Journal of Aquatic Research and Sustainability 1 (1): 8-14

Content available at Genesis Publishing Consortium Limited

Journal of Aquatic Research and Sustainability

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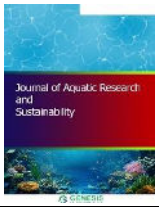

Original Research

Molecular identification of some species of dragonfly (Odonata: Anisoptera) using COXI gene and DNA barcodes from Basrah Province, Southern Iraq

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Article info

Article history
Received: 29 June 2024
Revised: 13 August 2024
Accepted: 18 August 2024
Published: 30 August 2024

Keywords
Anisoptera
Specific primer
COXI gene
DNA barcode
Iraq
Odonata

Abstract

The taxonomy of the Odonata group is still poorly understood; therefore, molecular identification has been developed to address the shortcomings of traditional taxonomy, such as misidentification and inconsistencies in reference databases. The mtCOXI gene has been used in animal barcoding studies because of its wide range of phylogenetic signals. Odonata, a diverse group of aquatic insects, has been studied at the molecular level to understand the evolutionary relationships between their species and global species. In the current study in Basrah Province, Iraq, we extracted mitochondrial DNA from adult and nymph Dragonflies and designed species-specific primers to distinguish some of the endemic species at the molecular level. This study identified five species, *Crocothemis erythraea*, *Diplacodes trivialis*, *Orthetrum sabina*, *Trithemis annulata* and *Hemianax ephippiger*. The DNA sequences were deposited in the NCBI database for the first time. The nucleotide sequences of the mtCOXI genes were analyzed via BLAST. The similarity results ranged from 91.8% to 100%, indicating related species on the basis of the branches of the phylogenetic tree. The molecular identity of the selected species was confirmed, and DNA barcodes for Anisoptera species from Basrah Province were successfully developed and documented in GenBank and IBIN.

1. Introduction

The limitation of phenotypic taxonomy, which is based on the ability of morphological characteristics to distinguish species, often leads to incorrect identification, especially for cryptic species, which require high levels of expertise (Vega-Sánchez *et al.*, 2020). In recent decades, molecular taxonomy has helped to resolve the "classification crisis", during which many scientists have suggested the use of phylogenetic taxonomy instead of morphological identification (Kozlov *et al.*, 2016).

Odonata are a highly diverse group of aquatic insects. This order is primitively divided into two suborders, Anisoptera and Zygoptera. They are predatory insects that help control harmful aquatic organisms. During their larval stage (called naiads), they spend most of their time in various freshwater environments, such as shallow ponds, swamps, and rivers. When they reach adulthood, they transform into flying terrestrial insects with colors that vary from bright to pale. They are often found near wet areas, and some species are migratory (Attaullaha *et al.*, 2021).

DNA barcoding is one of the most common taxonomic systems that has been particularly successful in diagnosing and identifying new species from different groups (Koroiva *et al.*, 2022). Identification via this method, which relies on sequence information from short fragments of primary DNA sequences, can be performed simply and affordably, without the need for a taxonomist in the group.

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doi: https://doi.org/10.69517/jars.2024.01.01.0003

In addition, the success of DNA-based species identification strategies can provide comprehensive information about the geographic and genetic diversity of the population of interest (Gaytán-Pinzón *et al.*, 2022).

The DNA sequences of each species are unique; they can be viewed as "barcodes" and are used to resolve misidentifications, which may cause taxonomic inconsistencies inherent in the type of taxonomy practiced to date in reference databases (Salvi *et al.*, 2020). Several genes, including those encoding the mitochondrial protein COXI and 16S and 28S rRNA, have been selected for use in animal barcoding studies because they possess a wide range of phylogenetic signals and exhibit rapid nucleotide substitution rates, which help distinguish between cryptic species and reveal phylogenetic structures within a species (Jackson *et al.*, 2014; Luo *et al.*, 2022).

The COI universal primers are very powerful, allowing the construction of demonstrative sequences for most animal groups (Kim *et al.*, 2014), whereas Abdullah *et al.* (2024) reported that the use of specific primers provides more precise and reliable quantification results where the design of appropriate target-specific primers avoids primer matches with nontarget species and allows undesired amplification.

Previous diagnostic studies did not survey entire genera of Odonata, were based on a small number of characters or did not use established phylogenetic methods (Bybee *et al.*, 2021). Recently, the evolutionary relationships of the Odonata group have been studied on a molecular basis, with increasing emphasis on molecular genetics and even the lowest ordinal level (Dijkstra *et al.*, 2014). However, the lack of large-scale studies based on high-throughput data that can be linked to an evolutionary perspective has prevented comparative

questions about the evolutionary levels of these insects (Letsch and Gottsberger, 2016).

Libellulidae is the most species-rich family within Anisoptera, with more than 900 described species distributed worldwide and found in all aquatic habitats and biota. The incidence of sexual dimorphism is high in this family because it adapts physiologically and morphologically during its life cycle to facing unsuitable conditions in the environment (Casas *et al.*, 2018).

Some local studies have addressed the taxonomy and ecology of Odonata adults of several Anisoptera and Zygoptera species, such as Ali and Khidhir (2015), who studied Odonata adults in northern Iraq, and Abd and Al-Asady (2014). Al-Hashmi (2017) studied central and southwestern Iraq; however, for naiads of some Odonata species, there are morphological identification studies by Darweesh (2018) and Ahmed and Kareem (2019; 2020; 2024), who studied the morphological identification of Odonata adults and nymphs in Basrah Province, southern Iraq. However, no molecular studies on this insect group of Iraq have been recorded in public databases such as BOLD, with the exception of one study by Geraci *et al.* (2011), who documented information about the genetic sequences of some Odonata species in the marshes of Iraq at the National Center for Biotechnology Information (NCBI). Our study aimed to obtain accurate identification and more detailed confirmation of the molecular genetics of some endemic species of Anisoptera from Basrah Province as a base step for documenting the DNA sequence information of the Odonata mtCOXI gene in the national GenBank via the BOLD system and DNA barcodes.

2. Materials and Methods

2.1 Ethical approval

No ethical approval is required for this study.

2.2 Sampling

Samples of 50 individuals of dragonflies (order Odonata) belonging to 5 species were collected from wetland regions with geographical extents of 30°25 45.1'N–47°55 52.1'E in Basrah Province, southern Iraq, between May and December 2018 (Figure 1). Dragonfly adult samples were collected randomly from the field by hand or with aerial nets, whereas the nymphs were collected via an aquatic insect net or sieve (30 cm diameter and 1 mm mesh size) and preserved by freezing at -15°C after each species was placed in a separate collection bottle with the assigned date of collection and location (Ahmed and Kareem, 2019). Morphological identification was performed according to Degabriele (2013).

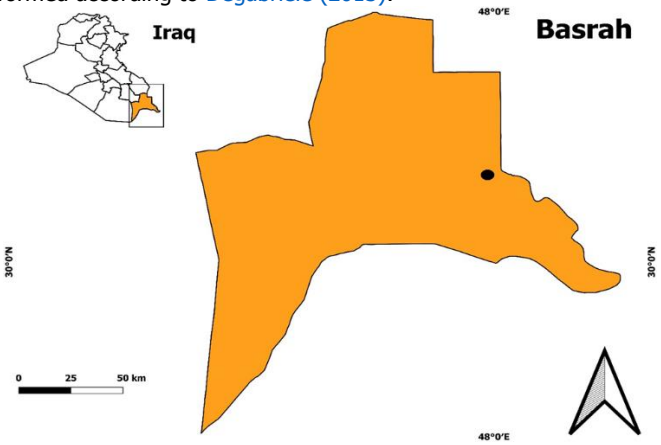

Figure 1. Samples were collected from Basrah Province, southern Iraq.

2.3 Primer design

Species-specific primers were designed by downloading sequences from selected species of Odonata on the National Center for Biotechnology Information (NCBI) website to choose nucleotide-based arrangements of mitochondrial COI gene markers (Lim *et al.*, 2011). The downloaded sequences are then imported into the BioEdit application; to perform multiple alignments on the acquired target and nontarget sequences, the ClustalW option in the BioEdit application was chosen, and pieces of 144--327 bp nucleotide bases are then entered into the Primer3Plus website to obtain some recommendations for forward and reverse primer pairs to be used.

We found that the optimum primer pair can be determined through criteria based on the %GC and Tm (melting temperature) values, where the web tool of NCBI's Basic Local Alignment Search Tool (BLAST) is the best option for performing primer specification validation, as summarized in Table 1.

Table 1. Details of selected species of Dragonflies, target genes and nucleotide sequence primers used in this study.

Species	Gene	Primer	Length	Product size	Tm.
Crocothemis erythraea	mtCOX1	F GTTATTGTAACCGCCCATGC R GGGTAGACTGTTCACCCAGT	20 20	224	59.7
Diplacods trivialis	mtCOX1	F CATCTTTGGAGCATGGGCAG R AGTCGTGGGAAGGCCATATC	20 20	231	59.5
Hemianax ephippiger	mtCOX1	F ACCTGATATGGCTTTCCCACG R GCAATTGCACCAGCAAGAGG	21 20	144	59.4
Orthetrum sabina	mtCOX1	F GGGCAGGTACTGGATGAACTG R AGATAGGGTCTCCTCCTCCCG	21 21	327	60
Trithemis annulata	mtCOX1	F CCGAATTGAACTAGGACAGCC R CCTGCCCCTCTTTCTACCAT	21 20	258	58.7

2.4 DNA extraction and amplification

Mitochondrial DNA was dissected following the manufacturer's instructions for a DNA extraction kit (gSYNC™ DNA extraction kit; Geneaid, Taiwan). From frozen samples of selected dragonflies, the thoracic and legs of adults and some nymphs were dried with liquid nitrogen and then crushed or ground as much as possible via a ceramic mortar; the powder was lifted to remove the remaining coarse parts. Each sample weighed 0.20 grams and was placed into a 1.5 ml microcentrifuge tube. The sample genetic material was then isolated or stored at -20°C if not used immediately. The extraction results revealed that the concentrations of the genetic material ranged from 6.2--58 ng/µl and that the purity ranged from 1.73-1.98 (Table 2).

Table 2. Concentration and purity of the genetic material (DNA) extracted via NanoDrop.

Sample	Purity	Conc. ng/µ	Sample	Purity	Conc. ng/µ	Sample	Purity	Conc. ng/µ
1	1.91	7.7	**11**	1.78	7.0	**21**	1.81	7.5
2	1.85	8.4	**12**	1.82	9.3	**22**	1.89	9.5
3	1.95	32	**13**	1.91	23	**23**	1.97	30
4	1.96	54	**14**	1.92	13	**24**	1.96	50
5	1.92	7.5	**15**	1.90	6.2	**25**	1.93	6.6
6	1.89	9.1	**16**	1.94	11.5	**26**	1.92	10.7
7	1.78	40	**17**	1.82	33	**27**	1.73	58
8	1.79	6.9	**18**	1.90	7.2	**28**	1.89	8.5
9	1.82	9.1	**19**	1.98	10.3	**29**	1.80	9.5
10	1.80	27	**20**	1.79	32	**30**	1.88	45

2.5 Molecular analysis, PCR and sequencing

In accordance with the methods of Lee *et al.* (2012), the DNA samples were analyzed via agarose gel (1%) electrophoresis and stained with ethidium bromide. Specialized primers were synthesized for each selected Anisoptera species from Basrah Province for the first time. The primers were prepared by the Bioneer Company as a dried product at different concentrations. The primers were dissolved in free water (DNase/RNase) to reach a final concentration equivalent to 100 pmol/microliter, and the stock solution was prepared at a concentration equivalent to 10 pmol/microliter for analysis. In addition, several experiments were conducted to obtain the Tm and Ta temperatures of the primers and to determine the optimal temperature that gave the best results via polymerase chain reaction (PCR).

The optimal concentration of the template DNA was determined by comparing the brightness of the amplified fragment with that of a band from a size marker (5000 bp) in agarose gel electrophoresis.

The amplified PCR products (20 µl for each species) were sequenced in both directions, forward and reverse, by using an automated sequencer Genetic Analyzer by Yang Ling Biotechnology Co. (China).

2.6 Phylogenetic identification

The PCR products were analyzed via Bioinformation BioEdit (software V.7.2.6, Network V.5, Mega 7.0), aligned and filtered, and then a Basic Local Alignment Search Tool (BLAST) search was performed on the online portal of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), the sequence matrices of the species were constructed for identity and homology of the analyzed sequences.

2.7 Molecular sequence products in GenBank

To obtain accession numbers from NCBI, representative sequences of the partial COI gene for each species of Anisoptera (nymph or adult) from Basrah Province were deposited for the first time in the DNA Data Bank of Japan (DJB) database (which is a biological database that collects DNA sequences located at the National Institute Genetics Institute in Shizuoka, Japan). It is a member of the International Nucleotide Sequences Collaborative Database (INSD) and exchanges its data with the European Biology Laboratory (EMBL) and the National Center for Biotechnology Information (NBCI) daily; thus, researchers can register and upload sequence data of their sample to GenBank at any of these centers, which will be saved in the global database.

Each DNA sequence of the diagnostic species from Basrah was matched with the recorded species in NCBI from different geographic regions of the world, the percentage of identity was determined via BLAST, and a phylogenetic tree was drawn. The evolutionary development of the species was compared with that of identical species in other geographical regions, and the genetic distance between them was calculated.

2.8 Barcodes of molecular sequence products

The DNA barcode images of the sequences submitted were developed via web-based https://ngdc.cncb.ac.cn/databasecommons/ database/id/4609 barcode at Insect Barcode Informatica (IBIn), NBAII database- India, by uploaded to the Barcode of Life Data (http://www.boldsystems.org). The required information for submission of the barcode of the target gene includes the order, family, and species of the insect for which the barcoding has been performed; the longitude and latitude of the location of the collected samples; the country; the barcode marker (name of the gene); the source (reference); the author name and institute address; the nucleotide sequence; and the image of the insect. Once the submission of the barcode record was performed, a unique barcode image was automatically created. Immediately after the submission of the barcode record, e-mail alerts are sent automatically to the experts through database administrative members.

3. Results

The five species of Anisoptera (Figure 2) that were morphologically identified as the species *Hemianax ephippiger* (Family: Aeshnidae), *Diplacods trivialis*, *Crocothemis erythraea* (naiad), *Orthetrum sabina* naiad, *O. sabina* adult and *Trithemis annulata* (Family: Libellulidae), and their scientific taxonomy are as follows,

Order: Odonata
Suborder: Anisoptera
Family: Aeshnidae
Genus: *Hemianax* Selys, 1883
1. *Hemianax ephippiger* Burmeister, 1883
Family: Libellulidae
Genus: *Crocothemis* Brauer 1868
2. *Crocothemis erythraea* (Brulle, 1832)
Genus: *Diplacodes* Kirby, 1889
3. *D. trivialis* (Rambur, 1842)
Genus: *Orthetrum* Newman, 1933
4. *Orthetrum sabina* (Drury, 1770)
Genus: *Trithemis* Brauer 1868
5. *Trithemis annulata* (Palisot de Beauvois, 1807)

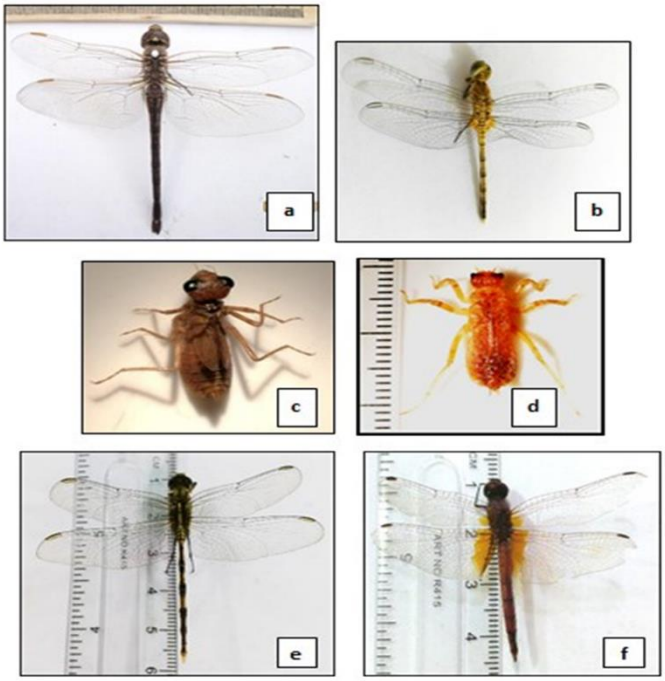

Figure 2. Species of Anisoptera (Odonata): a. *Hemianax ephippiger* (Family: Aeshnidae); b. *Diplacods trivialis*; c. *Crocothemis erythraea* (naiad); d. *Orthetrum sabina* naiad; e. *Orthetrum sabina* adult; f. *Trithemis annulata* (Family: Libellulidae) from Basrah Province.

The results of electrophoresis on agarose gels confirmed the success of amplifying the cytochrome oxidase mtCOXI gene via primers specific for each species of selected dragonflies, as the results revealed DNA bands with molecular weights ranging from 144--327 base pairs (Figure 3).

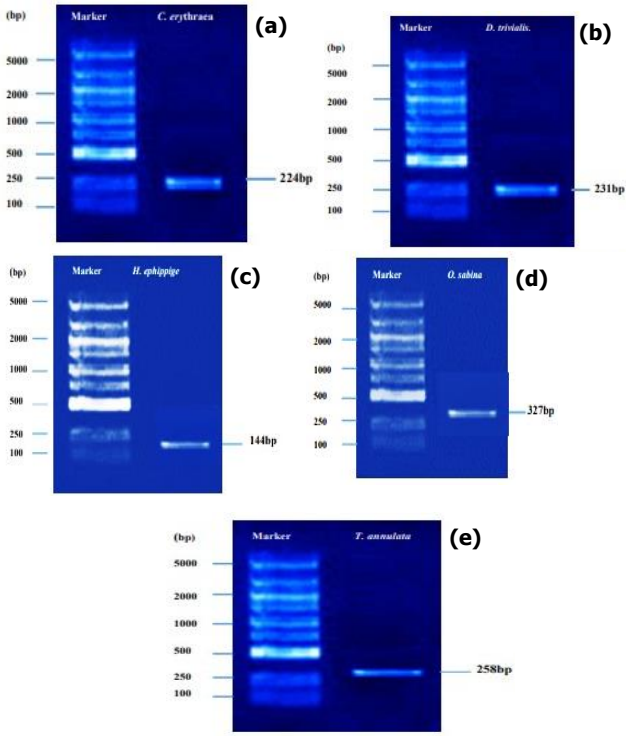

Figure 3. Amplification product of the mtCOX1 gene primer on an agarose gel for a. *Crocothemis erythraea*; b. *Diplacodes trivialis*; c. *Hemianax ephippiger*; d. *Orthetrum sabina* and e. *Trithemis annulata*.

3.1 Genetic analysis

Details of the alignment and filtering of the genetic sequence via Maga7.0 are shown in Figure 4, which illustrates the results of the DNA sequence of the COXI gene and the number of nucleotide bases for each selected species of Anisoptera from Basrah Province, Iraq.

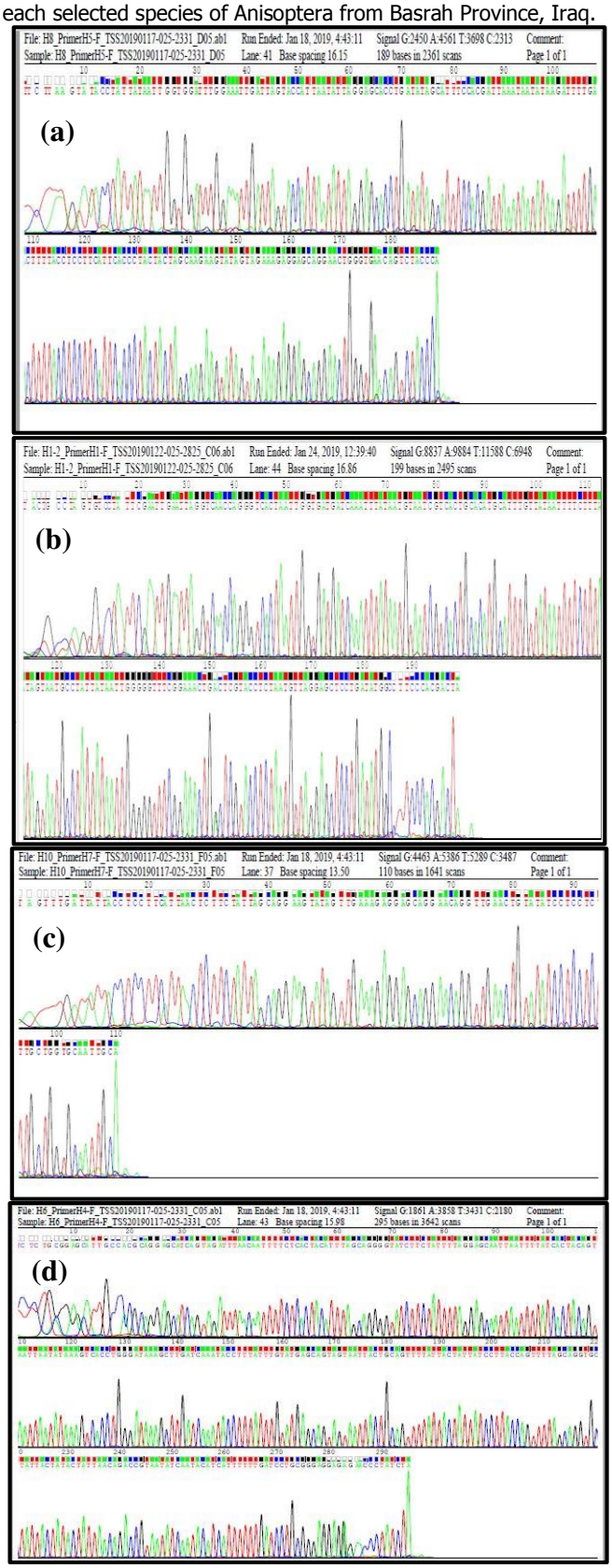

Figure 4. Genetic analysis of the mtCOXI gene in the species a. *Crocothemis erythraea*; b. *Diplacodes trivialis*; c. *Hemianax ephippiger*; d. *Orthetrum sabina*; e. naiad of *Orthetrum sabina* and f. *Trithemis annulata*.

The molecular identities of five species, *Crocothemis erythraea*, *Diplacodes trivialis*, *Orthetrum sabina*, *Trithemis annulata*, and *Hemianax ephippiger,* were established, and the sequences were deposited in the NCBI database for the first time (Table 3).

Table 3. Similarity percentage of mtCOX1 for the selected species of Anisoptera from Basrah Province, with the accession numbers of similar species in the GenBank database.

Species	Target gene	GenBank accession number	Similarity within species
Crocothemis erythraea	mtCOX1	KY847583	%100
Diplacodes trivialis	mtCOX1	KT957513.1	%91.8
Orthetrum sabina	mtCOX1	MG88491.1	%100
Trithemis annulata	mtCOX1	Ku566417.1	%99.10
Hemianax ephippiger	mtCOX1	AB708598.1 KC912217.1	%100 %99.06

The nucleotide sequences of the mtCOXI gene were subjected to BLAST, and DNA barcodes were successfully developed for selected Anisoptera species from Basrah. The results revealed 91.8% to 100% similarity to the NCBI database of Odonata species, and the molecular identity of our species was confirmed.

The total length of the product varied from species to species and ranged from 295-111 bp. The final analyzed sequences were submitted to GenBank with an accession number (Table 4).

3.2 Phylogenetic tree

The neighbor-joining (NJ) tree was inferred via MEGA 0.7 software to determine the evolutionary history, with evolutionary distances calculated via the composite maximum likelihood method (Ibragimov

and Has' Minskii, 2013). Units of the number of base substitutions per site were measured per site.

Table 4. The sequences of the Anisoptera species from Basrah Province were submitted to GenBank and IBIN.

Species	Target gene	Product size	GenBank accession numbers	IBIN (Barcodes ID)
Orthetrum sabina adult	*mtCOX1*	285 bp	LC476968 https://www.ncbi.nlm.nih.gov/nuccore/LC476968	NBAII-BC-858
Orthetrum sabina naiad	*mtCOX1*	295 bp	LC476969 https://www.ncbi.nlm.nih.gov/nuccore/LC476969	NBAII-BC-858
Crocothemis erythraea naiad	*mtCOX1*	189 bp	LC476970 https://www.ncbi.nlm.nih.gov/nuccore/LC476970	NBAII- BC-859
Trithemis annulata adult	*mtCOX1*	223 bp	LC476971 https://www.ncbi.nlm.nih.gov/nuccore/LC476971	NBAII- BC-860
Hemianax ephippiger adult	*mtCOX1*	111 bp	LC477206 https://www.ncbi.nlm.nih.gov/nuccore/LC477206	NBAII- BC-861
Diplacodes trivialis adult	*mtCOX1*	199 bp	LC477207 https://www.ncbi.nlm.nih.gov/nuccore/LC477207	NBAII- BC-862

The analysis involved five nucleotide sequences, and any ambiguous positions for each sequence pair were excluded. Figure 5 shows the evolutionary relationships between the species of selected odonates in the present study and their corresponding species in countries around the world. Two main branches were divided into five secondary branches. The first branch was divided into two branches. The first branch included the species *Diplacodes trivialis* from Iraq and the species *D. nebulosa* from Thailand. The second branch included two strains of the species *Trithemis annulata* from Iraq and Africa. The third branch isolated two strains of *Crocothemis erythraea* from Iraq and Namibia. The fourth branch included two strains of *Orthetrum sabina* from Iraq and Singapore. The fifth branch included *Anax ephippiger* from Namibia and its synonymous *Hemianax ephippiger* from Iraq. The genetic ratio between species in the branches of the evolutionary tree ranged from 0.000--0.98, with a scale bar of 0.05. Evolutionary analyses were conducted in MEGA 7 as previously described (Kumar *et al.*, 2016).

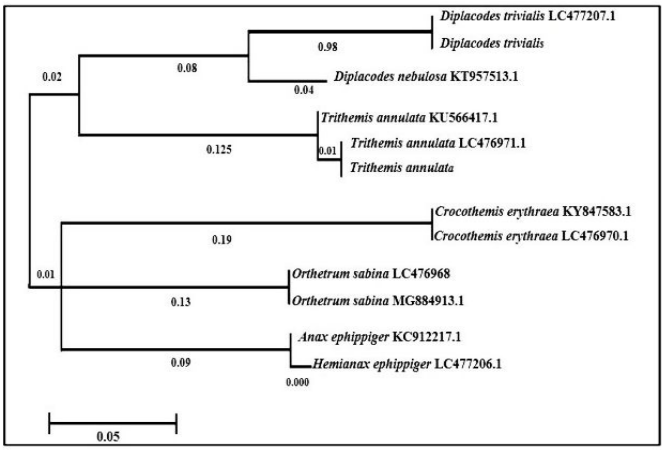

Figure 5. Phylogenetic tree of the selected Anisoptera species from Basrah Province related to global species via the NJ method.

3.3 DNA Barcodes

A barcode for the genetic sequence and a special reference number for each species were recorded in the NBAII database, as shown in Figure 6, on the basis of the documented information in GenBank.

4. Discussion

We designed a specific primer for each selected Anisoptera species from Basrah Province to provide more accurate and specific matches with target species. In agreement with recent molecular studies, this study revealed that the COX gene could help identify Odonata when it was used as the basis of the universal bioidentification system for animals at the regional scale (Jin *et al.*, 2022; Koroiva *et al.*, 2022).

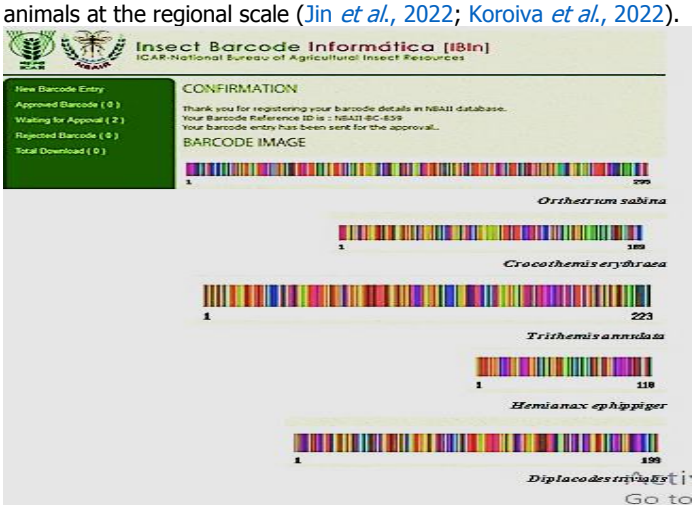

Figure 6. Barcodes of the five species of Anisoptera from Basrah Province.

The sequences of the products in our study were of good quality when DNA from the thorax and legs of Anisoptera species was isolated to match the identities of the selected species via both morphological and molecular identification. Although most taxonomic studies depend on phenotypic or morphological characteristics to diagnose the species of the Odonata order, which includes approximately 6,000 described species, we have confidence that the most accurate and important probability justifiable identification lies in the use of DNA sequences as taxonomic barcodes.

DNA analysis methods provide important insights into the distribution of genetic diversity around the world (Zheng *et al.*, 2023). Larvae of Libellulidae unknown by phylogenetic relationships can be identified with certainty and in a reliable manner (Huang *et al.*, 2020); all the sequences and species were identified via the BLAST tool on NCBI, which is available on the BOLD website (www.barcodinglife.com), for morphological identification with DNA barcoding identification.

In our study, we performed a BLAST search at NCBI to identify the specific species. For *C. erythraea,* the similarity was 100% with Namibia species, where this species is common worldwide, and this fact could be seen for *O. sabina*, which is 100% identical with the same species in Singapore. *T. annulata* was present in 99.10% of the African population, whereas *H. ephippiger,* which belongs to the family Aeshnidae, was 100% identical to the same species from Japan and 99.06% identical to the species from Namibia.

However, we found a close match for *Diplacodes trivialis*, but the maximum genetic similarity was 91.8% with *D. nebulosa* from Thailand, and the e value was 0. These species are phenotypically very similar, but *D. nebulosa* has not been recorded in Iraq until now, and the ratio of genetic similarity may be due to the presence of some strains of this species in the Arabian region. Furthermore, our findings support that the NJ, BLAST, and BOLD methods are dependable for the analysis of DNA barcodes.

Our results of sequence analysis revealed that a high frequency of identical amino acids remained in the ClustalW results because the selected species belong to Odonat and Anisoptera, where the two families Libellulidae and Aeshnidae are closely related. To resolve the relationships among Odonata species morphologically, many researchers have attempted to identify different characteristics to identify odonates on the basis of wing venation and copulatory organs (Sacchi and Hardersen, 2013), but no one has reached considerable conclusions. Bybee *et al.* (2021) reported that the genomic data they obtained were able to resolve parts of the topology of the studied

families of Odonata and shed light on possible evolutionary scenarios that may have shaped the evolutionary lineage of this taxon.

The structure of the phylogenetic tree for the selected dragonfly species from the Basrah region revealed that the DNA sequences of *Diplacodes trivialis* and *Trithemis annulata* were highly similar and close, whereas those of *Crocothemis servilia*, *Orthetrum sabina* species and *Hemianax ephippiger* were more similar, although those of *H. ephippiger* from different families were similar. Therefore, all the selected species belong to the same suborder, which is related to the DND contents.

Close species with high levels of similarity sequences are almost close taxonomically, whereas divergence is shown in the distant taxa groups, which means that the genetic information and signals are different in diverse regions, as shown by (Kumar *et al.*, 2016).

Our results are consistent with previous findings of Casas *et al.* (2018), who used the mitochondrial COXI gene to estimate genetic relationships between different members of the Libellulidae family. Additionally, many genetic studies have addressed the role of odonates in genetic diversity from an evolutionary point of view and morphological variation and confirmed the species diagnosis (Jisha Krishnan and Sebastian, 2015 a;b).

Finally, the identification via molecular data of these species was fully identical to that of the four species of *Crocothemis erythraea*, *Diplacodes trivialis*, *Hemianax ephippiger*, and *Trithemis annulata,* which commonly have a high level of dimorphism in body coloration between males and females, especially in the family Libellulidae, as mentioned by Corbet (1999). Therefore, the problem of misdiagnosis can be solved by molecular analysis, and this is what we observed through our study.

5. Conclusions

The use of DNA barcoding has proven to be a valuable tool for identifying dragonfly species, especially in Libellidus. Our work represents an initiative for the molecular identification of dragonflies in Iraq, helping to accurately document the local fauna of Odonata in the national genus GenBanck.

Acknowledgments

The authors are grateful to Dr. Ali J. Al-hassona for laboratory assistance and the members of the Department of Biology, College of Pure Science, and the Marine Science Center, University of Basrah, for providing the laboratory facilities during the study.

Data availability

Not applicable.

Informed consent statement

Not applicable.

Conflict of interest

The authors declare that they have no conflicts of interest.

Authors' contribution

Sample collection, species identification, DNA extraction and write the manuscript: HKA and DKK**. Supervision:** DKK. All the authors critically reviewed the manuscript and agreed to submit a final version of the article.

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