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Molecular Identification and Phylogenetic Analysis of *Hydra vulgaris* from Greater Zab River, Iraq

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RESEARCH ARTICLE

Molecular Identification and Phylogenetic Analysis of *Hydra vulgaris* from Greater Zab River, Iraq

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ABSTRACT

No genomic data for *Hydra vulgaris* was recorded in Iraq. DNA barcoding is a modern technique used for accurate identification that is more important than previous studies utilizing morphology characters. This study aims to investigate the identification of *H. vulgaris* based on genomic analysis using the mitochondrial gene Cytochrome Oxidase I (*COI*) and construct a phylogenetic tree as the first study in Iraq. In our current investigation, we found species of *Hydra* from the Greater Zab River during June–July 2022, with average Water temperatures (25.1–29.6˚C), Hydrogen potential (pH) 7.5–7.8, and Dissolved Oxygen (DO) 6.3–7 mg/l. As a result, the acquired sequencing data for *H. vulgaris* was registered in the GenBank database (OP521891.1) for the first time. There were minimal genetic differences across the same strains (zero – 0.09); however, there was maximum divergence (0.424–0.508) to other species due to geographic distribution. Also, applying phylogenetic methods such as Maximum Likelihood, Maximum Parsimony, and Neighbor-Joining, it was verified that the *Hydra* species belongs to the *'vulgaris'* group. According to aquatic parameters, only water temperature shows no significant correlation according to months ($P < 0.05$). In contrast, pH and Dissolved oxygen show significant ($P > 0.05$) differences according to period studies and are correlated with species. Finally, it should be noted that this particular strain is used as a bioindicator for ecology, adapted to environmental assessment and geographical location.

Keywords: COI primer, Greater Zab, Hydra vulgaris, Iraq, Molecular phylogeny

Introduction

Hydra vulgaris, its ecological importance, and the challenges of identifying it in Iraq used as a bioindicator for environmental assessment, acute toxicity, and regeneration tests.[1,2] Its freshwater polyps and the most diverse group of cnidarians, worldwide distribution except the Antarctic area and Oceanic islands.[3,4] *H. vulgaris* (previously *Hydra magnipapillata*) is a non-symbiotic brown hydra lineage.[5]

Taxonomizing hydrozoans is a complex task, owing to their morphological transparency, few differentiating features, considerable phenotypic variability, and the presence of multiple species that have been incorrectly classified or synonymized.[6,7] It considered the challenges associated with taxonomic identification based on morphological characteristics, which may be problematic when dealing with larval forms.[8] Recently, DNA sequencing is a powerful tool that provides access to numerous characteristics that may aid in differentiating between similar taxa.[6] The mitochondrial Cytochrome C Oxidase subunit 1 (*COI*) gene has gained prominence as a universal set of primers for gene amplification for all invertebrates, and it can yield more sequence variation between species.[6–9] The classification of *Hydra* is based on morphological and molecular phylogenetic analyses, which have four distinct groups: common hydra (*H. vulgaris*), gracile hydra (*H. braueri*), green hydra (*H. viridissima*), and stalked hydra (*H. oligactis*).[10] Generally, in Iraq, the DNA barcoding knowledge of Invertebrates, especially freshwater *H. vulgaris*, is inadequate and has been ignored. We think there are a lot of gaps in this field, the lack of genetic data and the potential for hidden diversity, and it is obvious that the variety and distribution of the species in *Hydra*

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from the majority of the places have not been thoroughly examined, only one has been reported based on morphology to date.[11]

For the lights of above reasons, we conducted this research to investigate the identification of *H. vulgaris* based on genomic analysis using the mitochondrial gene (*COI*) and construct a phylogenetic tree in Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor-Joining method (NJ) analyses to reconstruct the evolutionary history of current species as an alternative method of species identification in Greater Zab River as the first study in Iraq.

Materials and methods

Study site

Randomly taking water from a small pond on the Greater Zab River near Khabat District, Erbil City, during June – July (2022), searching for invertebrates; as a result, it was noted the Hydrozoa species attached shrubs, rocks, and attached shells of *Physa acuta* (Gastropoda),[12] directly at the study site examined water temperature and pH were measured by portable HANNA instruments (HI 9811-5, Romania), while dissolved oxygen was measured by the Winkler method in the laboratory depending on A.P.H.A.[13] The specimens were kept in a container filled with pond water transported to the Advance Invertebrates Laboratory and placed in a specific aquarium with an aerator. A post hoc test (Duncan) was applied to determine significant differences between months and environmental parameters. All data are expressed as means. A P value of 0.05 was considered as the limit for statistical significance. The pearson correlation was calculated.

DNA extraction, PCR amplification and sequencing

Hydra polyps were fixed and preserved at ethanol and centrifuged (Eppendorf microfuge, Germany) at 10000 rpm/2 min., the supernatant (ethanol) was eliminated, and the precipitated sample was digested for 24 hrs. by adding Lysis buffer solution containing proteinase-K (1 $mg.ml^{-1}$). The genomic DNA extraction was performed using the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). Partial sequences of *COI*; forward (LCO1490) and Reverse (HCO2198),[14] were amplified by PCR (Polymerase Chain Reaction-USA) mixture (20 μl) included: 10 μl master mix, 1.5 μl for each primer (LCO1490 & HCO2198), 2 μl DNA template and 5 μl double distilled water (ddH2O) were denatured at 95 C°/4 min, followed by 35 cycles of (95C°/40 s of denaturation, 48C°/60 s of annealing, and 72C°/1 min of extension), and final elongation step at 72C°/7 min. The products underwent electrophoresis (Biotech Fischer GmbH, Reistkirchen, Germany) on 1.3% agarose gels,[15] the expected product size for PCR amplification (690 – 710 bp). Following, the products were sequenced[16] via Applied Biosystems' ABI 3730XLs nucleotide sequencer at Macrogen Inc.-Korea. Sequences were checked for errors in Bio edit program, subsequently uploaded to the NCBI GenBank database using Blast n (Basic Local Alignment Search Tool for Nucleotides). Additionally, 18 sequences from GenBank were gathered for the constructing of the phylogenetic tree.

Phylogenetic analysis

The MUSCLE program within EMBL-EBI was used to adjust and align all DNA sequences using the ClustalW method for aligning homologous nucleotides. Large insertions observed in mt DNA strains were removed. For phylogenetic relationships among *Hydra* species, we used Neighbor-Joining (NJ), Maximum Likelihood (ML), and Maximum Parsimony (MP) methods.

Additional evaluations of *COI* variations and associations among species through applying the Neighbor-Joining method. Collapsed the bootstrap replicates of branches less than 50%. For the computation of evolutionary distances, the p-distance method was utilized. The Maximum Likelihood method and the General Time Reversible model were ultimately obtained. The initial trees for heuristic search were determined by selecting the topology with the highest log likelihood value from a matrix of pairwise distances obtained using the Maximum Composite Likelihood (MCL) method and applying the Neighbor-Join. Alongside the branches, the percentage of trees in which the associated taxa clustered is indicated (NJ/ML/MP). The MEGA11 software was used for evolutionary analyses.[17]

Results and discussion

Mitochondrial DNA COI from freshwater Hydrozoan was sequenced, and the results confirmed the present *H. vulgaris* Fig. 1. Polyps brown without a distinct stalk, 6–7 Moniliform tentacles shorter than the body column. Adult polyps measured about 6 – 11 mm in the relaxed state and 0.5–3 mm when fixed; without buds, the length reached 18 millimeters.

The taxonomy of hydra is confusing due to the large number of species that have been described, but a recent estimate of valid species is 12–15.[18] Conducting molecular biology research is important

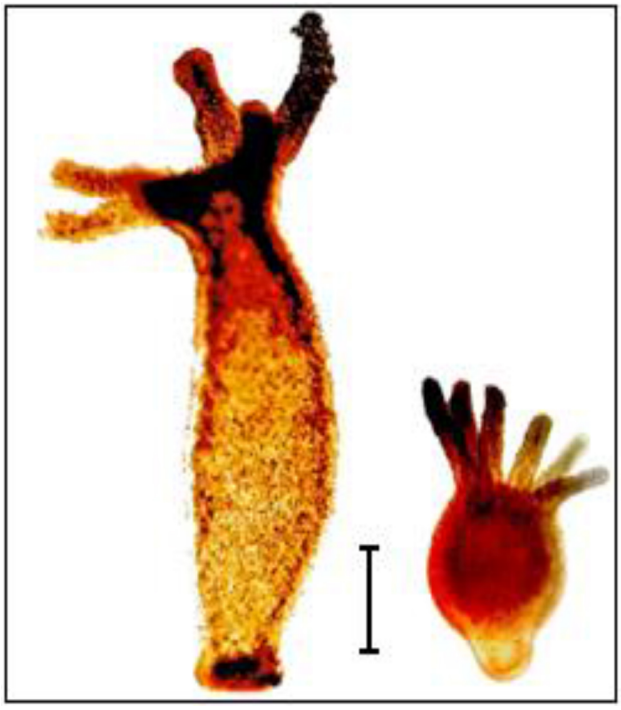

Fig. 1. Photo of whole mount of *Hydra vulgaris* (scale bar = 150 μm).

for Iraqi fauna and its presence on the world map. The present investigation was the first to study partial sequences of *COI* isolated from *H. vulgaris* and used as a marker for phylogeny from the Greater Zab River in the Kurdistan region, Iraq. Our sequencing data was deposited in GenBank under the following accession number (OP521891.1) for *H. vulgaris*, with multiple strains of *Hydra* and other species used for constructing trees using NJ/ML/MP methods. Fig. 2 shows the phylogenetic tree of *Hydra* species which is split off into various clades. The results of the present study (OP521891.1) were under the results found in the USA by Martinez[18] (GU722887.1), identical to Switzerland (MF135297.1), and as a sister group to the other species data. However, the addition of more loci could alter this pattern. The single Asian strain included in the analysis appears as a sister taxon of the European–North American group.[18] There have been relatively few reported phylogenetic analyses of species in the genus *Hydra*, Campbell[19] proposed that hydras are naturally into four groups of species; *braueri*, *oligactis*, *viridissima*, and *vulgaris*.[4] Using the *COI* gene dataset, phylogenetic analysis has established the existence of four hydra groups, supporting the previous results,[5] and the outgroup *Obelia dichotoma* (KX665200.1) reported by Cunaha[20] showed greater divergence with *H. vulgaris.* Cnidaria, like the freshwater polyp *Hydra*, is a monophyletic organism that exhibits considerable diversity in morphology.[21] Asian and European hydra of the vulgaris group form one clade with relatively little genetic variation with relatively high support. A well-supported clade includes all vulgaris hydra from Eurasia. *Hydra* was first described as *H. vulgaris* (Pallas,1766) and has often been called *H. vulgaris* collected in Europe, *H. orientalis,* collected in India, and *H. magnipapillata,* collected in Japan as well as a host of other names.[18]

Kimura 2 parameter model (K2P) was employed in phylogeny to account for intra and interspecific differences among strains. Intraspecific genetic diversity was (zero) with *Hydra vulgaris* GU722887.1 and MF135297.1; and (0.087) with *H. vulgaris* MG421276.1. This finding indicated that *Hydra* species from the present location have a low genetic variation and high similarity among them. In contrast, the maximum genetic distance (0.508) was observed with the outgroup *Obelia dichotoma* KX665200.1. The genomic phylogenetic study also indicated that there was relatively little genetic divergence among strains Table 2. This result is similar to Kawaida,[22] but morphological features like total polyp size, colour, emergence pattern of tentacles on buds and consistency in gonad production vary.[23] While large divergence with Europe, Japan, and USA species refers to the geographic distribution of many individuals.[24] The international commerce in aquatic animals may have helped *Hydra* species expand to all continents and may help to explain why individuals (or strains) with the same genetic makeup have been found on several continents.[24] These cases are likely to be the result of transport by humans. Many fish, aquatic invertebrates and plants are moved around the world for agricultural, sport and aquarial purposes and it is not surprising that hydra could ride undetected.[18] Furthermore, it demonstrates that current species is a member of the "*vulgaris*" and that it is separate from the *H. japonica* or *H. canadensis* clades, which arrived in Asia due to intercontinental drifts. The inability of morphological characteristics to categorize this strain unmistakably into one of the species "*vulgaris*" group shows the value of genetic phylogeny as a technique for identifying the relationships between closely related species.[21]

According to the study site parameters, water temperature fluctuated during the summer season ranging between 25.1–29.6C° Fig. 3, showing no significant ($P < 0.05$) correlation according to months Table 1; this range is comparable to Ali.[11] The temperature effects on chemicals may occur directly through altering the physicochemical behavior of an invertebrate.[25] *Hydra* species reproduce quickly dependent on environmental factors, such as optimum conditions, an acceptable temperature, and abundant prey.[26] Also, they greatly influence the occurrence

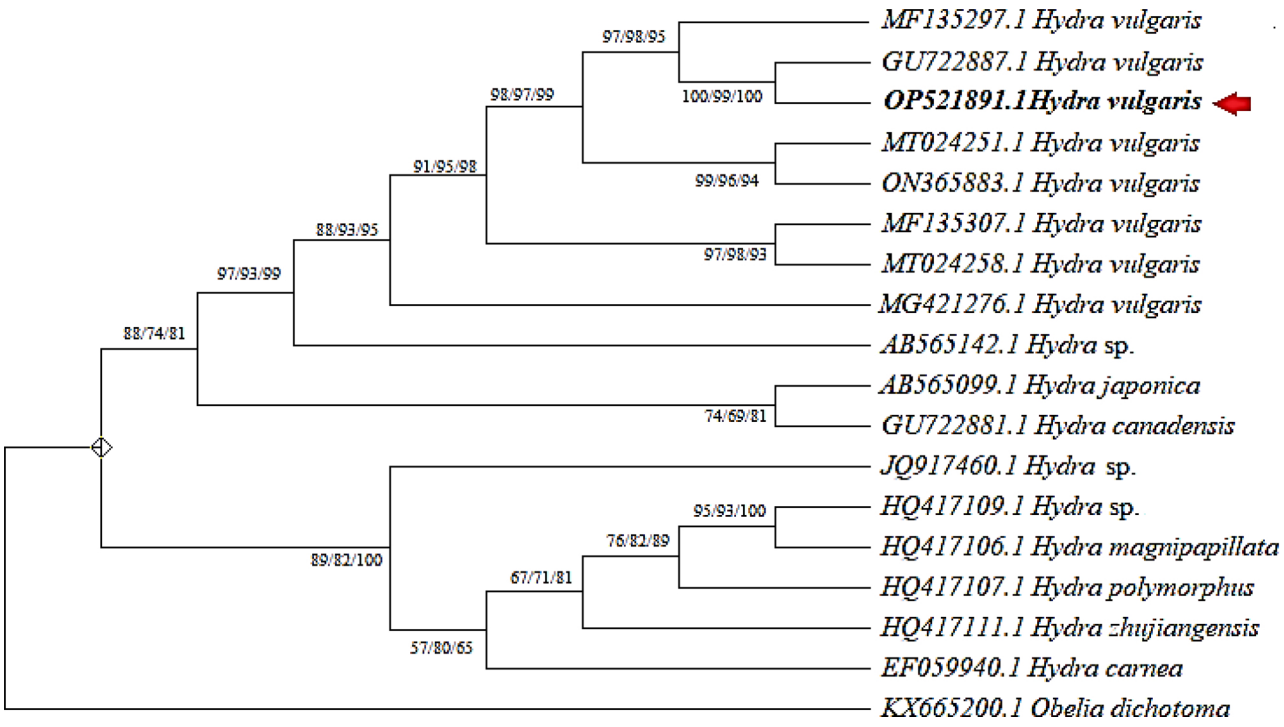

Fig. 2. Phylogenetic tree using Neighbor-Joining method, Maximum Likelihood method and General Time Reversible model, and Maximum Parsimony method obtained by Subtree Pruning Regrafting (SPR). Bootstrap values at the branch points above/below each node are inferred from NJ, ML, and MP methods, respectively.

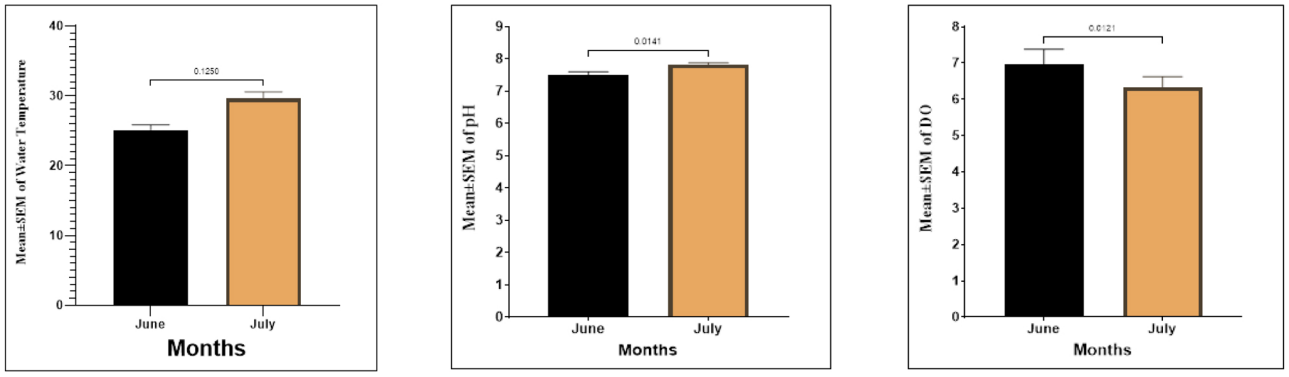

Fig. 3. Monthly variations according to water temperatures, pH, and dissolved oxygen (mean ± SE).

of organisms in the ecosystem.[27] On the other hand, modification of temperatures (upper and lower) due to initiation signals of gametogenesis depends on the *Hydra* species. Nonetheless, water temperature in summer months can reach above 25C°,[28] warm exposure might allow polyps to accumulate more resources during the summer, allowing them to invest more in sexual reproduction.

During the study period, pH was 7.5–7.8, showing significance ($P > 0.05$) according to the months study Table 1. This result is similar to Bashê[29] recorded (7.3–8.1) Fig. 3. The data indicates that there is a noticeable rise in pH levels throughout the summer season. This may be ascribed to the elevated temperatures, which in turn result in an increased rate of evaporation of the surface water. Consequently, there is also an observed increase in the concentration of carbonates.[30] In natural water, the pH value is usually between 6.5 and 8.5.[31]

The Dissolved Oxygen is critical for all living organisms.[32] It ranged from 6.3–7 mg/L Fig. 3. In contrast to water temperatures, Dissolved oxygen shows significant ($P > 0.05$) according to the period study Table 1. This data was comparable to the authors recorded at the same location.[29] *H. vulgaris* needs a minimum of 6 mg/L of DO.[33] Natural

Table 1. Statistical analysis among monthly variations with some physio-chemical properties of Greater Zab river, data represented as mean ± SE.

	Parameters		
Months	Water temperature (C°)	pH	Dissolved Oxygen (mg/L)
June	25.1 ± 0.7382[a]	7.5 ± 0.108[b]	6.975 ± 0.407[a]*
July	29.58 ± 0.9911[a]	7.825 ± 0.06292[a]	6.325 ± 0.3065[b]

* Same letters mean non-significate while different letters (a and b) mean significant.

Table 2. Neighbor-Joining, Maximum Likelihood, and Maximum Parsimony analysis of *COI* gene sequences reveal close clustering of the current *H. vulgaris* strain with established "*vulgaris*" group members, indicating low intraspecific genetic variation in the Greater Zab River.

Species	*Hydra vulgaris OP521891.1*	*Hydra vulgaris GU722887.1*	*Hydra vulgaris MF135297.1*	*Hydra vulgaris MT024251.1*	*Hydra vulgaris MF135307.1*	*Hydra vulgaris MT024258.1*	*Hydra vulgaris ON365883.1*	*Hydra vulgaris MG421276.1*	*Hydra sp. JQ917460.1*	*Hydra sp. AB565142.1*	*Hydra_sp. HQ417109.1*	*H. polymorphus HQ417107.1*	*Hydra japonica AB565099.1*	*Hydra carnea EF059940.1*	*H. magnipapillata HQ417106.1*	*H. canadensis GU722881.1*	*H. zhujiangensis HQ417111.1*	*Obelia dichotoma KX665200.1*
Hydra vulgaris OP521891.1																		
Hydra vulgaris GU722887.1	0.000																	
Hydra vulgaris MF135297.1	0.000	0.000																
Hydra vulgaris MT024251.1	0.005	0.005	0.005															
Hydra vulgaris MF135307.1	0.006	0.006	0.006	0.008														
Hydra vulgaris MT024258.1	0.006	0.006	0.006	0.008	0.000													
Hydra vulgaris ON365883.1	0.011	0.011	0.011	0.006	0.014	0.014												
Hydra vulgaris MG421276.1	0.087	0.084	0.084	0.082	0.084	0.084	0.090											
Hydra sp. JQ917460.1	0.424	0.434	0.434	0.432	0.436	0.435	0.431	0.454										
Hydra sp. AB565142.1	0.009	0.009	0.009	0.011	0.011	0.011	0.018	0.094	0.422									
Hydra_sp. HQ417109.1	0.448	0.457	0.457	0.455	0.457	0.457	0.455	0.473	0.087	0.450								
H. polymorphus HQ417107.1	0.465	0.472	0.472	0.470	0.472	0.472	0.467	0.485	0.094	0.464	0.084							
Hydra japonica AB565099.1	0.004	0.004	0.004	0.007	0.007	0.007	0.013	0.096	0.422	0.004	0.450	0.464						
Hydra carnea EF059940.1	0.426	0.435	0.436	0.434	0.438	0.437	0.433	0.456	0.026	0.427	0.070	0.094	0.427					
H.magnipapillata HQ417106.1	0.448	0.457	0.457	0.455	0.457	0.457	0.455	0.475	0.089	0.444	0.009	0.089	0.444	0.075				
H. canadensis GU722881.1	0.120	0.117	0.117	0.116	0.119	0.119	0.123	0.116	0.478	0.118	0.497	0.509	0.120	0.484	0.499			
H. zhujiangensis HQ417111.1	0.460	0.468	0.469	0.466	0.469	0.468	0.467	0.483	0.110	0.462	0.091	0.112	0.462	0.099	0.096	0.503		
Obelia dichotoma KX665200.1	0.184	0.184	0.184	0.182	0.184	0.184	0.186	0.191	0.484	0.183	0.512	0.523	0.187	0.495	0.516	0.191	0.508	

factors, such as ambient water temperature, salinity, altitude, organic pollution, dissolved oxygen consumption by aquatic organisms, and river water level, determine the amount of DO in the water. These are impacts of humans, such as urban development and agriculture.[32] The lower DO concentrations due to microorganisms that break down the organic compounds have a higher oxygen requirement when exposed to higher amounts of raw organic material. An extra investigation that explicitly examines the positive relationship between macroinvertebrate biodiversity and dissolved oxygen.[34] Many researchers conducted studies on the environmental effects, such as Cera,[35] and an analysis revealed a potential link between modified chemo-physical factors (pH, water temperature, and conductivity) and *H. vulgaris* disturbed test findings. According to Rocío,[36] there is very little knowledge about the relationship between these cnidarians and the aquatic environments they inhabit and the ecological roles with which they are intertwined. The fact that the chemicals in freshwater come from a variety of sources should be considered. Many of these are waste items that are regularly released into waters as a result of anthropogenic activity (such as factories, agriculture, and urban discharges).[37]

Conclusion

The first genome assembly of *H. vulgaris* in Iraq, represented in the current study, using recent molecular techniques *COI*, and phylogenetic approaches, clarifies some of the evolutionary relationships. For intraspecific or interspecific comparisons, identifying shared or divergent processes and functions among species, and other related tasks, such investigations depend on the accurate and unambiguous classification of the examined individuals to species. Furthermore, understanding the ecological role of *H. vulgaris* in the ecosystem and the fact that it exists elsewhere in the country advances our understanding of the aquatic ecosystems of the region and, consequently, the appropriate preservation of its water resources.

Summarize keys table

A.P.H.A.	American Public Health Association
Blast n	Basic Local Alignment Search Tool for Nucleotides
COI	Cytochrome C Oxidase subunit 1
DO	Dissolved Oxygen
K2P	Kimura 2 parameter model
MCL	Maximum Composite Likelihood
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MP	Maximum Parsimony
NCBI	National Center for Biotechnology Information
NJ	Neighbor-Joining
pH	Potential of Hydrogen
PCR	Polymerase Chain Reaction
SPR	Subtree Pruning Regrafting

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Authors' declaration

- Conflicts of Interest: None.
- I hereby confirm that all the Figures and Tables in the manuscript are mine. Furthermore, any Figures and images, that are not mine, have been included with the necessary permission for republication, which is attached to the manuscript.
- No human studies are present in the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee at University of Salahaddin.

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التعريف الجزيئي والتحليل الوراثي لهيدرا فولكاريس من نهر الزاب الكبير، العراق

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الخلاصة

لا توجد بيانات جينية مسجلة ل *Hydra vulgaris* في العراق. تعد تقنية تشفير الحمض النووي (DNA) تقنية حديثة تستخدم لتحديد الهوية بدقة وهي أكثر أهمية من الدراسات السابقة التي تستخدم للصفات المورفولوجية. تهدف هذه الدراسة إلى التحقيق في تحديد هوية *H. vulgaris* على أساس التحليل الجينومي باستخدام جين الميتوكوندريا سايتوكروم اوكسيديز Cytochrome Oxidase I (*COI*) وبناء شجرة النشوء والتطور كأول دراسة في العراق. في بحثنا الحالي، وجدنا أنواعًا من هيدرا من نهر الزاب الأكبر خلال الفترة من يونيو إلى يوليو 2022، بمتوسط درجات حرارة الماء (25.1-29.6 درجة مئوية)، درجة الحموضة (7.5-7.8)، والأكسجين المذاب (6.3-7 ملغم / لتر). ونتيجة لذلك، تم تسجيل بيانات التسلسل المكتسبة لـ *H. vulgaris* في قاعدة بيانات GenBank(OP521891.1)لأول مرة. كان هناك الحد الأدنى من الاختلافات الجينية عبر نفس السلالات (صفر-0.09)؛ وعلى العكس من ذلك، كان هناك أقصى قدر من الاختلاف (0.424-0.508) بالنسبة للأنواع الأخرى بسبب التوزيع الجغرافي. وبتطبيق أساليب النشوء والتطور أيضًا مثل الحد الأقصى للاحتمال والحد الأقصى من البخل والانضمام إلى الجار، تم التحقق من أن أنواع الهيدرا تنتمي إلى مجموعة "الشائعة". وفقا للمعايير المائية، لا تظهر أي علاقة معنوية لدرجة حرارة الماء وفقا للأشهر ($P < 0.05$). في المقابل، يظهر الرقم الهيدروجيني والأكسجين المذاب اختلافات كبيرة ($P > 0.05$) وفقًا لفترة الدراسة وارتباطه بالأنواع. وأخيرا، تجدر الإشارة إلى أن هذه السلالة بالذات تستخدم كمؤشر حيوي للبيئة، تتكيف مع المعايير البيئية والموقع الجغرافي.

الكلمات المفتاحية: جين *COI*، هيدرا فولكاريس، السلالة الجزيئية، الزاب الكبير، العراق.