

BULLETIN OF THE IRAQ NATURAL HISTORY MUSEUM

Iraq Natural History Research Center & Museum, University of Baghdad

<https://jnhm.uobaghdad.edu.iq/index.php/BINHM/Home>

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Online ISSN: 2311-9799, Print ISSN: 1017-8678


Bull. Iraq nat. Hist. Mus.

(2024) 18 (1): 105-120.

<https://doi.org/10.26842/binhm.7.2024.18.1.0105>

ORIGINAL ARTICLE

MOLECULAR STUDY OF SOME SPECIES OF CENTIPEDES IN IRAQ WITH NEW RECORD OF *LITHOBIUS CRASSIPES* L. KOCH, 1862 (CHILOPODA, LITHOBIOMORPHA, LITHOBIIDAE)

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Received: 20 August 2023, Revised: 7 Dec. 2023, Accepted: 28 Dec. 2023, Published: 20 June 2024



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ABSTRACT

The first molecular research on Iraqi centipede fauna is presented in this article. Between October 2022 and May 2023, during various climatic circumstances, centipedes were collected from several locations in four provinces of Iraq. Three families, represented by four genera, underwent molecular identification, and five species were found. From the order Scolopendromorpha family Scolopendridae, two species were recorded, *Scolopendra morsitans* Linnaeus, 1758, and *S. cingulata* Latreille, 1829, *Cormocephalus* sp.; while from the order Lithobiomorpha, family Lithobiidae, one species was recorded for first time in Iraq; *Lithobius crassipes* L. Koch, 1862 from the order Geophilomorpha family Himantariidae, one species *Bothriogaster signata* Kessler, 1874. DNA was extracted from the specimens, the mtDNA fragment from the Cytochrome C Oxidase Subunit I (COI) gene was amplified by using the PCR technique with appropriate primers, and subsequently, the Basic Local Alignment Search Tool (BLAST) tool, which is accessible at the NCBI, was used. Additionally, a phylogenetic tree was built, and a distant comparison was shown.

Keywords: Centipedes, Iraq, *Lithobius*, Molecular, New record.

INTRODUCTION

About 3000 species of Chilopoda have been described, and they are more frequently found in tropical and subtropical countries. Their rational evolutionary history, which has a fossil record lasting 420 million years, can explain their widespread global distribution. Recent investigations have demonstrated a hard phylogeny that supports theories about their biology that are based on evolution utilizing both morphological and molecular evidence (Edgecomb and Giribet, 2007).

The inexistence centipedes are classified into five monophyletic orders. Only two species of the order Craterostigmomorpha (Pockock, 1902) are extant species, and they are both from New Zealand and Tasmania, respectively (Vélez *et al.*, 2012). In contrast, the orders Lithobiomorpha (Pockock, 1895) and Scutigleromorpha (Pockock, 1895) contain

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approximately 1,100 valid species each and are both found in temperate regions of the world. With over 1,300 genuine species in 14 recognized families, the order Geophilomorpha (Pocock, 1896) is the most diverse and extensive group. With more than 700 recognized species, the order Scolopendromorpha (Pocock, 1895) is diverse in lower latitudes (Edgecombe and Giribet, 2007; Edgecombe and Bonato, 2011).

There are few studies on this field in Iraq. In the first papers on Chilopoda from Iraq were published by Brolemann (1921, 1922), when he studied material from Amara and Jebel Hamrin, he made a note including 14 species from the five orders. Even though Chamberlin and Hoffman (1958) studied material from different parts of Iraq and described three new species *Lithobius cuklavus* (Chamberlin, 1958), *M. integer* (Chamberlin, 1944) and *Ottobius irikensis* (Chamberlin, 1958); moreover, it recorded more than 11 species from 4 orders.

Advanced, Würmli (1975) recorded *Thereuonema turkestanica hamrinensis* Verhoeff, 1905, under the synonym *T. microstoma* (Meinert, 1886). While Dobroruka (1976) described two new species from Baghdad, Lewis (1986, 2001) studied the scolopendromorph fauna of Iraq, and recorded several scolopendromorphs from different regions of Iraq.

Lithobius irikensis Chamberlin, 1958, was introduced by Zapparoli (1993) as a new combination for the *Ottobius irikensis* described by Chamberlin and Hoffman (1958), and Zapparoli (1995) synonymized *Eupolybothrus syngenes* Verhoeff, 1907, under *E. litoralis* (L. Koch, 1867) as well. The first record of the morphology, description, and distribution preferences of the fauna of Scolopendromorpha centipedes in Kurdistan and Iraq was supplied by a number of faunistic papers (Ahmed and Hussien, 2017). Nine species from 183 taken from various locations in the central part of Iraq were recorded for the first time (Aldoori, 2020).

In Basrah, south of Iraq, Al-Simary *et al.*, (2022) gathered samples from eight stations in various regions. Out of the 105 specimens, five species (belonging to four orders and five families) were recognized. Each species' taxonomic information was also provided. The most recent and latest annotated checklist of Chilopoda from Iraq contains 27 species from 13 genera, 8 families, and 4 orders (Dyachkovi *et al.*, 2023).

In the current paper, the specimens of local centipedes were identified using genetic techniques. Utilizing the neighbor-joining method, a phylogenetic tree was created utilizing several other samples from GenBank to enhance molecular information for the centipedes in Iraq, by the present molecular study, and establish phylogenetic relationship patterns for the collected species, using molecular data from the Cytochrome c oxidase subunit (COI) analyses.

MATERIALS AND METHODS

Specimens' collection: The specimens collected from different geographic locations in Iraq include four provinces Baghdad, Wasit, Salahaddin, and Diyala in the middle of Iraq. All studied specimens were hand-collected in one place. The collection period was from October

2022 to May 2023, using a forceps and direct hand pickup to collect them from the soil, and under stones and remnants of mud pieces and tree roots, specimens were stored in glass tubes, then transferred to the laboratory for the purpose of killing them, and then preserved in 70% ethanol. Taxonomic nomenclature and species identification were based on the following literature: Schileyko (2007), Siriwut *et al.* (2015), and Ganske *et al.* (2018).

Specimens preparing for molecular studies: The following steps were adopted for this purpose:

- 1- DNA extraction: Utilizing the Geneid DNA extraction kit, DNA was extracted from 11 samples using a modified version of the manufacturer's usual recommended approach. The ultimate legs of centipedes were detached from their bodies and repeatedly rinsed with distilled water to remove excess alcohol. DNA was then isolated using a DNeasy Blood and Tissue kit from the dehydrated muscle tissues of the ultimate legs.
- 2- Primers Preparing: Specific primers were used to obtain and amplify the partial mitochondrial Cytochrome Oxidase subunit I gene (COI), the forward LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Baker *et al.*, 2019) with the reverse HCO2198, (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Oeyen, 2014; Kang *et al.*, 2017).
- 3- DNA Amplification: Specific primers were used to obtain and amplify the partial mitochondrial Cytochrome Oxidase subunit I gene (COI). Polymerase Chain Reaction (PCR) typically used 5µl of DNA extract as a template for the PCR reaction, as mentioned above. After the optimization condition of the PCR cyclor program (BIONEER, Crop, Daejeon, Korea), the reactions were amplified for 35 cycles.
- 4- Gel Electrophoresis: The amplification of 11 sample specimens' products was confirmed by using 1.5% agarose gel electrophoresis in a 1 X TBE buffer and then stained to visualize with 4 % ethidium bromide in UV light at 320 nm, the molecular weight of the specific target bands is 710 bp.
- 5- Sequencing of PCR products and phylogenetic analysis: Using the partial sequence and the Sanger Sequencing Technique (Sanger *et al.*, 1977), the forward primer LCO 1490 was applied, for sequencing, Macrogen Company, Korea, received the PCR of the mitochondrial Cytochrome Oxidase subunit I (COI) gene product to validate the identification of the 11 specimens, the phylogenetic tree was prepared using a Mega X program.

RESULTS AND DISCUSSION

From the family Scolopendridae, two species were recorded, *Scolopendra morsitans*, *S. cingulate*, and the genus *Cormocephalus*, while from the families Lithobiidae, and Himataridae, one species *Lithobius crassipes*, and *Bothriogaster Signata*, respectively, were recorded (Tab. 1).

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Table (1): Include families, genera and species in the study.

Family	Genus	Species
Scolopendridae	<i>Scolopendra</i> Linnaeus, 1758	<i>Scolopendra morsitans</i> Linnaeus, 1758
		<i>S. cingulata</i> (Latreille, 1829)
	<i>Cormocephalus</i> Newport, 1844	<i>Cormocephalus</i> sp.
Lithobiidae	<i>Lithobius</i> Leach, 1814	<i>Lithobius crassipes</i> (L. Koch, 1862)
Himataridae	<i>Bothriogaster</i> (Sseliwanoff, 1879)	<i>Bothriogaster Signata</i> (Kessler, 1874)

Order, Scolopendromorpha**Family, Scolopendridae****Genus, *Scolopendra*** Linnaeus, 1758**Species, *Scolopendra morsitans*** Linnaeus, 1758**Synonyms:** *Scolopendra morsitans amazonica* Bücherl, 1946

- S. morsitans procera* Haase, 1887
- S. morsitans calcarata* Daday, 1891
- S. morsitans fasciata* Attems, 1930
- S. morsitans scopoliana* C.L. Koch, 1841
- S. brandtiana* Gervais, 1837
- S. bilineata* Brandt, 1840
- S. crassipes* Brandt, 1840
- S. erythrocephala* Brandt, 1840
- S. elegans* Brandt, 1841
- S. fulvipes* Brandt, 1841
- S. angulipes* Newport, 1844
- S. leachii* Newport, 1844
- S. scolopendra longicornis* Newport, 1844
- S. platypoides* Newport, 1844
- S. tuberculidens* Newport, 1844
- S. algerina* Newport, 1845
- S. fabricii* Newport, 1845
- S. formosa* Newport, 1845
- S. richardsoni* Newport, 1845
- S. tigrina* Newport, 1845
- S. varia* Newport, 1845
- S. tongana* Gervais, 1847
- S. infesta* Koch, 1847
- S. planipes* Koch, 1847.
- S. pella* Wood, 1861
- S. porphyra* Wood, 1861
- S. brachypoda* Peters, 1862
- S. mossambica* Peters, 1862
- S. compressipes* Wood, 1862
- S. carinipes* Humbert & Saussure, 1870
- S. afzelii* Porat, 1871
- S. lineata* Saussure & Zehntner, 1902
- S. spinosella* Saussure & Zehntner, 1902
- Trachycormocephalus jodhpurensis* Khanna, 1977

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Material examined (28♂♂, 19♀♀): Baghdad Province: Al-Jadriyah, 33°16'46.6"N 44°23'06.4"E, 7♂♂, 4♀♀ 8.ii.2023; Al- Mahmudiyah 6 ♂♂ 4♀♀ 3.iii.2023, (33°3'42"N 44°21'15"E); Al- Madain, 33.0993° N, 44.5807° E, 5♂♂, 5♀♀ 22.iii.2023; Al-Tarmiah, 33.6732° N, 44.3615° E, 6♂♂, 4♀♀ 8.iv.2023. Wasit Province, Al- Azizia, 32°54'38.5"N 45°03'46.8"E, 4♂♂, 2♀♀ 12.ii.2023

Distribution: It's widespread in the world, including North and South America, the Caribbean Islands, Europe, Africa, the Arabian Peninsula, Pakistan, India and the Indian Ocean Islands, Southeast Asia, Australia, and New Zealand (Akkari *et al.*, 2008; Lewis, 2010; Siriwut *et al.*, 2015). In Iraq, Chamberlin (1944) recorded several species belonging to three orders; which represent one of them as being the first record which is *S. morsitans*.



Plate (1): Female of *Scolopendra morsitans*.

Species, *Scolopendra cingulata* Latreille, 1829

Synonyms: *Rhombcephalus parvus* Newport, 1845

- R. viridifrons* Newport, 1844
- S. banatica* C.L.Koch, 1847
- S. cingulatoides* Newport, 1844
- S. doriae* Pirota, 1878
- S. fulva* Gervais, 1847
- S. graeca* C.L.Koch, 1847
- S. hispanica* Newport, 1845
- S. italica* C.L.Koch, 1837
- S. nigrifrons* C.L.Koch, 1847
- S. obscura* C.L.Koch, 1863
- S. penetrans* C.L.Koch, 1847
- S. pulchra* C.L.Koch, 1847
- S. savignyii* Newport, 1845
- S. thracia* Verhoeff, 1928
- S. venefica* L.Koch, 1856
- S. violantis* Pirota, 1878
- S. zonata* C.L.Koch, 1847
- S. zwickiana* C.L.Koch, 1863

Material examined (2♀♀): Baghdad Province, Al-Jadriyah, 33°17'17"N 44°23'35"E, 8.ii.2023.

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Global distribution: Mediterranean area Portugal, Spain, France, Slovenia, Croatia, Bosnia-Herzegovina, Serbia, Hungary, Romania, Bulgaria, Albania, Greece, Turkey, Sicily, Corsica, Sardinia, Crete, Cyprus, (absent in Balearics), Ukraine, S. Europe, Russia in The Caucasus, Tajikistan, Lebanon, Palestine, Jordan, Egypt, Syria, Iran. Madagascar, the Andaman, and Nicobar Islands (Wang and Mauriès, 1996). It was listed for China, referring to Haase (1887). In Iraq, Chamberlin (1944) was also the first who recorded this species.



Plate (2): Female of *Scolopendra cingulate*.

Genus, *Cormocephalus* Newport, 1844

Cormocephalus sp.

Material examined (1♀): Baghdad Province, Al- Yusufiyah, 33.0791° N 44.2520° E, 3.i.2023.

Global distribution: Southern Europe, Africa, Madagascar, Philippines, New Guinea, New Britain, the Solomon Island, Micronesia, India, Bangladesh, Sri Lanka, New Caledonia, Australia (including Tasmania), New Zealand, the Galapagos Islands, Central America, the West Indies, and South America (Koch, 1983; Edgecombe *et al.*, 2019). In Iraq, Al-Doori *et al.* (2020) recorded *Cormocephalus nigrificatus* (Verhoeff, 1937) from Al-Qadisiyah Province which represents the only species that belongs to this genus (Dyachkov *et al.*, 2023). Since the only specimen obtained was damaged in its last segment, we could not diagnose the model at the species level.



Plate (3): Female of *Cormocephalus* sp.

Order, Lithobiomorpha

Family, Lithobiidae

Genus, *Lithobius* Leach, 1814

Species, *Lithobius crassipes* L.Koch, 1862

Synonyms: *Archeobius ulterior* Chamberlin, 1952

Archilithobius longipes Attems, 1907

Lithobius atrifrons Silvestri, 1896

L. ephessoensis Matic, 1980

L. (Monotarsobius) ephessosensis Matic, 1980

L. (Monotarsobius) ercijasus Verhoeff, 1943

L. (Monotarsobius) olympicus Verhoeff, 1941

L. podokes Attems, 1903

L. riggioi Matic, 1968

L. stictonotus Manfredi, 1957

L. (Monotarsobius) ulterior Chamberlin, 1952

Material examined (1♂): Baghdad Province, Al Husseinia, 33°32'48.0"N 44°25'00.0"E, 23.ii.2023.

Global distribution: Wang (1963) verified only one specimen of *Monotarsobius crassipes* from Shao Tso Kiang in Taiwan (Chao, 2010), also established the presence of this species in Taiwan. as well, it distributes in Albania, Algeria, Austria, Belgium, Bosnia-Herzegovina, Bulgaria, the Canary Islands Central European Russia, Finland, Croatia, Denmark, Germany, Great Britain, France, Greece, Hungary, Ireland, Italy, Luxembourg, Macedonia, Madeira, the netherlands, Norway, Poland, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Tunisia, Turkey, and Ukraine.(Zapparoli, 2006). In Iraq, there are three species recorded from this genus, including: *Lithobius microps* Meinert, 1868; *L. aeruginosus* (L. Koch, 1862), and *L. fossipes* Brölemann, 1922 (Aldoori, 2020; Dyachkovi *et al.*, 2023), in addition to *L. ferganensis* (Trotzina, 1894), while this species was recorded for the first time for the centipedes in Iraq.



Plate (4): Male of *Lithobius crassipes*.

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Order, Geophilomorpha**Family, Himantariidae****Genus, *Bothriogaster*** Sselivanoff, 1879*Bothriogaster signata* (Kessler, 1874)**Synonyms:** *Bothriogaster affinis* Sselivanoff, 1879*B. africanus* Sselivanoff, 1881*B. arasana* Verhoeff, 1943*B. cyrenaica* Verhoeff K.W., 1908*B. egyptiacus* Attems, 1896*B. meinerti* Sselivanoff, 1879*B. porigera* Verhoeff, 1901*B. thesei* Attems, 1902*B. tunetanum* Verhoeff, 1899*B. wohlberedi* Verhoeff, 1934*Himantarium taeniatum* Meinert, 1886*Notiphilus sanguineus* C.L. Koch, 1847*N. taeniatum* C.L. Koch, 1847

Material examined (2♂♂, 1♀): Saladin Province, Al-Hamra Village, 34°45'15"N43°36'22"E, 2♂♂, 22.iii.2023, Muhazam Village, 34°43'40"N43°38'36"E, 1♀, 22.ii.2023.

Global distribution: Eastern Mediterranean Region (Zapparoli, 1991; Stoev, 2000), through Western Asia, Central Asia, Turkmenistan, Uzbekistan, Tajikistan, Kazakhstan, (Kessler, 1874), Balkan Peninsula, Cyprus, North Africa, and Russia (Volgograd Region and South Kazakhstan regions). A new checklist of centipedes from Iran in the Alborz Mountains (Zarei and Rahimian, 2020). In Iraq, Attems (1929) was recorded this species.



Plate (5): female of *Bothriogaster signata*.

Molecular identification

Conventional PCR results: COI (710-bp) fragment was amplified by the primer pair (LCO1490 F/ HCO2198 R). Consistently across the widest variety of invertebrates, including centipedes (Folmer, et al., 1994). Results indicated a success rate of 11/11 effective DNA amplification. The mitochondrial DNA-COI gene's highly conserved regions were amplified using DNA amplification. When products of mitochondrial DNA fragments run in 1.5% agarose gel during gel electrophoresis, the particular band with the PCR test was seen in Plate (6).

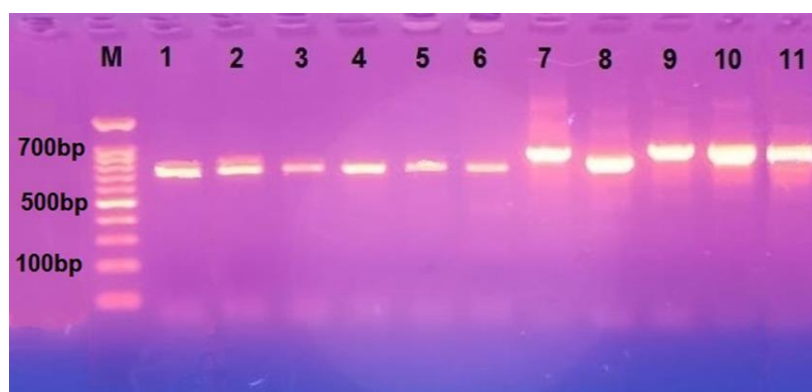


Plate (6): Amplification of polymerase coding region (710bp) COI of specimens by using conventional PCR. The replicons run on agarose gel 1.5% and visualized with trans illuminator, M: DNA ladder (100bp).

Sequencing Analysis: According to our investigation, 62 specimens of Chilopoda have been collected since its initiation (October 2022) to (May 2023). DNA was extracted from 11 centipedes' specimens; the results of the DNA sequences showed only six specimens were successfully sequenced, the nucleotide sequences of specimens were analysed for alignment, and the Basic Local Alignment Search Tool (BLAST) program is available online at the National Centre for Biotechnology Information (NCBI).

The acquired sequences' phylogenetic analysis showed that they were closely related to the query sequences collected from the GenBank. The GenBank records for several of these sequences revealed that they were classified as belonging to species that are phylogenetically remote from the locations of their local documented distribution. The bit score is a statistical indicator of moral similarity, and a greater value denotes a high level of resemblance. The definition of the expectation value (E) is that it provides an assessment of the frequency with which it is anticipated to encounter such coincidences and lowers the E-value.

This suggests that there was a significant degree of similarity between sequences, which increases confidence; if it is very close to or equal to zero, then the sequences are the same as those in Table (2). The specimen *Lithobius crassipes* and the specimen *Bothriogaster signata* had 100 % identity by 660 bits (357) and 0.0 expect, and 607 bits (672) and 1e-176 expect,

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respectively. These two samples had a position of 654 in GenBank query. The species *lithobius crassipes* (Family: Lithobiidae) is a new record for the Iraqi centipede fauna.

Table (2): Sequencing ID in GenBank, show score, expect, and compatibility of mitochondrial DNA sequences for six centipedes' specimens.

Specimen No.	Genus-Species	GenBank ID.	Score (bits)	Expect	Identity	Position in GenBank
1	<i>Scolopendra morsitans</i>	KR705661.1	531	2e-153	82%	49-665
2	<i>S. morsitans</i>	KR705664.1	564	2e-163	82%	34-650
3	<i>S. cingulata</i>	JN688413.1	446	4e-126	82%	18-532
5	<i>Lithobius crassipes</i>	KX458776.1	660	0.0	87%	50-649
7	<i>Cormocephalus</i> sp	MZ503647.1	525	4e-144	81%	34-680
9	<i>Bothriogaster signata graeca</i>	AY288749.1	607	1e-176	81%	31-645

The study's sequence specimens and the same species' deposited (COI) gene copies in the NCBI database were compared, and the results revealed a high identity of more than 80% with the same reference species with accession numbers (Tab.3). The specimen from Baghdad that was recognized as *Scolopendra morsitans* demonstrated identification (97%) when compared to *Scolopendra morsitans* ON 923747 Vietnam and KR 705665 Thailand. This specimen has a different accession number due to the nucleotide polymorphism, which is supported by the alignment matrix. Additionally, the *Scolopendra cingulata* specimen revealed an identity of 82% and a score of 446 consistent with 95% identity to the specimen with accession numbers JN688413 Greece, as shown in the phylogenetic tree in Diagram (1).

In general, the Scolopendridae family is a genera-rich family that made its species identification using molecular characteristics an approach in alpha taxonomy. The molecular approach, which offered 97%, 100% compatibility to the genus *Lithobius crassipes* and *Bothriogaster signata*, however, revealed that the two families, Lithubiidea and Himataridae, have many similar traits and have a rich-genera distribution. The specimen *cormocephalus* sp. has an identity of 81% with a score of 525 bits (284), and expect 4e-144 belongs to the family Scolopendridae that contains the sample with an identity of 97 % with MZ305647 Australia.

Phylogenetic tree: The similarity matrix of COI gene local sample isolates in Table (2) serves as an analysis of the data gathered. With the phylogenetic tree, a distance comparison is also shown (Diag. 1). In the phylogenetic tree of our study, we noticed that local *Lithobius crassipes* is grouped into one branch with the same species from Canada, France, and

Germany, and also *Bothriogaster signata* is grouped into one branch with the same species from Australia. Although local species *Scolopendra morsitans*, *S. cingulate*, and unidentified species of the genus *Cormocephalus* sp. did not unite with its counterparts in one group, their relationships were very clear that they are close to its counterparts. We also find that this tree is very similar to the phylogenetic tree presented by Wang *et al.* (2022), where the relationship of order Lithobiomorpha with Geophilomorpha is closer than the relationship with Scolopendromorpha. The relationships identified in the present study are consistent with the conclusions obtained from a previous study (Hu *et al.*, 2020; Wang *et al.*, 2022).

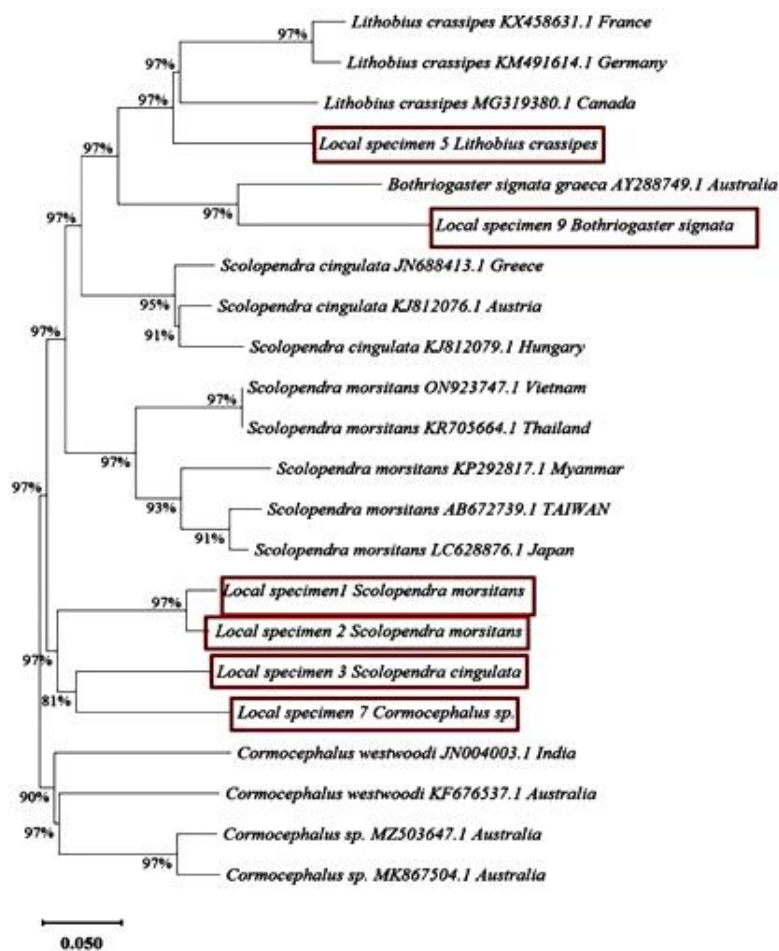


Diagram (1): phylogenetic tree constructed of Cytochrome Oxidase I gene sequences centipedes from different provinces in Iraq; local specimens with red marks, a measure of distance between taxa is indicated by the scale bar below the tree.

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CONCLUSIONS

In the present article, 5 Chilopoda species from 3 families and 3 orders are known from Iraqi fauna. This study characterizes the first molecular study of centipedes in Iraq. The phylogenetic relationship is made by the sequence of the partial mtDNA-COI gene, and a phylogenetic tree was also constructed. The analysis resulted in a record of the species *lithobius crassipes* (L.koch, 1862) (Chilopoda, Lithobiomorpha, Lithobiidae) for the first time in Iraq. Our study identified a close relationship between Lithobiomorpha and Geophilomorpha. The distance comparison was also provided with the phylogenetic tree.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare. We "the authors" have followed and signed the scientific research ethics announced by the journal.

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دراسة جزيئية لبعض أنواع مئوية الاقدام (Centipedes) في العراق مع تسجيل
جديد للنوع *Lithobius Crassipes* L. Koch, 1862
(Chilopoda, Lithobiomorpha, Lithobiidae)

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الاستلام: 2023/8/20، المراجعة: 2023/12/7، القبول: 2023/12/28، النشر: 2024/6/20

الخلاصة

قدمت هذه الدراسة أول عمل جزيئي حول حيوانات مئويات الأقدام في العراق. جمعت مئويات الأقدام من تجمعات مختلفة في 4 محافظات عراقية للفترة ما بين تشرين الأول 2022 ومايو 2023 في ظروف مناخية مختلفة. تم إجراء التعريف الجزيئي لـ 3 عائلات ممثلة بـ *Scolopendra morsitans* وأنواع 5 من رتبة Scolopendromorpha، سجل كل من: *Cormocephalus* sp. و *S. cingulata* Latreille, 1829 و Linnaeus, 1758 مرة في العراق النوع *Lithobius crassipes* (L. Koch, 1862). ومن رتبة Geophilomorpha، سجل نوع واحد وهو *Bothriogaster Signata* Kessler 1874. استخلص الحمض النووي من العينات، وضخم جزء mtDNA، من جين COI (Cytochrome C Oxidase Subunit I) باستخدام البادئ المحدد، وبعد ذلك تم تطبيق برنامج أداة المحاذاة المحلية الأساسية (BLAST) التابع للموقع NCBI. رسمت شجرة النشوء والتطور باستخدام مقارنة المسافة.