, while primer ISSR 1 have amplified the 6 bands (Table 1). As shown in Fig. 5 with the red arrows, there are several specific amplified loci appeared as a unique positive or negative band that may have impact to use for differentiating between the two subspecies.

Use of the ten primers allowed us to use UPGMA to cluster the 13 *T. chloris* individuals into two groups (Fig. 8). The first cluster was for the nine samples of *T. c. abyssinicus*, while the second cluster was for the four samples of *T. c. kalbaensis*. Interestingly, the cluster number one for the *T. c. abyssinicus* was divided into two sub-clusters according to the sample's area of collection (Figs. 1 and 8). The capability of ISSR markers to separate the two subspecies confirmed the possibility of ISSR technique to distinguish the genetic identification of *T. chloris* subspecies. These results demonstrate the potential and efficacy of ISSR profiles to accurately assess genetic identification between *T. chloris* subspecies.

4. Conclusion

The present study result concludes as partial sequences of three mitochondrial genes were used to determine the molecular relationship between two Arabian subspecies of Collared Kingfisher *T. chloris* (purportedly *T. c. abyssinicus* and *T. c. kalbaensis*). A SNP exists in the *12S* and *ND1* gene regions sequenced between the two subspecies, which suggests as these two genes could be used to differentiate two subspecies. Therefore, the current research finds a molecular method through DNA Barcoding to identify the two different subspecies and shows that *COI* does not effort but that *12S* and *ND1* work. Interestingly, the sequences of the *COI* gene for the two Arabian subspecies differ by only 1.28% from Sacred Kingfisher *T. sanctus*, which implies that the Arabian subspecies are closely related to Sacred Kingfisher. Finally, the use of the highly polymorphic nuclear markers shows a great ability to distinguish between the subspecies of the two samples with accuracy.

Declaration of Competing Interest

The authors declare no conflicts of interests.

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