ZANCO Journal of Pure and Applied Sciences
The official scientific journal of Salahaddin University-Erbil
https://zancojournals.su.edu.krd/index.php/JPAS

**ISSN (print ):**2218-0230**, ISSN (online):** 2412-3986**, DOI: http://dx.doi.org/10.21271/zjpas**

RESEARCH PAPER

# Molecular identification of some earthworm species (Annelida; Oligochaeta) In Kurdistan-Iraq

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**A B S T R A C T:**

A total of 256 specimens of earthworms were collected from four provinces in Kurdistan region of Iraq includes Erbil, Sulaymaniyah, Duhok and Halabja, during a period from July 2018 to April 2019.

DNA barcoding was used for the first time in Iraq for identification of earthworms to species level. A modified CTAB method was used for isolation and extraction of DNA. Mitochondrial COI region was amplified and sequenced to determine the differences among species.

Results revealed that six species belong to three genera of two families; Lumbricidae and Megascolecidae were successfully identified and the similarity of all species was 95% or more. which are *Amynthas morissi* (Beddard, 1892) *; A. luridus* (Shen et al., 2019) *; A. gracilis* (Kinberg, 1867) *; Aporrectodea trapezoids* (Duges, 1828) *; A. longa* (Ude, 1888) *;* and *Healyella syriaca* (Rosa, 1893). Depending on the present results the species (*Haelyella syriaca*, *Amynthas gracilis* and *Amynthas luridus*) are considered as new records to Iraqi fauna. The phylogenetic tree analysis using maximum likelihood programs shown that there are seven clades. This study could be a suitable research program for future studies on the earthworms in Iraq.

KEY WORDS: Earthworms, COI, Lumbricidae; Megascolecidae.
DOI: http://dx.doi.org/10.21271/ZJPAS.32.6.5
ZJPAS (2020) , 32(6);46-53 .

## 1. INTRODUCTION

Earthworms belong to the phylum Annelida, class Oligochaete, they are widely distributed and more than 5500 aquatic and terrestrial species have been identified (Escudero et al., 2019). Their body is cylindrical and divided into segments by intersegmental grooves (Ahmed, 2015). The body cavity filled with coelomic fluid that extended from head to the anus; externally they bear little setae or short bristles (Dominguez and Edwards, 2011, Xiao, 2019).

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**Article History:**
Received: 02/06/2020
Accepted: 23/07/2020
Published:2 0/12 /2020

Clitellum which found in adult worms only is a glandular swelling and saddle-shape usually occupying some segments and has a role in reproduction process (Brusca and Brusca, 1990, Csuzdi and Zicsi, 2003). These characteristics are important for morphological identification to species level.

Although, this worms have great role in soil studies and as soil biomarkers, recently many researchers proved that they have a helpful role in therapeutic processes of human diseases like treatment of diabetes, some types of cancer or as antibiotics (Liu et al., 2011, Liu et al., 2017, Augustine et al., 2017, Cooper et al., 2004). The earthworm glycolipoprotein (as known G-90) is a blend of macromolecules with some biological properties including mitogenicity, anticoagulation, fibrinolysis, bacteriostatic and antioxidatiaon (Goodarzi et al., 2016).

Researchers which use the morphological characteristics for identification of earthworms, they critics the DNA barcodes for taxonomical studies because depended on a single mitochondrial region for identification and it can be misleading, but mitochondrial DNA(mtDNA) can be officiated at any stage of life cycle and the codes not change easily (Will and Rubinoff, 2004).

Phylogenetic studies for identification of animals verified that mtDNA have been widely used because it evolves more rapidly than nuclear DNA, thereby resulting in the accumulation of differences between closely related species (Moore, 1995, Mindell, 1997). Divergence of sequences among species is much higher than within species and mtDNA genealogies generally capture the biological discontinuities recognized by taxonomists as species. Cytochrome-C oxidase I gene (COI) describe as a DNA barcode for identification species of which is usually found in all animals, and comparison of sequences are simple because insertion and deletion are rare (Hebert et al., 2003, Azeez and Mohammed, 2017). Several phylogenetic analyses of Oligochaete taxa included a combination of several molecular markers (for instance nuclear18S, 28S rDNA, mitochondrial 12S rDNA, COI) and morphological features also confirmed for species identification (Bozorgi et al., 2019).

This present study aimed to introduce the use of molecular techniques for identification of earthworm species in Iraq. It was constructed on an earthworm collection from samplings in different soils in Kurdistan/Iraq.

## 2.MATERIAL AND METHODES

### 2.1. Sample collection

During July 2018 to April 2019 a total of 256 specimens of earthworms were collected from four provinces in Kurdistan region of Iraq (Erbil, Sulaymaniyah, Duhok and Halabja) (Fig. 1). Collection was carried out by using Hand-storing method (Sims and Gerard, 1985, Farhadi et al., 2013), which the worms can be obtained simply by digging up the soil. The adult earthworms obtained from sites with their activity such as moist soil near ponds, gardens, farm areas, and forests. The samples of earthworms spread in plastic jars with the same soil from the area where the worms were obtained. Ten to fifteen worms were collected from each area, after that the worms were transported to laboratory and placed in bigger plastic jars. Leaf litters and manure added frequently to each plastic jars and the humidity of the plastic jars was kept between 70% to 80% by rushing water daily. However, each plastic jar was covered by textile net to avoid worm escaping. The adult worms were washed with tap water to remove soil particles and placed in Petri dishes (Ansari and Saywack, 2011). Finally, the worms killed in 30% ethanol and preserved in 95% ethanol for DNA extraction (Klinth et al., 2019).

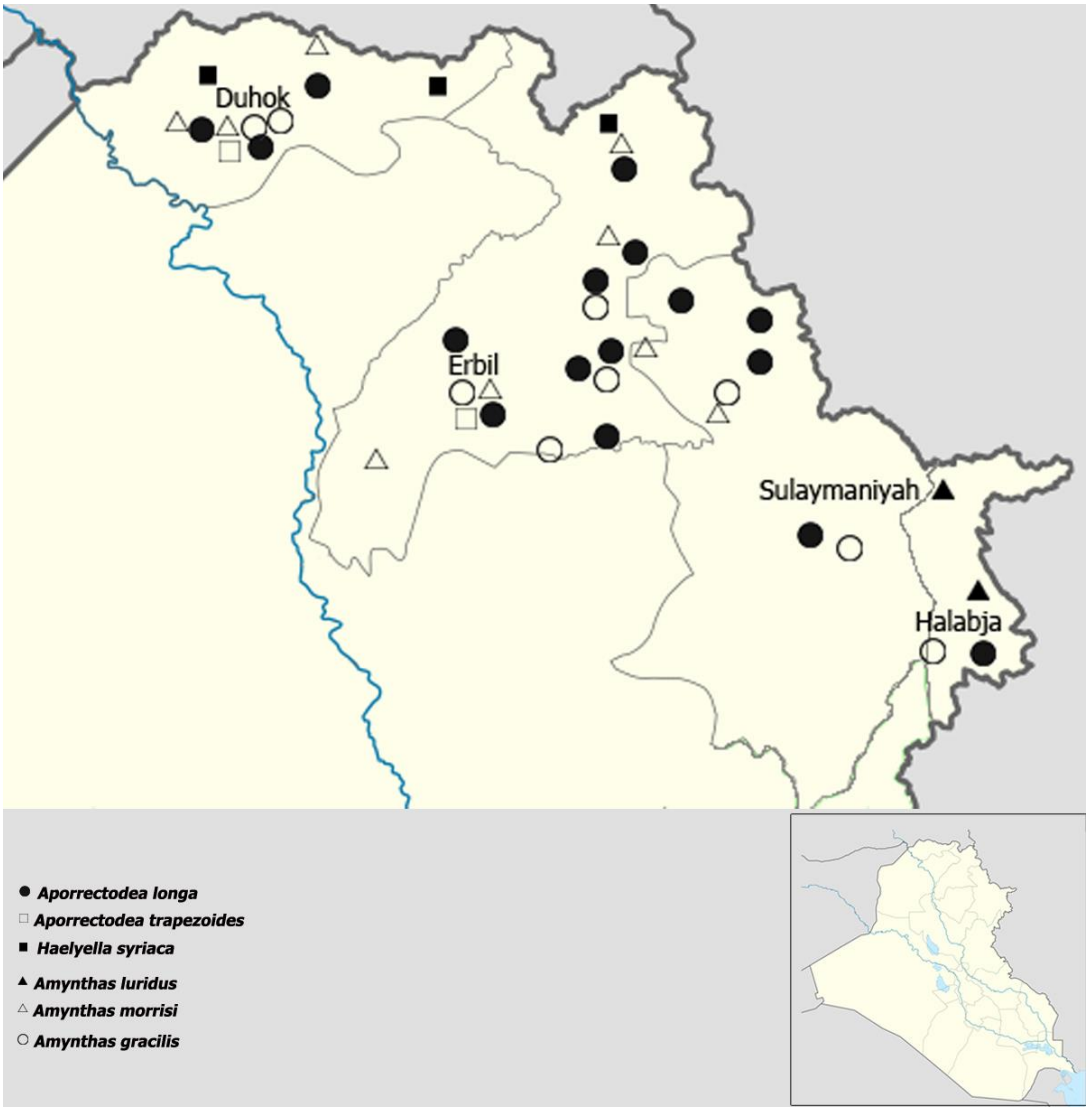


**Figure 1**. Distribution of earthworm species in Kurdistan Region of Iraq.

## 2.2. DNA Extraction

After morphological identification of the specimens (Fig. 2.) the molecular study performed, the isolation of high quality of DNA is an essential for molecular study. In present study a modified CTAB (Cetyltrimethyl ammonium bromide) method for isolate high quality of DNA from different species of earthworm's tissue (Agustí et al., 2000, Juen and Traugott, 2006). The CTAB method generally used for extraction DNA from plant tissue which described by Murray and Thompson (1980) (Murray and Thompson, 1980). The CTAB genomic DNA isolation method include a cell lysis along with Chloroform; Isoamyl and use of proteinase K (Doyle and Doyle, 1987).

In present study the DNA extraction for amplification of the mitochondrial COI gene region was performed in Ankara university, science college, Biology faculty. The mitochondrial COI region was amplified with primer LCO1490 and HCO2198 (Vrijenhoek, 1994, Huang et al., 2007, Escudero et al., 2019).

The preserved earthworms were placed on slides and 1 cm from caudal tissue at anterior part appended to prevent contamination by gut content (Huang et al., 2007).These tissues were divided to smaller pieces and transferred to clean and dry Eppendorf tubes. After adding 3ml of CTAB solution to each tube the tissues were crashed as much as possible, however more CTAB solution (3ml) were added. BME (Beta-mercatoethanol) a lysis buffer used to remove and destroy all proteins, lipids and other content (Ince et al., 2011, Horne et al., 2004), Fifty ml of BME buffer was added and placed in water bath for 1hr. to destroy the tissues and extract all mitochondria.

To each tube 500ml of CIAA (Chloroform: Isoamyl alcohol) were added and shacked so it became milky. All samples centrifuged for 15min. at 13,000 rpm at 4c° to add extra stability to DNA. The DNA extractions poured into other Eppendorf tubes with 500ml of CIAA and centrifuged again for best results.

At next step we add 500ml of Isopropanol to each sample and placed in -80c°, after freezing all samples centrifuged at 13,000rpm for 10min., when the centrifugation ended the supernatant and the DNA located at bottom of the tube were discharged.

For cleaning the DNA, 500ml of 100% alcohol added, centrifuged at 13,000 rpm for 3min., this step repeated twice. After all the supernatant discharged. Finally, the DNA diluted with TE buffer (PH 8) and left overnight.


**Figure 2:** Earthworm species identified in this study; *Aporrectodea longa* (A), *Aporrectodea trapezoides* (B), *Haelyella syriaca* (C), *Amynthas morissi* (D), *Amynthas gracilis* (E), *Amynthas luridus* (F).

**2.3. PCR (Polymerase chain reaction)**

The extracted DNA was amplified using PCR machine. Amount of 12.5 ml was added of PCR mixture into a tube with 9.5 ml of water, 1 ml of primer will be added and mix it well, after that 1 ml of extracted DNA added. The solution must mix well and take 1 ml of it, putted in an Eppendorf tube for PCR running, however the run for 30 cycles at 94 c° for 45s, at 49-52 c° for 40-60s, while at 72 c° for 90s (Huang et al., 2007).

Electrophoresis was used to ensure that the DNA completely extracted from earthworms. Both TAE (Tris-acetate- Ethylenediaminetetraacetic acid) buffer and agaroses prepared to run the electrophoreses, 50 ml of TAE buffer diluted in 950 ml of distal water, after that 0.8 % of agarose gel was dissolved in diluted TAE buffer. The solution was heated until boiling and shacked well. When the solution reached the boiling degree, transferred to an electrophoresis glass and let to solidify. After that the solidified gel added into electrophoresis machine and 5 ml DNA also added with the marker.

Finally, the gel stained with bromide (1 mg per 1 ml of D.W) and was observed under the U.V. light, if there is a band meaning the extraction is perfect and there was DNA. The band on the gel clearly seen so there were a sufficient amount of DNA, finally the extractions sent to BM Labosis company in Ankara/Turkey for complete sequencing.

**3.RESULTS**

The DNA sequencing process results subjected to Nucleotide Basic Local Alignment Search Tool

(BLAST) in NCBI GenBank. The best match species are shown in Table 1. The results were three genera and six species belong to two families; Lumbricidae and Megascolecidae (Asian family).

The results of phylogenetic tree analyses using maximum likelihood method revealed in (Fig.3.) shows seven clades, two other results taken from NCBI for each species for compare. The first clade was *Amynthas luridus* having a moderate support bootstrap value reaches 67%. *Aporrectodea longa* is the second clade with a high bootstrap value reaches 100%. While *Healyella syriaca* include two clades, each with 97% bootstrap value. The fourth clade *Amynthas gracilis* with bootstrap reaches 90% and at fifth clade *Amynthas morrisi*, the bootstrap with marked sample that got from GenBank reaches 100%. The last clade includes *Aporrectodea trapezoides* has a high bootstrap reaches 99%.

According to above results Lumbricidae family involve three species; *Aporrectodea longa*, *Aporrectodea trapezoids* and *Haelyella syriaca.* Megascolecidae family also comprise three species; *Amynthas morrisi*, *Amynthas gracilis* and *Amynthas luridus*. Depending on the present results the species (*Haelyella syriaca*, *Amynthas gracilis* and *Amynthas luridus*) are first records in Iraq.

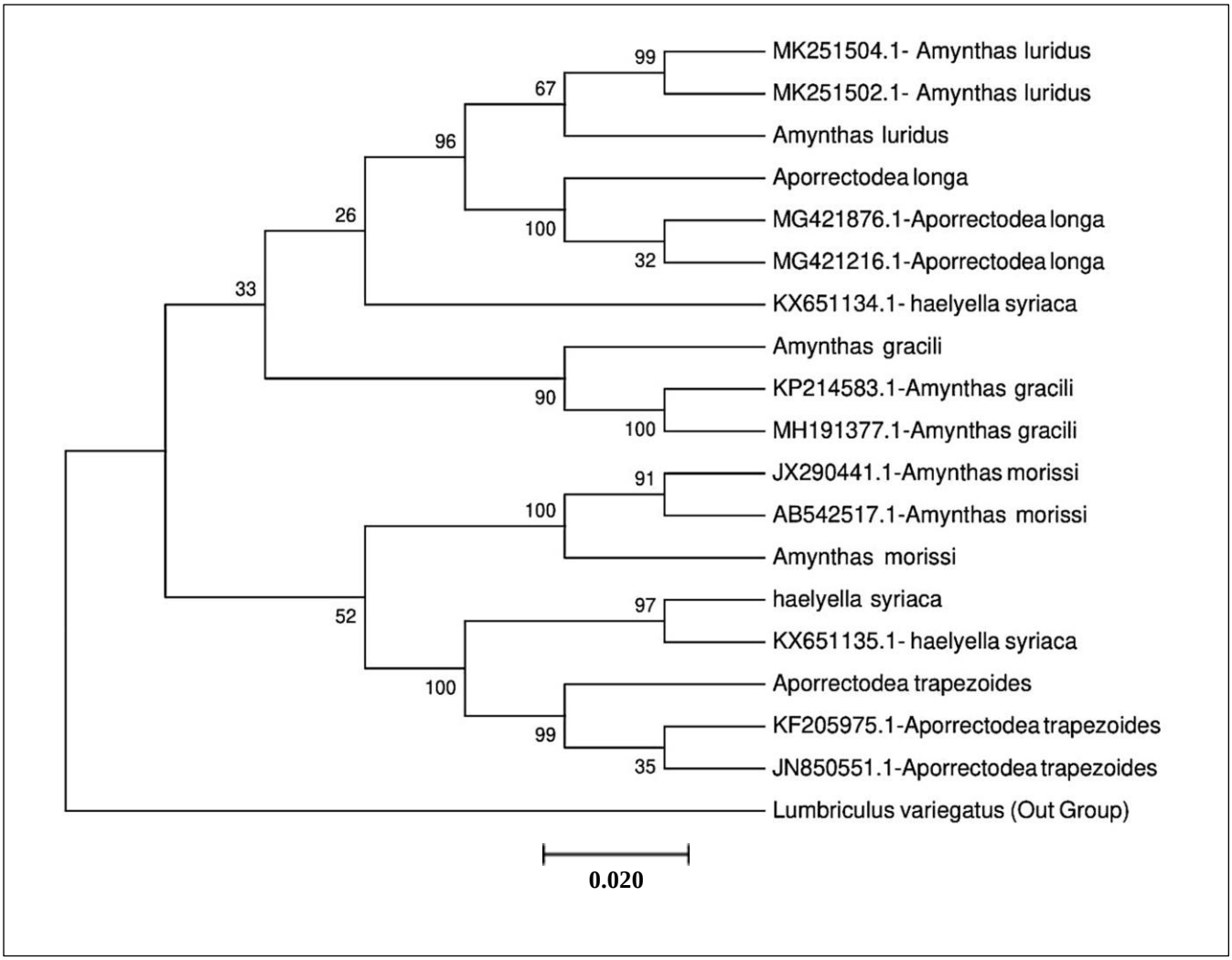


**Figure 3.** Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-6713.35) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with

superior log likelihood value. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 557 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

**Table 1:** Representative of specimen collected in sampling sites in Kurdistan Region/Iraq with annotated sequences in GenBank.

| Earthworm species | Province | Query Cover % | Identic Number % | GENBank Accession Number | GENBank earthworm Species Identification | Country Identification |
|---|---|---|---|---|---|---|
| ***Amynthas morissi*** | Sulaymaniyah Duhok Erbil | **100** | **96.49** | **JX290441.1** | ***Amynthas morissi*** | **Taiwan** |
| | | **99** | **96.49** | **AB542517.1** | ***Amynthas morissi*** | **Japan** |
| ***Healyella syriaca*** | Duhok Erbil | **100** | **95.13** | **KX651135.1** | ***Healyella syriaca*** | **USA** |
| | | **99** | **90.45** | **MG941001.1** | ***Healyella syriaca*** | **USA** |
| ***Amynthas luridus*** | Sulaymaniyah Halabja | **100** | **95.09** | **MK251502.1** | ***Amynthas luridus*** | **Taiwan** |
| | | **100** | **95.09** | **MK251504.1** | ***Amynthas luridus*** | **Taiwan** |
| ***Aporrectodea trapezoides*** | Duhok Erbil | **100** | **100** | **KF205975.1** | ***Aporrectodea trapezoides*** | **China** |
| | | **100** | **100** | **JN850551.1** | ***Aporrectodea trapezoids*** | **Portugal** |
| ***Amynthas gracilis*** | Sulaymaniyah Duhok Erbil Halabja | **100** | **94.79** | **KP214583.1** | ***Amynthas gracilis*** | **UK** |
| | | **100** | **94.61** | **MH191377.1** | ***Amynthas gracilis*** | **India** |
| ***Aporrectodea longa*** | Sulaymaniyah Duhok Erbil Halabja | **100** | **100** | **MG421876.1** | ***Aporrectodea longa*** | **Canada** |
| | | **100** | **100** | **MG421216.1** | ***Aporrectodea longa*** | **Canada** |

## 4.DISCUSSION

This study based on DNA barcodes for identification of earthworms, it represents the results of the first molecular based study for identification of earthworms in Iraq. Our results reveal that DNA barcoding is an auspicious tool for studying earthworm biodiversity: previous studies in the area reported three species from Kurdistan region, while our rather limited sample yielded nine (only six of them are represents here because the other three species gave inadequate DNA sequencing results, so they must be undergoing further molecular studies to confirm their classification accurately). There are two explanations for this, first, barcoding permits identify juvenile or scantily preserved specimens. Second, many earthworm species look alike, and so the species indicating small minority of the sample bend to be grouped with more numerous species and unobserved.

In the study of BLAST, *Aporrectodea longa* (Ude, 1888) and *Aporrectodea trapezoides* (Duges, 1828), analysis of similarity in BLAST showed 100% similarity with those sequences annotated in GenBank at COI region. Conversely, *Amynthas morrisi* (Beddard, 1892) showed only 96% similarity with annotated sequences. While, *Amynthas gracilis* (Kinberg, 1867) and *Amynthas luridus* (Shen et al., 2019) showed lower similarity which both of them 95%. The last

species is *Haelyella syriaca*, (Rosa, 1893) the result of analysis is between 90% and 95% similarity with annotated sequences in GenBank. Those species belong into two families Lumbricidae and Megascolecidae. These two families ranked as a first and second most widely distributed and abundant families in temperate zones (Simberloff and Rejmánek, 2011). *Amynthas gracilis* is highly associated with *Amynthas corticis* (Sims and Gerard, 1985).
The evolutionary divergence based on the Maximum Likelihood method shows different clade groups, regarding the present study the tree divided into seven clades. *Amynthas luridus*, related with annotated sample from Taiwan at bootstrap 67% (which a moderate value) and this may be due to the geographical distribution differences between them (Shen et al., 2019) Environment and mutation also have role in divergence among species and individual of same species at different habitat and countries (Csuzdi et al., 2017, Escudero et al., 2019).

However, *Aporrectodea longa* and *Amynthas morissi* included into different clades and this has agreement with results obtained by each of (Porco et al., 2018, Chang et al., 2009). *Amynthas gracilis* also located fourth clade with bootstrap reaches 90% , results obtained from NCBI which carried out by (Pop et al., 2005) were similar with ours . *Healyella syriaca* included into two clades, the local species and one species from NCBI which located at sixth clades related at high bootstrap value reaches 97 %. The present findings confirmed that the suggestive bootstrap values of the attained tree were previously documented with same COI mtDNA marker which is existing in NCBI.

## 5.CONCLUSION

Six species belong to three genera of two families; Lumbricidae and Megascolecidae were successfully identified and the similarity of all species was 95% or more. *Haelyella syriaca*, *Amynthas gracilis* and *Amynthas luridus* are first records in Iraq. The phylogenetic tree analysis using maximum likelihood programs revealed that there are seven clades. This study could be a suitable research program for future studies on the earthworms in Iraq.

## ACKNOWNLEGMENT

We will express our gratitude to those who helped us, and special thanks to Salahaddin University-College of Science/Biology department for their support during the period of achievement of this research.

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