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DNA barcoding of termite Anacanthotermes *sp. Jacobson, 1905 in Erbil governorate - Iraq.*

Submitted to the Department of (biology) in partial fulfilment of the requirements for the degree of BSc in (biology).

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بسم الله الرَّحْمَنِ الرَّحِيمِ

﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا العِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ﴾

صدق الله العظيم

SUPERVISOR CERTIFICATE

This research project has been written under my supervision and has been submitted for the award of the degree of BSc. in Biology with my approval as a supervisor.

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Date: /04/2024

I confirm that all the requirements have been fulfilled.

DEDICATION

I dedicate this work to:

- My dear parent, who always prayed for me and supported me in everything, and my sisters and brothers, who are beside me.
- My supervisor Dr. Sarwat Ekram Al-Qassab
- My best friend who helped me.

Zhikal

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ABSTRACT

DNA barcoding was applied to identify a termite belonging to the genus *Anacanthotermes* sp. Jacobson, 1905 collected from a village near Erbil city – Iraq, in March 2023. Sequencing resulted in (97%) of identities to the Accession number (OQ440393.1), which is available on NCBI webpage.

Keywords: *Anacanthotermes*, Isoptera, DNA barcoding, Biodiversity.

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1. INTRODUCTION

Termites are small to medium-sized orthopteroid insects that are cryptic inhabit (Darlington, 2021). All species live in eusocial colonies and feed primarily on cellulose (Nalepa, 1994). Although referred to in older literature as “white ants,” termites are unrelated to ants. Termites belong to the Phylum Arthropoda, Class Insecta (= Hexapoda), and Order Isoptera. The name of the Order is derived from the Greek words “iso” (equal) and “ptero” (wing), which describe the similar length and shape of both the fore and hind wings of the reproductive alates. Order Isopoda is divided into two families: The Family Rhinotermitidae is divided into seven small and closely allied subfamilies, and the Family Termitidae is divided into four large and diverse (Donovan, 2000). Mature colonies are composed of task-specific castes that typically include one or more pairs of reproductive, about 0 - 25% soldiers, and a majority of immature or sterile workers (Smith et al., 2008). During part of the year, colonies may also contain some maturing or fully winged reproductive (alates, imagos) destined to leave their colony in brief, but often intense, dispersal flights (Pervez, 2018). Termites cause huge losses to agricultural crops, forest trees, and buildings made of wood. The global economic losses of Subterranean termites are estimated at 22 billion US dollars, including the costs of chemical control and the restoration of damaged buildings (Su, 2003).

This study aimed to identify some termite species using molecular techniques that were collected from a village located northeast of Erbil governorate – Iraq.

2. MATERIALS AND METHODS

2.1. Collection of samples

Termite specimens were collected by hand from a side of a hill outside Bragh village, Erbil governorate - Iraq. The samples were preserved in 96% ethanol and kept in (-20° C) freezer. Photos of the dorsal and ventral sides of the samples were taken using Celestron Handheld Digital Microscope Pro (China).

2.2. Molecular Techniques

2.2.1. DNA extraction by using a BETA BAYREN extraction kit (Germany)

Grind the tissue (50-100 mg) wet weight 10-20 mg lyophilized tissue) using a mortar and pestle or homogenizer.

A. Sample preparation from Tissue

1. Add 300 µl of BDL to the sample and vortex for a few minutes.

2. Add 12 µl of proteinase K solution (20 mg/ml) to the sample tube, mix by vortex, and incubate at 58° C until the sample is completely lysed.

3. Using a pipette, transfer the lysate into a microcentrifuge tube.

B. Binding DNA to column

5. Add 400 µl BDB and mix by vortexing (2 X 5 sec).

6. Incubate for 5 min on ice.

7. Transfer all of sample into a column positioned on top of the collection tube.

8. Centrifuge at (8,000 RPM) for 2 min. Discard the flow through. Reassemble the spin column with its collection tube

C. Column wash 1st wash

9. Add 400 µl Buffer BDW1 to the BETA BAYREN DNA spin column.

10. Centrifuge at (10,000 RPM) for 2 min.

11. Discard the flow through. Reassemble the spin column with its collection tube 2nd wash.

12. Add 400 µl Buffer BDW2 to the column and centrifuge for 1 minute at (10,000 RPM).

13. Discard the flow through. Reassemble the spin column with its collection tube.

14. spin the column for 2 minutes to thoroughly dry the resin. Discard the collection tube.

D. Elute DNA

15. place the spin column in a microcentrifuge tube.

16. Add 50 µl BDE for tissue and bacteria or 100 µl for blood (preheated to 70° C) to the column.

17. incubate at rm temperature for 1 min.

18. Centrifuge at (13,000 rpm) for 2-3 min.

(**optional**): An additional elution may be performed if desired by repeating steps 19-22. collect the second elution into a microcentrifuge tube. The yield can be improved by an additional 20-30% when this second elution is performed.

E. Storage of DNA

The purified DNA can be stored at -20° C for a few weeks.

2.2.2. PCR preparation

The DNA barcoding for terrestrial termite was achieved based on the amplification and sequencing of mitochondrial DNA Cytochrome Oxidase subunit I (*COI*) gene (680-720 bp long) (Folmer et al., 1994) by using primer pairs: LCO1490: (**5`-GGTCAACAAATCATAAAGATATTGG-3`**) and HCO2198: (**5`-TAAACTTCAGGGTGACCAAAAAATCA-3`**) (Macogen, Korea). Master mix (Ampliqon PCR Enzymes & Reagents, Denmark) was used to amplify the partial sequences of (*COI*). The amplification was done in a total volume of (25 µl) as shown in Table (1):

Table (2.1) PCR material mixture.

Master Mix	12.5 µl
Primer F	1.5 µl
Primer R	1.5 µl

Templet DNA	2 µl
ddH_20	7.5 µl
Total volume	25 µl

PCR was carried out in (PCRmax Alpha thermal cycler, UK) and PCR thermal reaction applied according to (Hajibabaei et al., 2006) as shown in Table (2.2)

Table (2.2) shows PCR reaction

94 °C	2 min	
94 °C	30 sec	5 cycles
45 °C	40 sec	
72 °C	1 min	
94 °C	30 sec	35 cycles
51 °C	40 sec	
72 °C	1 min	
72 C	10 min	

2.2.3. Agarose gel electrophoresis

PCR samples were run in (1.5%) of agarose gel electrophoresis as following:

1. **Prepare Gel**: Mix agarose powder with TAE buffer, heat to dissolve, DNA Safe stain (Danmark) add, pour into mold, and let it solidify.
2. **Load Gel**: Set up gel in electrophoresis tank, insert comb to create wells, pour in TAE buffer, and load samples into wells

3. **Run electrophoresis**: Apply (80) voltage across the gel, allowing DNA fragments to migrate through the gel based on size using GeneRuler 50 bp DNA Lader marker (Thermo Inc., USA).
4. **Visualize DNA:** After electrophoresis, visualize DNA bands under UV light.
5. **Analyze results**: Determine size and quantity of DNA fragments by comparing to size marker.

2.2.4. Gel Extraction Kit

For sequencing, QIAquick Gel Extraction Kit (Qiagen, Germany) was used as following:

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colourless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 pl). The maximum amount of gel per spin column is 400 mg. For 2% agarose gels, add 6 volumes of Buffer QG.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2-3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.

4.Add one gel volume isopropanol to the sample and mix.

5. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply a vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 pl, load and spin/apply vacuum again.

6. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 ul Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

7. To wash, add 750 pl Buffer PE to QlAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube. Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2-5 min after addition of Buffer PE. Centrifuge the QlAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

8. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, add 50 pl Buffer EB (10 mM Tris-CI, pH 8.5) or water to the center of the QlAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 pl Buffer EB to the center of the QlAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QlAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

10. If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

2.2.5 DNA sequencing

DNA sequencing was performed utilizing an ABI 3730XLs nucleotide sequence analyzer through Macrogen Inc. (Korea). All obtained DNA sequences were edited using Chromas software and aligned with (ClustalW algorithm), available in MUSCLE program within EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/muscle/).

To verify the closest species match for DNA sequences obtained in this research, Basic Local Alignment Search Tool for nucleotides (Blastn) implemented in the NCBI GenBank database was used to evaluate all sequences.

3. RESULTS

In the present study, termite belong to genus ***Anacanthotermes* sp**. Jacobson, 1905, Family Hodotermitidae was identified.

3.1 Taxonomy of termite genus of genus *Anacanthotermes*:

Table (3.1) shows the taxonomy of genus *Anacanthotermes*.

Scientific classification	
Domain	**Eukaryota**
Kingdom	**Animalia**
Phylum	**Arthropoda**
Class	**Insecta**
Order	**Isoptera**
Family	**Hodotermitidae**
Genus:	***Anacanthotermes*** **(Jacobson 1905)**

3.2 Morphological description

As a termite species, *Anacanthotermes* consists three types of members: worker (Figure 3.1), soldier and alate (Figure 3.2 & 3.3). Soldier mouth part is usually used for classification of termites.

Body: The entire body of *Anacanthotermes* is divided into the head, thorax, and abdomen. These segments work together to ensure the termite's survival, colony function, and ecological interactions.

Head of *Anacanthotermes* contains essential sensory organs, mouth parts, and structures for communication and feeding. Key features include: Antennae: Used for detecting chemical cues, communication, and navigation. Compound Eyes: Facilitate vision. Mandibles: Strong jaws for chewing wood and other organic matter. Labrum: A flap-like structure covering the mouth. Clypeus: The front part of the head.

Maxillary Palps: Sensory structures near the mouth. Labial Palps: Additional sensory structures. Hypopharynx: Involved in food manipulation and transport.

Thorax: The thorax is the middle segment of the termite's body. It consists of three integral segments: Prothorax: The first segment, which bears the first pair of legs. Mesothorax: The middle segment, supporting the second pair of legs and wings (if present). Metathorax: The posterior segment, carrying the third pair of legs and wings (if present). The thorax plays a pivotal role in termite mobility and daily activities.

Abdomen: The abdomen is the posterior part of the termite's body. Key features include: Spiracles: Tiny openings for gas exchange. Tergites and Sternites: Hard plates covering the dorsal and ventral sides of the abdomen. Rectum: Involved in waste elimination. Malpighian Tubules: Excretory structures. Glands: Produce pheromones for communication. Reproductive Organs: Located in the posterior abdomen. Digestive System: Processes cellulose from wood. Hindgut: Further digestion and absorption of nutrients occur here (Rerat, A., 1978)

Fig. (3.1) shows ventral side of the termite worker

Fig. (3.2) show the dorsal side of the termite alate.

Fig. (3.3) shows the ventral side of the alate termite.

3.3. Molecular identification

3.3.1. Gel electrophoresis

PCR successfully generated a 658 bp a target *COI* gene for termite as shown in Figure (3.4).

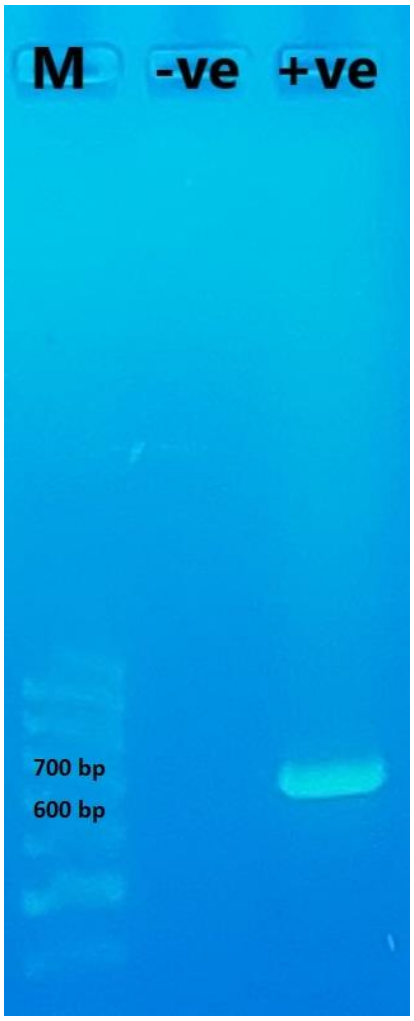

Fig. 3.4 shows gel electrophoresis run of the target *COI* gene (658 bp). M = GeneRuler 50 bp DNA ladder marker, (-ve) = ddH_2O, (+ve) = Isopod DNA.

3.3.2 Sequencing analysis

A chromatogram of sequencing belongs to mitochondrial DNA *COI* gene obtained from Macrogen Inc. shown in figure (3.5). The nucleotide of the present termite sequencing of *COI* gene reveals that this termite belongs to genus *Anacanthotermes* sp. with identities of (97%) to Accession number (**OQ440393.1**) by using Blastn tool which available NCBI webpage (figure 3.6).

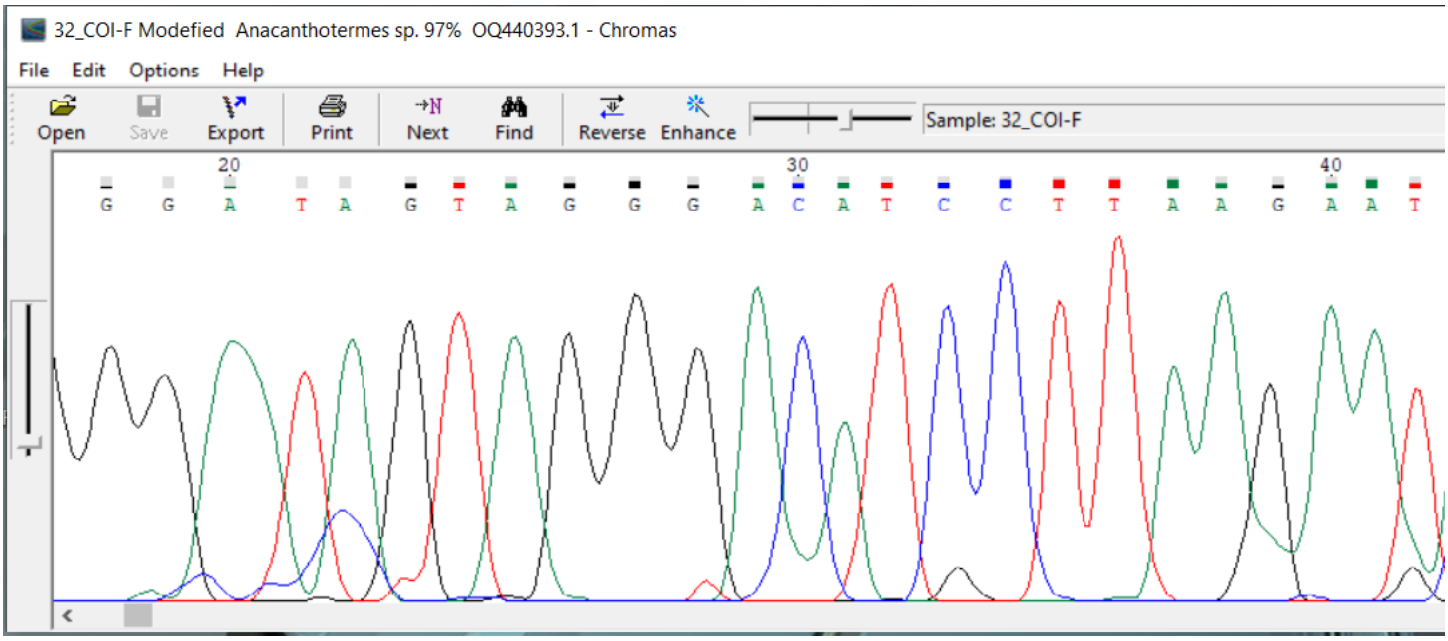

Fig. (3.5) shows the chromatogram of the sequence of the termite

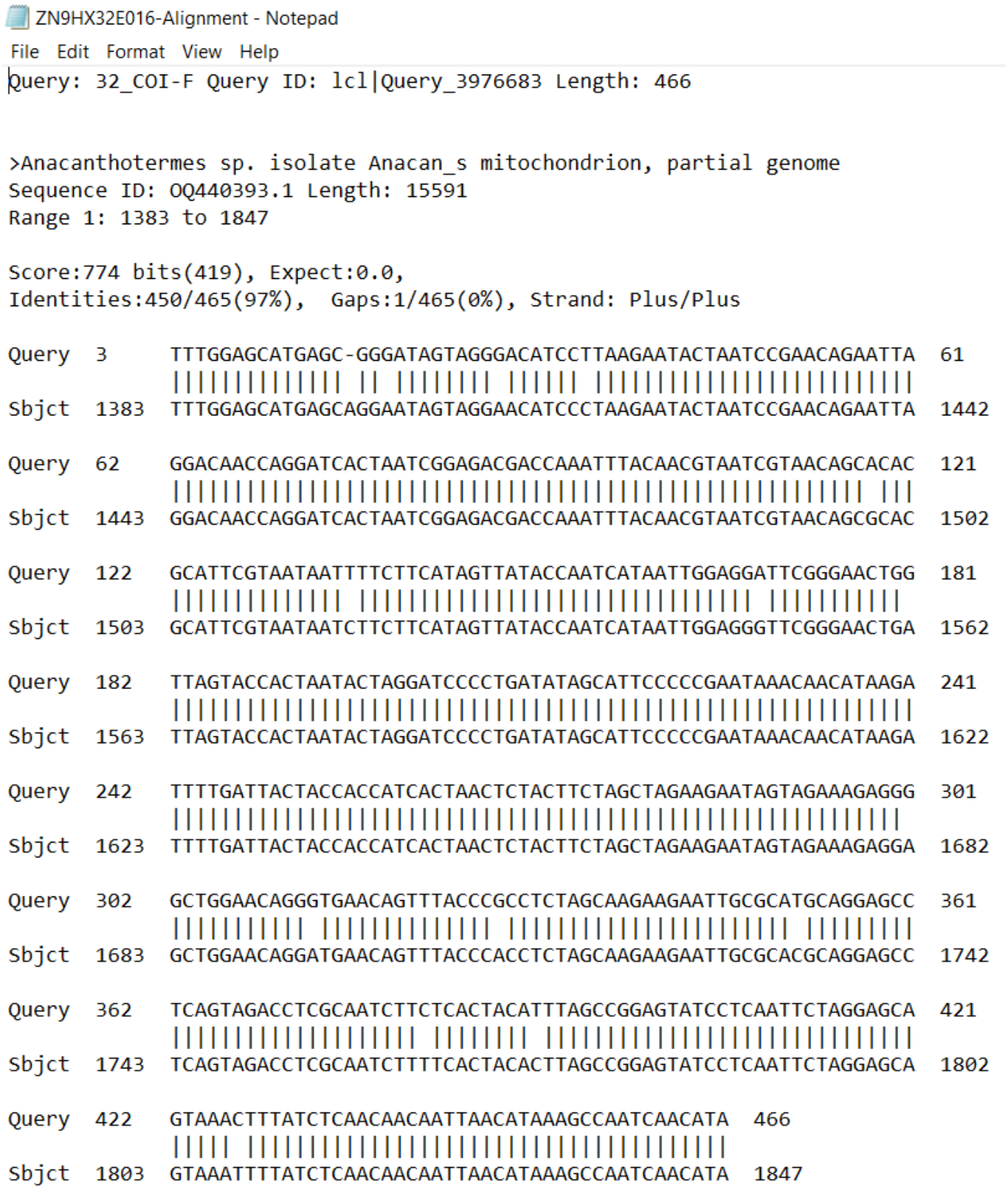

Fig. (3.6) Two sequence alignment between present (Query) and OQ440393.1 **(subject)**

4. DISCUSSION

Termites are important economic insects that attack wood and wood products in tropical, subtropical, and temperate regions (Su and Scheffrahn, 2001; Rouland-Lefèvre, 2011). In the Arab world, the number of known species does not exceed 24, Twelve of which belong to the Termitidae family (Al-Mallah, 2010). Of the diagnosed species of termites in Iraq, seven species belong to three families and three species belong to the Termitidae family (Arab Organization for Agricultural Development, 1976, Al-Alawi, 1987; Zarzis and Muhammad, 1992).

Anacanthotermes is a harvesting termite restricted to arid and semi-arid habitats. However, although sandy or gravelly soils are preferred, there must be some clay content in the soil as well as enough groundwater to allow some vegetation to grow. Nests are always subterranean, with or without surface structures. They consist of diffuse systems of small cells at various depths with interconnecting galleries. Storage cells with dry forage occur within the nest (Harris, 1967; Roonwal, 1975).

In current study, the species of *Anacanthotermes* couldn't be identified due to the lack of the soldier termite sample, which their mandibles mainly used as species identifiers.

DNA analysis of the present termite affirms it belongs to *Anacanthotermes* sp. using *COI* gene. The genus *Anacanthotermes* Jacobson, 1905, currently consists of 16 species, according to the Encyclopedia of Life (EOL) webpage (https://eol.org/pages/8976932).

In Iraq, four species of *Anacanthotermes* were recorded which are *A. sawensis* Al-Alawi et al., 1990, *A. septentrionalis* (Jacobson, 1905), *A. ubachi* (Navás, 1911) and *A. vagans* Hagen, 1858 (Krishna et al., 2013).

Anacanthotermes baluchstanicus Akhtar, 1974, *A. iranicus* Raven & Akhtar, 1993, *A. esmailii* Ghayourfar, 1998 were recorded in Iran.

5. CONCLUSIONS

Anacanthotermes sp. was identified by using *COI* gene target collected from northeast Erbil governorate - Iraq.

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