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Identify *Lumbricus Rubellus* According DNA Barcoding

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Abstract: The species of earthworms is usually determined through careful observation of morphological features, is often accomplished by closely examining morphological characteristics, which are frequently sexual characteristics only seen in mature individuals. Earthworm species identification as such, it is sometimes hard to identify juveniles or cocoons, which might introduce bias into studies that record species richness and abundance. A viable method for species discrimination is DNA barcoding, which uses a brief, standardized DNA fragment for species identification, use of DNA remains the best in determining the species. *Lumbricus rubellus* have been found on in the common epigeic earthworm from Iraq in Al-Diwaniyah city, the presence of this worm is recorded for the first time in Iraq, specifically in the city of Diwaniyah. This study tested sequence data for the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene in order to identify the utility of DNA barcodes in the identification of earthworm species.

Keywords: *lumbricus rubellus*, DNA sequencing, COX1, soil.

RESEARCH PAPER

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INTRODUCTION

The first person to refer to earthworms was the scientist Charles Down (1809–1882) before the Geological Society in 1837, and the first to classify earthworms and consider his classification system successful, in the early seventies (Stürzenbaum *et al*., 2009). Earth worms are the most common creatures in the soil, and belong to the phylum Annelida, classified as clitellata, oligochaeta (Nouri-Aiin *et al*., 2021). They are particularly widespread in temperate and tropical climates (Kiyasudeen, 2016). From a biomass and activity perspective, earthworms are without a doubt the most significant soil invertebrates in the majority of soil types across the world (Cunha., 2016). Darwin described earthworms as the engineers of the ecosystem (Feller *et al.*, 2003) while Aristotle called them the intestines of the earth (Yadav *et al.*, 2017).

A more precise knowledge of species diversity and its relevance to ecosystem services is needed, as evidence of the sensitivity of soil fauna components to various human activities grows (Norris., 2012) and (Vos *et al*., 2014). Ignoring cryptic species might cause ecological complexity to be underestimated, which could complicate conservation and biomonitoring efforts (Cahill *et al.*, 2023).

Although *L. rubellus*, the earthworm, is endemic to Western Europe, it is currently considered an invasive species due to its global proliferation in temperate northern regions (Onrust *et al*., 2019*). L. rubellus* inhabits soils with a high content of organic matter. Worms like to burrow in soft soil that has enough moisture to allow for exchange gasses, it feeds on decomposing organic matter (Klein *et al*., 2020).

In most situations, it is hard to identify juveniles of closely related species (such as members of the genus Lumbricus) due to their lack of diagnostic characteristics (Richard *et al*., 2010). Juveniles of closely related species are difficult to identify due to their lack of distinguishing characteristics(Sims *et al.*, 1985). Thus, taxonomists are limited to giving generic identifications for juveniles, which complicates soil investigations aimed at evaluating species richness (Giska *et al*., 2015).

Among the study's objectives are the following: Molecular diagnosis through DNA extraction, using PCR technology, and relying on DNA sequencing to diagnose earthworms found in the city of Diwaniyah and registering the sample in the Gene Bank.

Collecting Samples:

76samples of Earthworm were collected from the soil of three locations in the city of Diwaniyah (Figure 1), located in the north of city32°00′12.72″N44°52′56.78″E, middle of city 31°59′05.1 ″N44°55′21.44′′E and south of Diwaniyah city 31°58′5826.9″N44°57′06.72″E, in August 2022.

The samples were collected by digging the soil to a depth of 20-25 cm using a shovel and placed in plastic containers measuring 9*7* 16 cm, the forms were sent to the College of Science for the purpose of molecular examination and diagnosis.

Figure 1: Earthworm type *lumbricus rubellus*

MATERIALS AND METHODS

DNA Extraction and primer designed

DNA was extracted from earthworms according to the method provided by the company Geneaid Biotech Ltd.USA with a DNA extraction ki gSYNC™. The concentration and purity of the extracted DNA were measured using a Nanodrop.

DNA was extracted by the company Geneaid Biotech Ltd. USA with a DNA extraction ki gSYNC™. The current investigation in order to amplify the mitochondrial COX1 gene (5'TACCGCTCATGCATTCGTAA3') (5'CGCATCCCTCTTCATCGTAT3') producing 357bp fragment, Primers were designed to identify the earthworm *Lumbrica rubellus* by using the NCBI-Genbank (JX531570.1) database and the primer 3 plus online design program.

PCR: Upon completion of the polymerase chain reaction method, after that reaction were sent to Bioneer, where the COX1 gene was compared with the COX1 gene sequences found on the NCBI website in order to determine the confirmatory diagnosis of the sample. The results are read according to the Blast program on the NCBI website. Using MEGA6 program, the genetic relationship tree was analyzed. Finally, the sample was registered in the NCBI Genbank submission.

RESULT AND DISCUSSION

There is difficulty in accurately classifying earthworms depending on the depth of location, size and color of the earthworm, because some features may be changing depending on the maturity of the earthworm. Therefore, the molecular tool has been used to determine the genus as well as the species of earthworms (Ansari and Saywack.,2011; Jorge Escudero *et al*., 2022).The results of gel electrophoresis of DNA samples extracted from *Lumbricus rubellus* using the primer for the COX1 gene showed that the DNA bands were (357 bp) and that the marker used was (100-2000pb).The concentration and purity of the extracted DNA was measured using a Nanodrop device, where the DNA concentration was between (177.6 - 829.3) nanograms/microliter, while the purity was between (2.02 - 2.21).

The DNA sequencing method was carried out to genetic identification earth worm species analysis in cytochrome c oxidase subunit I (cox1) gene in local *Lumbricus rubellus* IQD isolate and NCBI-Genbank related country *Lumbricus rubellus* isolates. The phylogenetic tree genetic relationship analysis was showed that The *Lumbricus rubellus* IQD isolate was showed closed related to NCBI-BLAST *Lumbricus rubellus* isolate 14 Austria isolate (DQ092902.1) at total genetic changes (0.06-0.01%) as showed in figure (2). The homology sequence identity between local *Lumbricus rubellus* isolate IQD isolate and NCBI-BLAST *Lumbricus rubellus* isolate isolates were showed genetic homology sequence identity ranged from (88.45 -99.70%). Finally, local *Lumbricus rubellus* isolate were submitted into NCBI Genbank and identified by accession numbers(OP967515).

In a comparative study, a study was conducted in Australia to diagnose earthworms using four mitochondrial genes (12S rRNA, 16S rRNA, COI, and COII) with regard to identifying earthworms, calculating earthworm strains, and discovering cryptic species (Klarica *et al.*, 2012) the study concluded that the four genes are suitable for identifying species, but COI The best gene for coding genes and processing genetic lineages. In a British study, the goal was to examine the genetic structure of earthworm species, *Allolobophora chlorotica*, *Aporrectodea longa*, *Aporrectodea rosea* and *Lumbricus rubellus* all of them comprising highly divergent lineages with species-level divergence in the mitochondrial cytochrome oxidase I (COI) gene Phylogenetic analyses of mitochondrial COI and 16S genes showed the presence of five highly divergent lineages (King *et al.*, 2008). Earthworms have been diagnosed based on one nuclear (H3) and one mitochondrial (COI) marker in northern Europe where *Lumbricus rubellus* and *L. terrestris* and *L. herculeus* have been identified (Martinsson and Erséus., 2017).

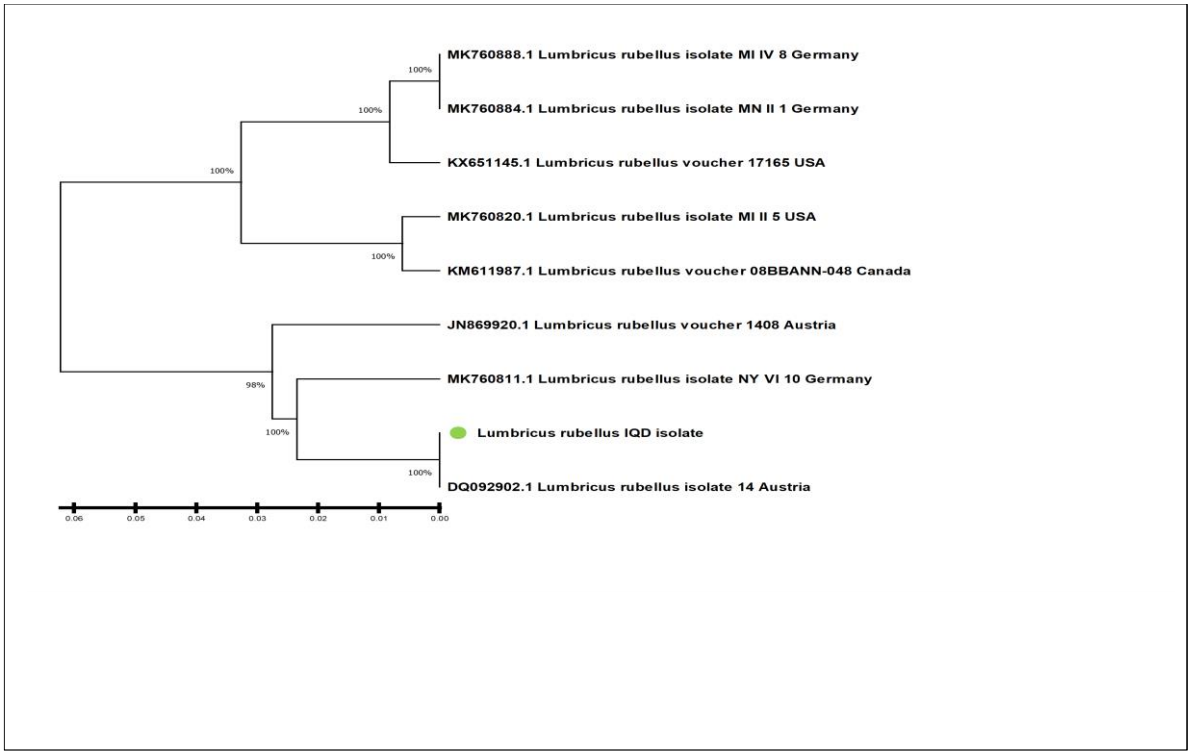

Figure 2: Phylogenetic tree analysis based cytochrome c oxidase subunit I (cox1) gene partial sequence in local *Lumbricus rubellus* IQD isolate that used for genetic species confirmative detection analysis

Soil contaminations could also affect the distributions of genetic lineages in natures. If the degrees of sensitivity to soil pollutions differ among DNA lineages, some lineage will be lost in pollute spots, which were reducing variation and is consistent with genetic erosion hypothesis. Andre *et al.*, have investigated the highly differentiated population of *L. rubellus* from a Pb-polluted habitat close to Cwmystwyth, UK. The predominant linages differed by study sites depending on the levels of contamination. This pattern supported the loss of distinct DNA lineage due to pollutions. In our research, phylogenetic tree analysis in the least polluted sites. In contrast, it was not found at any of the polluted Al-Diwaniyah city.

In ecotoxicology, earthworm is used for standard toxicity test. The recommended and most commonly used species are *L. rubellus*. However, the taxonomy of these species is not clear because of cryptic diversity. The earthworm *L. rubellus* has been suggested to be a species complex. Römbke *et al.*, reported two distinct DNA clusters of *L. rubellus* that were separated by a distance of 11.2 %. Based on the assumption that an uncorrected distance > 10 % indicates species level differentiation, these authors hypothesized that *L. rubellus* consisted of cryptic species. This result calls the quality and the comparability of ecotoxicological test into question because cultures of earthworms are rarely barcoded. Nuclear markers were not applied to confirm the DNA clustering of the *L. rubellus* reported by Römbke *et al.*, although previous analysis of nuclear 28S gene indicated possibility that *L. rubellus* from Ireland might be a cryptic species. Therefore, the findings of our study are particularly relevant because we showed that high DNA divergence, even values exceeding 15 %, did not necessarily indicate the presence of cryptic earthworm species. Thus, in addition to crossbreeding experiments, we recommend the use of nuclear data to test for cryptic species in *L. rubellus*.

CONCLUSION

The study concludes that a viable method for species discrimination is DNA barcoding, which uses a brief, standardized DNA fragment for species identification, use of DNA remains the best in determining the species. *Lumbricus rubellus* have been found on in the common epigeic earthworm from Iraq in Al-Diwaniyah city, the presence of this worm is recorded for the first time in Iraq, specifically in the city of Diwaniyah. This study tested sequence data for the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene in order to identify the utility of DNA barcodes in the identification of earthworm species.

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