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Molecular identification of seven earthworm (Annelida: Oligochaeta) species using DNA barcoding of the 18S rRNA gene region and their relationship with soil properties in Babylon province, Iraq

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Abstract

Objective

This investigation aimed to employ molecular techniques to diagnose and classify earthworm species present in Babylon Province, Iraq, and to assay the extent to which environmental factors influence their distribution and abundance. Given the limited investigation on Iraqi earthworm fauna, this work sought to provide a clearer understanding of species diversity in the region and to assess how variations in soil characteristics may affect their ecological presence.

Materials and methods

Fieldwork was operated at three ecologically distinct stations within Babylon Province, each representing unique environmental and land-use situations. Sampling was carried out across three seasons-fall, winter, and spring-during the 2023-2024 period. Soil samples from each site were analyzed for temperature, pH, electrical conductivity (EC), total dissolved solids (TDS), and moisture content, providing a comprehensive profile of key physicochemical soil parameters. Earthworm specimens were collected manually and preserved for molecular analysis. DNA barcoding applying the 18S rRNA gene region was employed to diagnose the species, and the resulting sequences were submitted to the NCBI GenBank database for comparison and verification.

Results

Seven species were identified through molecular methods: *Aporrectodea tuberculata*, *Aporrectodea caliginosa*, *Dendrobaena platyura*, *Fitzingeria platyura*, *Hormogaster redii*, *Lumbricus rubellus*, and *Polytoreutus finni*. Statistical correlations revealed that soil moisture and temperature significantly influenced earthworm distribution, with population densities declining markedly during warmer periods. These discoveries suggest that earthworms in this region exhibit strong physiological responses to thermal and hydric stress, which in turn shapes their spatial and seasonal dynamics.

Conclusions

This investigation confirms the utility of DNA barcoding for accurate earthworm species identification and contributes new data on the biodiversity of Oligochaeta in Iraq. The observed correlations between environmental situations and earthworm populations highlight the importance of soil management practices in maintaining biodiversity. Future investigation incorporating wider genomic markers and additional sampling sites is recommended to further elucidate the ecological roles of these organisms across Iraq's diverse habitats.

Keywords: *Dendrobaena platyura*, ecosystem, molecular, Oligochaeta, 18S rRNA

Paper Type: Research Paper.

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Introduction

Agriculture plays a fundamental role in Iraq's economy, contributing approximately 2.8 percent to the Gross Domestic Product (GDP). However, challenges such as soil degradation, water scarcity, and excessive utilization of chemical fertilizers pose significant threats to this sector. Consequently, earthworms, as ecosystem engineers of soil, play a crucial role in improving soil structure and fertility, enhancing aeration and drainage, and reducing the need for chemical fertilizers. Earthworms are highly valued among invertebrates due to their essential role in manipulating soil and breaking down organic matter (Aira et al., 2007). They are considered environmental engineers because of their substantial contribution to preserving soil structure and composition (Singh et al., 2016). Their presence significantly affects the dynamics of soil nutrients by altering the soil's physical, chemical, and biological properties (Singh et al., 2016; Vršič et al., 2021). Earthworms positively influence the soil by creating the drilosphere layer, increasing porosity, and modifying soil structure (Blouin et al., 2013; Singh et al., 2018). They also regulate soil porosity by influencing the rate of organic matter decomposition and nutrient release (De Wandeler et al., 2016). These beneficial effects are crucial for plant growth and yield. Because of their ecological value, several earthworm species have been utilized in soil management and fertilization strategies. One such example is vermicomposting, which efficiently recycles organic waste and produces nutrient-rich vermicompost (Ansari & Ismail, 2012). The distribution of earthworms is influenced by several environmental factors, including temperature, pH, organic matter content, moisture, and soil texture (Moreno & Mischis, 2003).

Earthworms are the most prevalent members of the subclass *Oligochaeta*, class *Clitellata*, and phylum *Annelida*, with more than 6,500 defined species worldwide (Csuzdi, 2012; Lalthanzara et al., 2018). Despite this diversity, many soil invertebrates remain poorly investigated, and a large proportion of species are affected by taxonomic uncertainty. Recognizing earthworm species can be challenging due to morphological similarities, variability associated with various life stages, and a lack of clear diagnostic features (Othman & Ahmed, 2020). Additionally, phenotypic plasticity and environmental influences further complicate classification. To address these challenges, molecular discrimination techniques have been increasingly employed. These methods are now considered reliable tools for determining genetic diversity and overcoming taxonomic limitations (Jorge Escudero et al., 2019). Genetic diversity plays a critical role in the evolution and adaptability of populations. It promotes the emergence of beneficial traits, supports evolutionary processes, and enables adaptation to changing environmental situations (Javanmard et al., 2008; Mohammadabadi et al., 2021). Moreover, recognizing gene polymorphisms is essential for disease detection, treatment, and understanding immune response variability (Mohammadabadi & Tohidinejad, 2017; Saadatabadi et al., 2023). Molecular techniques are also beneficial for characterizing populations and breeds, and for informing conservation and breeding programs (Mohammadifar & Mohammadabadi, 2017; Noori et al., 2017; Jafari Ahmadabadi et al., 2023). Sustainable conservation strategies must be based on knowledge of population structures and genetic resources (Zamani et al., 2011; Molaei Moghbeli et al., 2013). Genetic diversity is therefore an essential component of genetic improvement, conservation, adaptation, and resilience (Askari et al., 2011; Mohammadabadi et al., 2024). Likewise, identifying gene polymorphisms is important for defining individual genotypes and their associations with disease resistance and immune system responses (Norouzy et al., 2005; Shokri et al., 2023; Mohammadabadi et al., 2024). Deoxyribonucleic acid (DNA) barcoding, which utilizes a specific segment of the mitochondrial cytochrome c oxidase subunit I (COI) gene, is a widely utilized molecular approach for species discrimination. The COI gene serves as a genetic marker in molecular systematics and taxonomy (Hebert et al., 2003). According to Moritz and Cicero (2004), the COI gene region provides a reliable means of specimen discrimination and the discovery of novel species. Multiple investigations support the utilization of this gene region as an effective DNA barcode for differentiating animal species (Hebert et al., 2003; Bozorgi et al., 2019).

In Iraq, molecular studies on earthworm discrimination have been relatively limited. Ahmed et al. (2015) operated one of the earliest studies on phylogenetic relationships within the genus *Aporrectodea* applying specimens collected from various locations in Baghdad. Othman and Ahmad (2020) carried out the first investigation applying DNA barcoding to diagnose earthworm species in the Kurdistan region, where they identified six species belonging to three genera and two families: *Lumbricidae* and *Megascolecidae*. These investigations provided the foundation for more comprehensive molecular surveys in the country. Therefore, the present investigation aims to highlight the utilization of molecular techniques to diagnose earthworm species in Babylon Province, Iraq, and to evaluate the potential of these tools to explore species diversity and operate COI sequencing (Decaëns et al., 2013). The agricultural sector in Iraq benefits both environmentally and economically from the activity of earthworms. Their burrowing enhances soil structure, boosts aeration, and promotes nutrient cycling—all essential processes in maintaining soil fertility, particularly in degraded or arid agricultural lands. Earthworms naturally enrich the soil by decomposing organic material, making nutrients more accessible to crops and reducing the dependence on chemical fertilizers. Their contribution supports higher crop yields and promotes sustainable agricultural practices, helping to decline the environmental impact of farming. Furthermore, earthworm activity improves water retention in the soil, which is critical for efficient irrigation in water-scarce environments like Iraq. Overall, the presence of earthworms plays a fundamental role in ensuring long-term agricultural productivity, rural livelihoods, and food security. Recent investigations have significantly contributed to the understanding of earthworm diversity in Iraq. A 2023 survey in Babylon Province revealed the presence of nine various earthworm species, including *Aporrectodea tuberculata* and *Lumbricus terrestris*, drawing attention to the region's biodiversity. In the Kurdistan region, a 2020 molecular

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investigation involving 256 specimens from Erbil, Sulaymaniyah, Duhok, and Halabja was the first to employ DNA barcoding in Iraq for species-level discrimination. Similarly, earthworm discrimination studies operated in Baghdad Governorate in 2023 have provided updated insights into local species composition.

Despite these advances, several obstacles hinder accurate discrimination of earthworms in Iraq. The absence of a comprehensive national reference database and the limited number of taxonomic studies pose significant challenges. Morphological approaches alone are often insufficient for distinguishing closely related species, particularly in regions where taxonomic expertise is scarce. Furthermore, molecular tools such as DNA barcoding remain underutilized due to restricted access and limited infrastructure. Environmental and geopolitical situations have also constrained long-term ecological investigation, reducing opportunities for systematic biodiversity assessments (Othman & Ahmed, 2020). Addressing these limitations will require expanded investigation efforts, international collaboration, access to advanced molecular technologies, and more extensive field surveys. Vegetation cover at sampling sites may vary based on crop types, land utilization, and seasonal changes. Accurately documenting the crops grown and the soil texture at each site is essential to enhance the scientific value of such studies. Both factors significantly affect the density and diversity of earthworm populations. Soil texture influences water retention, aeration, and organic matter availability-key situations for earthworm survival. For example, loamy and silt loam soils generally support larger populations due to their balanced moisture and nutrient levels, while heavy clay soils can restrict movement and reduce aeration. Including this information can lead to a more comprehensive understanding of the environmental variables affecting earthworm distribution in the region.

Materials and methods

Description of the investigation area: This investigation was operated at three agriculturally significant sites within Babylon Province, Iraq (Figure 1). All selected stations are characterized as cultivated land and were chosen to represent the geographical diversity of the province:

Station 1 – *Albu Alwan Village* (32°35'47.43"N, 44°23'4.45"E) located in the northern part of Babylon.

Station 2 – *Al-Bu Nafi Village* (32°31'1.93"N, 44°33'3.70"E) situated in the central region.

Station 3 – *Al-Mu'aymira District* (32°22'3.16"N, 44°42'36.04"E) representing the southern region.

The selection of Babylon Province was based on its agricultural importance, diverse soil types, and pronounced seasonal variation, which are likely to influence soil biodiversity.

Sampling locations were strategically selected to ensure coverage of the province's north, center, and south. Additionally, environmental variables such as soil texture (loam, silt loam, and clay loam), organic matter content, moisture levels, and land-utilization practices were considered to understand their impact on earthworm distribution and diversity.

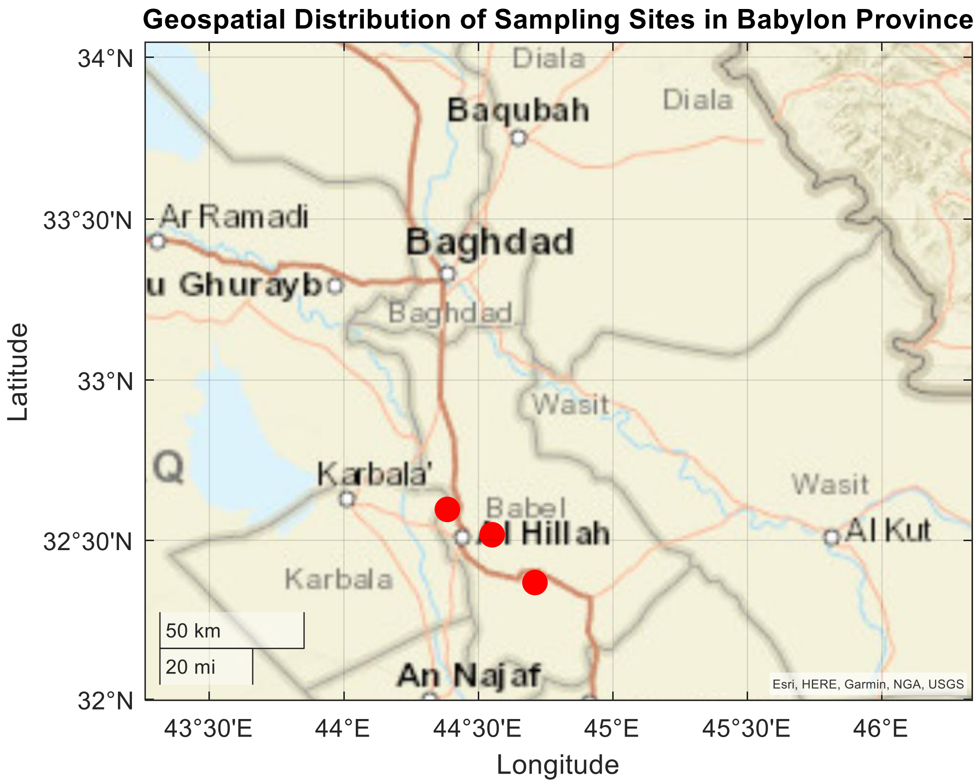

Figure 1. Sampling sites in Babylon province

Climatic and seasonal situations of the investigation area: The selected stations fall within an arid to semi-arid climatic zone, characterized by hot summers and mild winters. These climatic situations strongly influence soil properties and the biological activity of soil fauna, including earthworm populations.

- Precipitation Pattern: Rainfall in the region is sparse and irregular, with most precipitation occurring between November and April. Annual rainfall ranges from 100 to 200 mm.
- Evapotranspiration Rate: High temperatures and low relative humidity lead to elevated evapotranspiration rates, resulting in significant soil moisture loss during the summer season.
- Temperature Regime: According to the USDA Soil Taxonomy, the investigation area falls under the hyperthermic temperature regime, indicating persistently high soil temperatures throughout the year.

- Moisture Regime: The predominant soil moisture regime is classified as *aridic*, meaning the soil remains dry for most of the year, except during brief rainfall periods.

Collection of samples: Earthworms and associated soil samples were collected seasonally across three distinct periods: autumn 2023 (October–November), winter 2023 (December–February), and spring 2024 (March–April). The sampling methodology followed standardized procedures as outlined by Khidher (2017). At each of the three investigation sites, triplicate samples were collected per season, resulting in a total of nine replicates across the entire investigation period. Due to insufficient surface moisture, soil samples were excavated to a depth of 5-25 cm applying shovels, as recommended by Farhadi et al. (2013). Earthworms along with surrounding soil and organic material were placed in polyethylene containers measuring 40 × 30 cm. These containers were transported to the Department of Biology, College of Science, University of Babylon for further processing and species discrimination. To maintain suitable environmental situations for the survival of earthworms during transport and holding, each container was supplemented with manure, leaf litter, and irrigated daily to sustain a moisture level between 70% and 80%. Containers were securely covered with breathable textile nets to prevent the escape of earthworms while ensuring adequate airflow. The utilization of triplicate samples per site and season strengthened the reliability of the data and enhanced the robustness of the statistical analyses. This approach facilitated accurate evaluation of seasonal variations and the influence of environmental factors on earthworm distribution and abundance within the investigation area.

Molecular discrimination of earthworms: Genomic DNA was extracted from earthworm tissue samples applying the cetyl trimethyl ammonium bromide (CTAB) DNA extraction kit (Qiagen, Germany), following the manufacturer's protocol. To assess genetic diversity among earthworms, the 18S ribosomal RNA (18S rRNA) gene was selected due to its conserved nature and taxonomic relevance across eukaryotes. Custom primers were designed specifically for this investigation applying the Primer3Plus online tool (NCBI), targeting the 18S rRNA gene for accurate characterization (Table 1). These primers were not previously published and were optimized for the species under investigation. Sequence matching was operated to verify primer specificity and ensure alignment with the target genomic regions. DNA purity and concentration were assessed applying a NanoDrop 2000 spectrophotometer (Thermo Scientific), with absorbance measured at 260 and 280 nm according to standard protocols (Desjardins & Conklin, 2011; Scientific, 2009). The polymerase chain reaction (PCR) situations—including temperature profiles and cycling parameters—were specifically optimized for the earthworm species being investigated. An expected amplicon size of approximately 412 base pairs allowed for reliable verification of amplification success applying agarose gel electrophoresis. These primer sequences and PCR settings provide a reproducible and robust methodological framework for future molecular discrimination studies of earthworms in various ecological settings. The 18S rRNA gene was selected for its high conservation across eukaryotes, as well as for its mixture of conserved and variable regions, which make it ideal for distinguishing between species while maintaining broad phylogenetic utility. Its slow evolutionary rate supports detailed phylogenetic analysis, offering insights into interspecific relationships. Additionally, the gene's high copy number facilitates amplification from small or partially degraded DNA samples, improving detection accuracy. Due to its widespread utilize in soil biodiversity investigation, the utilization of 18S rRNA enhances the accuracy of species discrimination and supports meaningful comparison with prior studies. Overall, this molecular approach contributes to a more comprehensive understanding of earthworm diversity and their ecological roles in varying soil environments.

Table 1. Custom primers designed for the molecular characterization of earthworms based on the 18S rRNA gene

Primer Name	Sequence (5′–3′)	Target Gene	Product Size (bp)	PCR Conditions
Primer-F	GCT TGT CTC AAA GAT TAA GCC ATG CAT G	18S rRNA	412	Step 1: 95 °C for 2 min Step 2: 95 °C for 30 s
Primer-R	GCC TGC TGC CTT CCT TGG A	18S rRNA		Step 3: 62 °C for 30 s Step 4: 72 °C for 20 s Step 5: 72 °C for 5 min Step 6: 4 °C (hold)

Statistical analysis of data: Statistical analysis was performed applying SPSS software, version 23.0. (IBM Corp, 2015). Duncan's Multiple Range Test (DMRT) was applied to compare means, with statistical significance set at $p < 0.05$. This threshold is widely accepted in agricultural and ecological investigation for determining the presence of statistically significant differences among treatment means.

Results and discussion

A total of seven earthworm species—*Aporrectodea tuberculata*, *Aporrectodea caliginosa*, *Dendrobaena platyura*, *Fitzingeria platyura*, *Hormogaster redii*, *Lumbricus rubellus*, and *Polytoreutus finni*—were identified through molecular techniques based on genomic DNA amplification. The DNA concentrations extracted from the samples ranged from 7.9 to 9.4 ng/µL, with purity ratios (A260/A280) varying between 1.1 and 1.4, as measured applying a NanoDrop 2000 spectrophotometer (Thermo Scientific). The observed DNA purity ratios were lower than

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the ideal value of ~1.8, which is generally indicative of pure genomic DNA (Desjardins & Conklin, 2011). This deviation suggests possible contamination—likely from residual proteins, humic substances, or phenolic compounds commonly present in soil-derived biological samples. Proteins, for instance, absorb strongly at 280 nm, and their incomplete removal during DNA extraction can significantly lower the A260/A280 ratio. Similarly, the presence of humic acids and organic soil pollutants may interfere with absorbance readings and compromise DNA purity. Additionally, incomplete RNA removal could have also contributed to these lower values. To improve the quality of extracted DNA, future protocols may benefit from incorporating additional purification steps, such as phenol–chloroform extraction, proteinase K digestion, or column-based purification techniques. These steps are essential for ensuring higher-quality DNA suitable for precise downstream molecular analyses. Out of 40 total PCR-amplified products, only seven samples were selected for DNA sequencing, which was carried out by Macrogene (South Korea). The resulting sequences were subjected to BLAST similarity analysis, showing 83–100% identity with existing sequences deposited in GenBank (Table 2). The confirmed sequences were subsequently submitted to the NCBI GenBank, where they were assigned unique accession numbers. Traditional taxonomic discrimination of earthworms based on morphological features alone can be unreliable, particularly due to interspecific similarities and phenotypic variations during various developmental stages (Jorge Escudero et al., 2019). Immature or degraded specimens often lack distinguishable morphological traits, making them especially difficult to classify with certainty. In this context, DNA barcoding provides a robust and precise method for species-level discrimination by targeting conserved genetic regions, such as the 18S rRNA gene, as utilized in this investigation (Chang et al., 2009). Our discoveries underscore the effectiveness of molecular taxonomy in complementing and often surpassing classical methods of species discrimination. This is particularly important for ecological monitoring and biodiversity assessments, where precise discrimination is critical (Al-Habib & Othman 2021). The high genetic similarity with sequences in GenBank supports the accuracy of our discrimination and affirms the genetic distinctiveness of the species found in Babylon Province.

Importantly, this investigation represents the first molecular characterization of earthworm species in Babylon Province applying DNA barcoding, marking a foundational step for future soil biodiversity studies in the region. These molecular insights contribute to a better understanding of the ecological distribution of earthworms and their potential roles in various soil environments.

Table 2. Sequence alignment results of recorded earthworm species based on BLAST similarity analysis

Accession Number	Similarity (%)	Aligned Base Pairs	Identified Species (Barcoding)
LC822238	100%	356/356	*Aporrectodea tuberculata*
LC822239	98%	355/361	*Aporrectodea caliginosa*
LC822240	89%	308/347	*Fitzingeria platyura*
LC822241	99%	372/376	*Lumbricus rubellus*
LC822242	99%	371/374	*Dendrobaena platyura*
LC822243	86%	168/196	*Polytoreutus finni*

Among the seven identified species, *Aporrectodea tuberculata*, *Aporrectodea caliginosa*, *Dendrobaena platyura*, and *Lumbricus rubellus* emerged as the most abundant throughout the investigation period. The highest recorded density was attributed to *Lumbricus rubellus*, accounting for 37.1% of the total earthworm population in Station 2 during the spring season (Figures 2-4). This finding aligns with the observations reported by Klok et al. (2007), who noted that *L. rubellus* is particularly prolific under moderate temperatures and favorable moisture situations. It is well-established that earthworm density is influenced by climatic and edaphic factors, particularly temperature and soil moisture. High temperatures and reduced moisture levels typically result in a decline in earthworm activity, especially in arid and semi-arid regions. The present discoveries are consistent with previous studies (Lavelle & Spain, 2001; Al-Khafagi et al., 2013; Javidkar et al., 2020), which indicate that both seasonal temperature variations and water availability significantly affect earthworm distribution and behavior. During the experimental period, soil temperatures in Station 2 ranged from 17.3 °C in spring to 25.83 °C in autumn (Table 3). Statistical analysis revealed significant differences in soil temperature across the three stations throughout the investigation period. These differences reflect the geographical and climatic heterogeneity of the investigation area, consistent with discoveries by Manickam et al. (2014). Soil moisture content was notably low, ranging from 5.22% to 9.24% (Table 3), particularly during warmer months. Reduced moisture availability is known to negatively affect earthworm survival and activity (Hendrix & Edwards, 2004), which may explain the seasonal fluctuations observed in the current investigation. Soil pH also varied between stations and seasons, with the lowest recorded value of 5.66 in Station 2 during winter and the highest value of 7.06 in Station 1 during spring (Table 3). These values fall within the range considered suitable for earthworm habitation, typically between slightly acidic and slightly alkaline situations. This observation aligns with the discoveries of Sankar & Patnaik (2018), who reported that while most earthworms thrive in near-neutral soils, some species demonstrate a degree of tolerance to broader pH variations. In addition, electrical conductivity (EC) is commonly associated with the concentration of dissolved salts in soil, and a strong correlation typically exists between EC and total dissolved solids (TDS). In the current investigation, both EC and TDS values exhibited notable spatial and seasonal variation. EC ranged from a minimum of 420 µS·cm⁻¹ in Station 1 during winter to a maximum of 787.67 µS·cm⁻¹ in Station 1 during spring. Similarly, TDS values varied from 333.66 mg·L⁻¹ in Station 3 during winter to 683.66 mg·L⁻¹ in Station 3 during spring (Table 3).

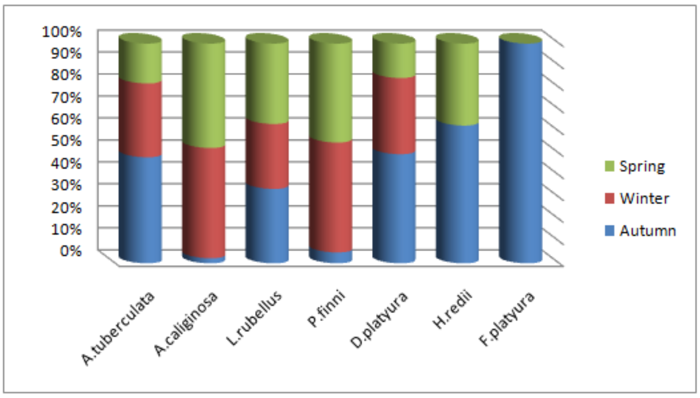

Figure 2. Seasonal variation in earthworm density at Station 1 throughout the investigation period

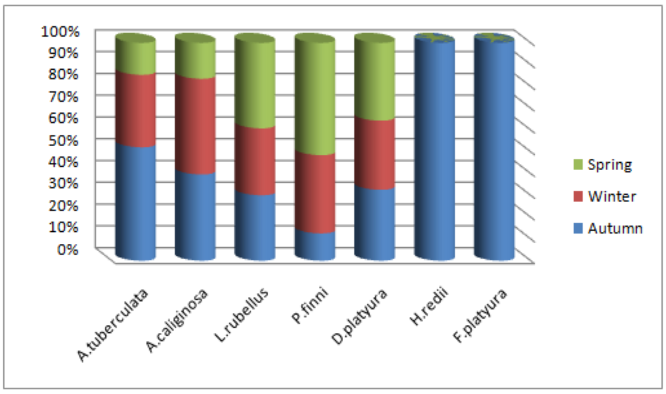

Figure 3. Seasonal variation in earthworm density at Station 2 throughout the investigation period

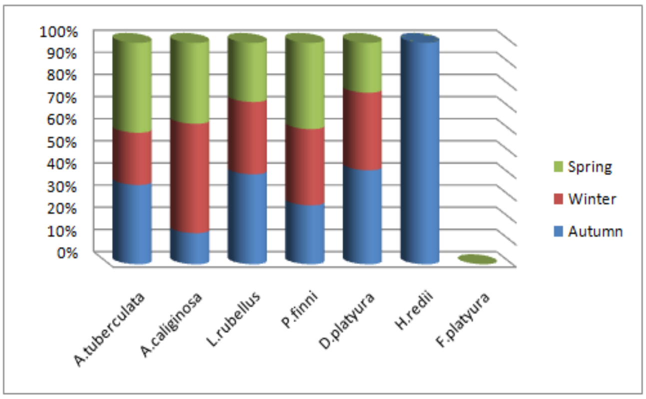

Figure 4. Seasonal variation in earthworm density at Station 3 throughout the investigation period

These seasonal fluctuations were statistically significant and likely influenced by environmental factors such as temperature and soil moisture content. Elevated values of EC and TDS during the spring season may be attributed to enhanced evaporation rates, reduced soil moisture, and higher mineralization of organic matter under warmer situations. During drier periods, the limited water availability may concentrate salts and dissolved ions in the soil, thereby increasing EC and TDS. This observation is consistent with discoveries from previous studies operated in various climatic regions, including those by Dhanasekaran et al. (2017) and Dastgeer et al. (2020), which also reported enhanced salinity indicators during dry or warmer seasons. The results highlight the importance of monitoring physicochemical parameters as they directly influence soil biota, including earthworm distribution and activity.

Table 3. Mean ± standard deviation of physicochemical parameters at investigation stations during spring, winter, and autumn seasons

Parameter	Season	Station 3	Station 2	Station 1
Soil Temperature (°C)	Spring	17.66 ± 0.57^{a}	17.3 ± 0.88^{a}	17.53 ± 0.83^{a}
	Winter	18.36 ± 1.06^{a}	18.83 ± 0.76^{b}	17.86 ± 0.41^{a}
	Autumn	24.66 ± 0.57^{B}	25.83 ± 0.28^{c}	23.66 ± 1.52^{b}
pH	Spring	7.03 ± 0.56^{a}	5.9 ± 0.17^{a}	7.06 ± 0.61^{a}
	Winter	6.7 ± 0.25^{a}	5.66 ± 0.47^{a}	6.66 ± 0.6^{a}
	Autumn	6.8 ± 0.45^{A}	6.26 ± 0.5^{a}	6.96 ± 0.11^{a}
Electrical Conductivity (μS/cm)	Spring	783.33 ± 336.5^{a}	688.33 ± 75.4^{a}	787.67 ± 116.98^{b}
	Winter	430.66 ± 73.22^{a}	456.66 ± 245.83^{a}	420 ± 83.54^{a}
	Autumn	612 ± 129.83^{A}	533.33 ± 70.94^{a}	446.67 ± 117.18^{a}
Total Dissolved Solids (TDS, mg/L)	Spring	683.66 ± 22.23^{c}	623 ± 42.14^{b}	671 ± 30.26^{b}
	Winter	333.66 ± 60.28^{a}	361.33 ± 163.9^{a}	375 ± 31.0^{a}
	Autumn	464.66 ± 46.5^{b}	342.33 ± 32.56^{a}	355.67 ± 65.89^{a}
Moisture Content (%)	Spring	6.54 ± 3.8^{a}	5.22 ± 1.01^{a}	5.23 ± 1.76^{a}
	Winter	8.71 ± 1.33^{a}	8.44 ± 1.6^{c}	9.24 ± 1.28^{b}
	Autumn	5.81 ± 0.58^{a}	6.66 ± 0.45^{ab}	6.94 ± 1.06^{ab}
Organic Matter (%)	Spring	2.5 ± 0.3^{a}	3.1 ± 0.4^{b}	2.8 ± 0.5^{a}
	Winter	3.2 ± 0.3^{b}	3.8 ± 0.5^{c}	3.4 ± 0.4^{b}
	Autumn	2.7 ± 0.2^{a}	3.3 ± 0.4^{b}	2.9 ± 0.3^{a}
Cation Exchange Capacity (CEC, cmol(+)/kg)	Spring	14.8 ± 0.5^{a}	16.4 ± 0.7^{b}	15.2 ± 0.6^{a}
	Winter	15.8 ± 0.4^{b}	17.5 ± 0.8^{c}	16.1 ± 0.5^{b}
	Autumn	15.1 ± 0.3^{a}	16.9 ± 0.6^{b}	15.7 ± 0.4^{a}

The values represent (mean ± SD). Similar adjacent letters indicate a non-significant differences P≥ 0.05 for each season.

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In Table 3, the utilization of both lowercase and uppercase letters denotes the results of statistical comparisons operated applying Duncan's Multiple Range Test (DMRT). Lowercase letters (e.g., *a, b, c*) are utilized to compare values within the same season across various stations (St1, St2, St3). Identical lowercase letters following the mean values indicate no significant difference among stations during that specific season ($p > 0.05$). Conversely, various lowercase letters suggest a statistically significant difference ($p < 0.05$) between the stations in that season. Uppercase letters (e.g., *A*, *B*, *C*) are utilized to compare values across various seasons within the same station. If all seasonal values for a station share the same uppercase letter, this indicates no significant seasonal variation ($p > 0.05$). various uppercase letters imply a statistically significant seasonal difference ($p < 0.05$) at that station. This notation method is a widely accepted convention in statistical analysis for distinguishing meaningful differences in datasets, helping to clarify whether observed variations reflect actual effects rather than random variability.

Figure 5 presents a phylogenetic tree illustrating the evolutionary relationships among various earthworm species, with the genera *Amyntas*, *Aporrectodea*, and *Haelyella* serving as primary focal points. In this tree, the nodes represent common ancestors, while the branches illustrate distinct evolutionary lineages. Bootstrap values at the nodes provide statistical support for the branching patterns, with values approaching 100 indicating greater confidence in the inferred evolutionary relationships. The tree reveals that *Amyntas luridus*, *Amyntas gracili*, and *Amyntas morissi* form well-supported and distinct clusters, suggesting close evolutionary relatedness within this genus. Similarly, species within the genus *Aporrectodea* are grouped together, yet remain clearly distinct from the *Amyntas* clade. Notably, *Haelyella syriaca* appears in two separate locations on the tree, indicating potential intraspecific genetic divergence or cryptic speciation within this taxon. The tree is rooted applying *Lumbricus variegatus* as an outgroup, providing a reference point for assessing the relative divergence among the ingroup species. The scale bar, representing a genetic distance of 0.020, quantifies the extent of evolutionary change. Overall, this phylogenetic analysis enhances our understanding of the genetic divergence and evolutionary history among various earthworm species.

It is also essential to consider environmental parameters—particularly soil pH—when analyzing earthworm distribution. Various species exhibit varying degrees of tolerance to soil acidity or alkalinity. Soils with low pH typically exhibit reduced microbial activity and nutrient availability, which may limit the presence or survival of certain earthworm species. In contrast, soils with pH values between 6.5 and 7.5-neutral to slightly alkaline-are generally the most conducive to earthworm activity and diversity. Furthermore, other soil characteristics, such as moisture content, organic matter, and soil texture, play crucial roles in influencing earthworm population density, burrowing behavior, and nutrient cycling. Regular assessment of these factors is recommended to gain a holistic understanding of earthworm ecology. Integrating these variables into a comprehensive analysis—supported by relevant scientific literature—not only strengthens ecological interpretations but also aligns the discoveries with the standards of academic investigation.

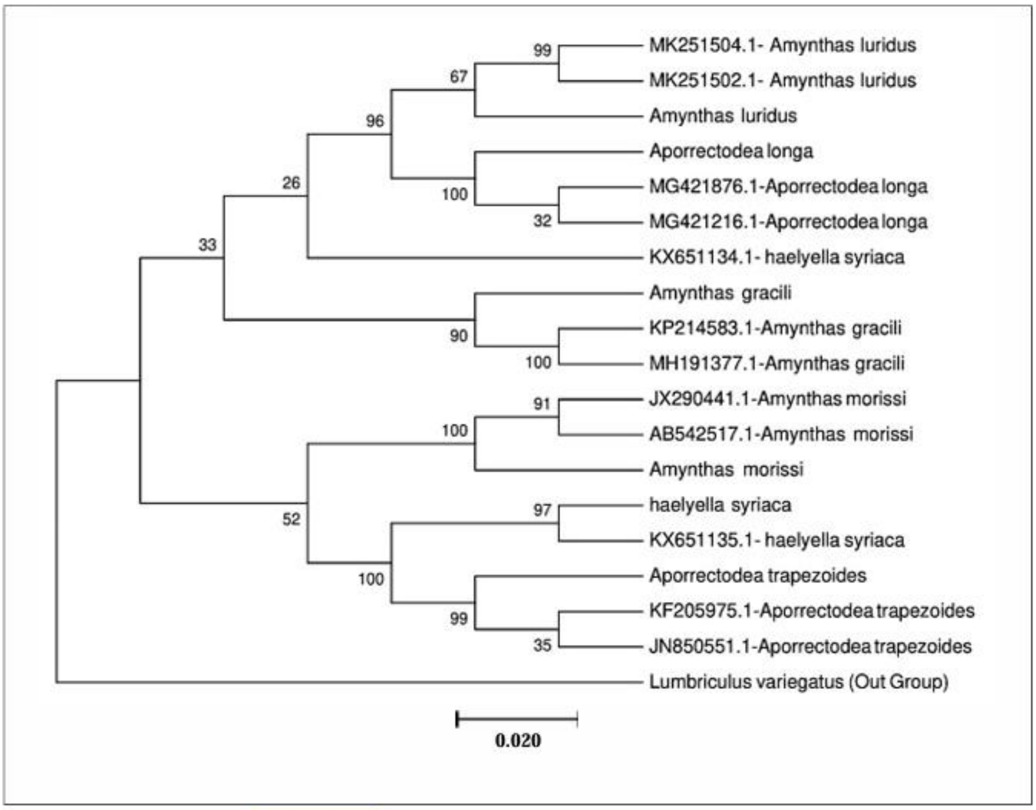

Figure 5. Phylogenetic tree analysis based on 18S rRNA gene sequences

Significant correlations with earthworm distribution-soil temperature: Earthworms are highly sensitive to soil temperature, making it a critical factor influencing their distribution and biological activity. The investigation observed seasonal variations in temperature across various sampling stations, with the lowest soil temperatures recorded in spring and the highest in fall. These thermal fluctuations are likely to affect earthworm density and species distribution. Lower soil temperatures may inhibit earthworm activity, while elevated temperatures can enhance metabolic rates and activity levels. Therefore, soil temperature, particularly during seasonal transitions, warrants close examination as a potential driver of earthworm population dynamics.

Moisture content: Earthworms require moist environments to survive and thrive, making soil moisture a fundamental determinant of their habitat preference. In the current investigation, moisture content ranged from 5.22% to 9.24%, with such variations likely exerting a direct influence on earthworm abundance. Low moisture levels—especially during arid periods—can reduce earthworm activity or result in mortality. Conversely, higher soil moisture supports enhanced earthworm density. A significant positive correlation between soil moisture and earthworm population density is expected, reinforcing the importance of water availability in earthworm ecology.

Soil pH: Soil pH is another critical parameter influencing earthworm distribution, as most species prefer neutral to slightly alkaline situations. Nonetheless, some species can tolerate mildly acidic environments. The pH range recorded in this investigation (5.66 to 7.06) falls within the generally favorable range for earthworm activity. If a significant correlation between pH and earthworm density exists, it may suggest species-specific preferences for slightly acidic, neutral, or slightly alkaline situations. Understanding these preferences could offer insights into the niche partitioning among earthworm species across various soil environments.

Electrical conductivity (EC) and total dissolved solids (TDS): EC and TDS are indicators of soil salinity and mineral content, both of which can significantly impact earthworm survival and distribution. High levels of EC and TDS, especially under dry-season situations, may induce osmotic stress or toxicity, leading to reduced earthworm populations. In contrast, lower values of EC and TDS often indicate more favorable situations for earthworms. A negative correlation between EC/TDS and earthworm density would suggest that elevated salinity is detrimental to earthworm survival, while a positive correlation might indicate that moderate mineral levels are beneficial. These relationships provide valuable insight into the environmental thresholds that influence earthworm ecology. Together, these discoveries underscore the multifactorial nature of earthworm distribution. Table 4 presents the statistical significance of correlations between earthworm density and the measured environmental variables, offering a comprehensive overview of the ecological drivers shaping species presence and abundance in the investigated habitats.

Soil properties: To assess the characteristics of the soil across the investigation sites, standard and widely accepted analytical methods were employed:

Organic matter content: The Walkley-Black method, also known as wet oxidation, was utilized to estimate the amount of organic carbon in the soil. This method is considered reliable for determining soil organic matter. The final organic matter content was calculated by multiplying the measured organic carbon by a conversion factor of 1.724, in accordance with established protocols.

Cation exchange capacity (CEC): The CEC of the soil was determined applying ammonium acetate extraction at pH 7.0, a well-established and effective technique for assessing the soil's total capacity to retain and exchange cations. This parameter is critical for evaluating soil fertility and nutrient retention potential.

Soil temperature: Soil temperature readings were obtained directly at the sampling stations applying calibrated glass thermometers (Kamp, England) with a range of 0 to 100°C. Measurements were taken consistently across all sites and seasons.

Table 4. Summary of significant correlations between environmental parameters and earthworm distribution

Parameter	Observed Range	Effect on Earthworm Distribution	Significance	Expected Correlation
Soil Temperature (°C)	17.3 – 25.83	Influences metabolic rates and activity; lower temperatures may limit activity, while moderate to higher temperatures enhance it	Significant variation across seasons and stations	Positive with moderate temperatures; negative at extreme ends
Moisture Content (%)	5.22 – 9.24	Essential for survival; low moisture leads to declined activity or mortality	Significant seasonal and spatial variation	Strong positive correlation with higher moisture levels
Soil pH	5.66 – 7.06	Most species favor neutral to slightly alkaline pH; extremes may reduce species diversity	Within favorable range for earthworm activity	Positive correlation with neutral to slightly alkaline situations
Electrical Conductivity (EC) (µS/cm)	420 – 787.67	Elevated salinity may cause osmotic stress, negatively affecting earthworm survival	Significant during dry seasons	Negative correlation with high EC values
Total Dissolved Solids (TDS) (mg/L)	333.66 – 683.66	High TDS can enhance soil salinity, leading to reduced earthworm activity	Significant during dry seasons	Negative correlation with high TDS levels

Electrical conductivity (EC), pH, and total dissolved solids (TDS): These parameters were measured applying a HANNA multi-parameter probe (Model Romania), following proper calibration procedures for each measurement, in accordance with APHA (2005) guidelines. Given that the investigation is based on conventional soil analysis methods for agricultural contexts, the saturated paste extraction method was deemed the most suitable for accurate readings.

Moisture content: Soil moisture was measured by drying a 50-gram composite sample in a laboratory oven at 105°C for 24 hours, following the method defined by Joel & Amajuoyi (2009). The sample was weighed before and after drying until a constant weight was achieved. The moisture content was calculated based on the weight loss, representing the water held in the soil.

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To enhance the ecological interpretation of this investigation, Table 5 provides valuable insights into the relationship between soil fertility, organic matter availability, and the diversity of earthworm species. The relative abundance (%) was calculated according to the method outlined by Salahi et al. (2017). This metric represents the proportion of a specific species or group within the total population at a given station or during a particular season. It is widely utilized in ecological and soil biodiversity studies to evaluate species dominance and community structure. The calculation is performed applying the following formula:

$$\text{Relative Abundance (\%)} = \left(\frac{\text{Number of individuals of a species}}{\text{Total number of individuals in the sample}} \right) \times 100$$

This approach provides valuable insights into species distribution patterns across various locations and seasons, helping to diagnose dominant species and assay the potential environmental factors influencing their abundance. The relative abundance values were compared across the three stations to evaluate spatial and seasonal variations in soil biodiversity.

Table 5. Organic matter and cation exchange capacity across stations and seasons

Station	Season	Organic Matter (%)	CEC (cmol(+)/kg)
Albu Alwan	Spring	2.8	15.2
	Summer	2.3	14.5
	Autumn	2.9	15.7
	Winter	3.4	16.1
Al-Bu Nafi	Spring	3.1	16.4
	Summer	2.6	15.2
	Autumn	3.3	16.9
	Winter	3.8	17.5
Al-Mu'aymira	Spring	2.5	14.8
	Summer	2.0	13.9
	Autumn	2.7	15.1
	Winter	3.2	15.8

Species abundance and environmental factors: Providing additional insights into the reasons behind the higher abundance of certain species at specific stations or during particular seasons will deepen the understanding of the ecological system. Key factors to consider include soil characteristics such as pH, moisture content, organic matter, and temperature. Moreover, accounting for seasonal climate patterns and agricultural practices could also help explain species prevalence.

Broader ecological factors: While soil properties play a crucial role, it would be beneficial to incorporate discussions on land management practices (e.g., plowing, fertilization, and irrigation), vegetation cover (natural vs. cultivated areas), and interactions with other soil organisms (e.g., microbial communities and predators). These factors collectively contribute to a more comprehensive understanding of earthworm distribution.

Establishing a connection between the discoveries and soil health and agriculture: The impact of this investigation would be further amplified by framing the results within the context of sustainable agricultural practices in Iraq. Discussing the relationship between earthworm diversity and abundance, and soil fertility, nutrient cycling, and crop yield, would make the discoveries more relevant for agricultural management. Furthermore, this perspective highlights the role of earthworms in improving soil health, reducing dependence on synthetic fertilizers, and enhancing ecosystem resilience. There are seasonal fluctuations in soil properties across all three sites in Babylon Province, influenced by ambient situations and agricultural activities (Table 6). Organic matter concentration peaks during the winter months due to slower decomposition, while it declines in the summer months owing to enhanced microbial activity. Soil porosity follows a similar pattern, with higher values observed in winter and spring, and lower values in summer due to soil compaction. Although there are slight variations in the amounts of clay, silt, and sand, soil texture remains relatively consistent across the sites. Most stations exhibit loam, silt loam, and clay loam textures, all of which influence water retention, aeration, and crop adaptability. These results underscore the importance of implementing soil management strategies to ensure soil fertility and sustainability throughout the year.

Table 6. Soil properties across three stations and seasons

Station	Season	Organic Matter (%)	Porosity (%)	Clay (%)	Silt (%)	Sand (%)	Soil Texture Class
Albu Alwan	Winter	2.5	45	35	40	25	Loam
	Spring	2.3	44	38	37	25	Loam
	Summer	2.0	42	40	35	25	Clay Loam
Al-Bu Nafi	Winter	3.1	47	30	45	25	Silt Loam
	Spring	2.8	46	33	42	25	Silt Loam
	Summer	2.4	43	35	40	25	Loam
Al-Mu'aymira	Winter	2.9	46	32	43	25	Silt Loam
	Spring	2.6	44	34	41	25	Loam
	Summer	2.2	41	36	38	26	Clay Loam

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Seasonal variations in soil properties: Significant differences were observed in organic matter, porosity, and soil texture, reflecting the influence of seasonal changes on soil health and fertility. These variations were evident when examining soil parameters across various stations and seasons. The highest organic matter content was recorded in the winter months at Al-Bu Nafi (3.1%), followed by Al-Mu'aymira (2.9%) and Albu Alwan (2.5%). This suggests that winter situations are more conducive to organic matter accumulation, likely due to lower rates of microbial decomposition and higher moisture retention. Conversely, organic matter levels were at their lowest during the summer months, with values dropping to 2.0% at Albu Alwan and 2.2% at Al-Mu'aymira. These reductions can be attributed to enhanced microbial activity and organic matter mineralization at higher temperatures. Soil porosity displayed a similar trend, with the highest value of 47% recorded at Al-Bu Nafi during winter, and the lowest value of 41% at Al-Mu'aymira in summer. This reflects the impact of seasonal drying and soil compaction on soil quality. Soil texture classifications indicated that Al-Bu Nafi and Al-Mu'aymira were predominantly silt loam during the winter and spring seasons, while Albu Alwan transitioned from loam to clay loam in summer, highlighting seasonal shifts in soil particle distribution. The enhance in clay content during summer, particularly at Albu Alwan (40%) and Al-Mu'aymira (36%), suggests heightened soil compaction and reduced aeration, which may limit earthworm activity and root growth. In contrast, the consistent silt loam texture at Al-Bu Nafi throughout the investigation period suggests a more stable soil structure, beneficial for moisture retention and biological activity. These seasonal fluctuations in soil properties, as demonstrated by the data, emphasize the critical role of seasonal variations in shaping soil characteristics, with important implications for agricultural production and ecosystem health.

Conclusions: Seven earthworm species were successfully identified in this investigation: *Aporrectodea tuberculata*, *Aporrectodea caliginosa*, *Dendrobaena platyura*, *Fitzingeria platyura*, *Hormogaster redii*, *Lumbricus rubellus*, and *Polytoreutus finni*. The similarity of species across sites was found to range from 83% to 100%. However, most species exhibited low densities, likely due to environmental stress resulting from changes in the chemical and physical characteristics of the soil, which may be attributed to pollution.

Authors' Contributions

Zainab Imran Issa, Wameedh A.K. Al-Yasari, and Maher Ali Alquraishi designed the experiment. Zainab Imran Issa collected samples and operated the experiments. Wameedh A.K. Al-Yasari analyzed the data and wrote the first draft of the manuscript. Maher Ali Alquraishi revised the manuscript. All authors reviewed and approved the final version of the manuscript for publication.

Data Availability Statement

The data can be provided by the corresponding author on reasonable request.

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Ethical Considerations

All procedures involving animals were performed in compliance with institutional ethical standards and national regulations for the care and utilize of laboratory animals.

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Conflict of Interest

The authors declare no conflicts of interest.

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مجله بیوتکنولوژی کشاورزی
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شناسایی مولکولی هفت گونه کرم خاکی (Annelida: Oligochaeta) با استفاده از بارکدینگ DNA ناحیه ژنی 18SrRNA و ارتباط آن‌ها با ویژگی‌های خاک در استان بابل، عراق

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چکیده

هدف: این مطالعه با هدف شناسایی و طبقه‌بندی گونه‌های کرم خاکی موجود در استان بابل عراق با استفاده از روش‌های مولکولی انجام شد و همچنین تأثیر عوامل محیطی بر پراکنش و فراوانی این گونه‌ها بررسی گردید. با توجه به کمبود پژوهش‌ها در زمینه کرم‌های خاکی در عراق، این تحقیق سعی دارد درک روشن‌تری از تنوع گونه‌ای در این منطقه ارائه داده و بررسی کند که تغییرات ویژگی‌های خاک تا چه میزان بر حضور بوم‌شناختی این ارگانیسم‌ها اثر می‌گذارد.

مواد و روش‌ها: کار میدانی در سه ایستگاه بوم‌شناختی متمایز در استان بابل که شرایط زیست‌محیطی و کاربری متفاوتی داشتند، انجام شد. نمونه‌برداری در سه فصل پاییز، زمستان و بهار در بازه زمانی ۲۰۲۳ تا ۲۰۲۴ صورت گرفت. نمونه‌های خاک از هر سایت برای اندازه‌گیری دما، pH، هدایت الکتریکی (EC)، مجموع جامدات محلول (TDS) و میزان رطوبت مورد تجزیه‌وتحلیل قرار گرفتند تا نمایه‌ای جامع از ویژگی‌های فیزیکوشیمیایی خاک ارائه شود. نمونه‌های کرم خاکی به صورت دستی جمع‌آوری و برای تجزیه و تحلیل مولکولی نگهداری شدند. برای شناسایی گونه‌ها از بارکد DNA ناحیه ژنی 18S rRNA استفاده شد و توالی‌های حاصل برای مقایسه و تأیید در پایگاه داده GenBank متعلق به NCBI ثبت گردیدند.

نتایج: هفت گونه *Aporrectodea tuberculata*، *Aporrectodea caliginosa*، *Dendrobaena platyura*، *Fitzingeria platyura*، *Hormogaster redii*، *Lumbricus rubellus* و *Polytoreutus finni* از طریق روش‌های مولکولی شناسایی شدند. همبستگی‌های آماری نشان داد که رطوبت و دمای خاک تأثیر قابل‌توجهی بر پراکنش کرم‌های خاکی دارند، به‌طوری‌که تراکم جمعیتی آن‌ها در دوره‌های گرم به‌طور محسوسی کاهش می‌یابد. این یافته‌ها نشان می‌دهد که کرم‌های خاکی این منطقه واکنش‌های فیزیولوژیکی شدیدی نسبت به تنش‌های گرمایی و رطوبتی از خود نشان می‌دهند که این موضوع الگوهای مکانی و فصلی آن‌ها را شکل می‌دهد.

نتیجه‌گیری: این مطالعه کارآمدی بارکدینگ DNA را برای شناسایی دقیق گونه‌های کرم خاکی تأیید کرده و داده‌های جدیدی را در مورد تنوع زیستی Oligochaeta در عراق ارائه می‌دهد. همبستگی مشاهده‌شده بین شرایط محیطی و جمعیت کرم‌های خاکی بر اهمیت مدیریت صحیح خاک برای حفظ تنوع زیستی تأکید دارد. انجام تحقیقات آینده با بهره‌گیری از نشانگرهای ژنومی گسترده‌تر و نقاط نمونه‌برداری بیشتر برای درک بهتر نقش‌های بوم‌شناختی این ارگانیسم‌ها در زیستگاه‌های متنوع عراق توصیه می‌شود.

کلمات کلیدی: *Dendrobaena platyura*، اکوسیستم، مولکولی، Oligochaeta، 18S rRNA

نوع مقاله: پژوهشی.

استناد: زینب عمران عیسی، وامید ع. ک. الیاسری، ماهر علی القریشی (۱۴۰۴). شناسایی مولکولی هفت گونه کرم خاکی (Annelida: Oligochaeta)با استفاده از بارکدینگ DNA ناحیه ژنی 18SrRNA و ارتباط آن‌ها با ویژگی‌های خاک در استان بابل، عراق. *مجله بیوتکنولوژی کشاورزی*، ۱۷(۲)، ۱۷۱-۱۹۶.

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