The First Molecular Phylogeny of *Orthochirus fomichevi* Kovařík, Yağmur, Fet & Hussen, 2019 (Scorpiones: Buthidae) from Duhok Province, Kurdistan Region, Iraq, as Inferred from Mitochondrial Cytochrome c Oxidase Subunit 1 Gene (June 2024 - March 2025)

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Abstract

Orthochirus fomichevi Kovařík, Yağmur, Fet & Hussen, 2019 comprises small-sized scorpions in the family Buthidae, which inhabit the Kurdistan region of Iraq and parts of Turkey. Despite the availability of some molecular data, the phylogenetic relationship and genetic variation within this species remain largely unexplored. Therefore, this study aimed to use the mitochondrial cytochrome c oxidase subunit I gene to build the phylogenetic relationships and estimate the evolutionary divergence of *O. fomichevi* collected from Duhok province, Kurdistan Region, Iraq. A maximum likelihood tree of *O. fomichevi* revealed that this species forms a highly-supported clade, distinct from other *Orthochirus* species. In contrast, *O. innesi* isolates from GenBank did not group under the same clade; instead, each formed a separate clade. Additionally, the *O. afghanus* isolate from GenBank failed to form a distinct clade and grouped with *O. persa* isolates from Iran. Mean genetic distances, calculated using the Kimura 2-parameter model, indicated that all analyzed sequences of *O. fomichevi* are genetically homogeneous and show greater similarity to *O. innesi* than to other species. Morphological analysis was in agreement with the molecular findings, further supporting the distinctiveness of *O. fomichevi*. In contrast, the *O. innesi* isolates were genetically highly divergent from each other, indicating that they belong to different taxa. Furthermore, *O. afghanus* and *O. persa* isolates from GenBank were genetically very close to each other, indicating that they belong to the same species. Overall, this study presents the first molecular phylogeny of *O. fomichevi*, confirming its distinct genetic identity within the genus *Orthochirus* and highlighting the potential cryptic diversity and misidentifications in *O. innesi*, *O. afghanus*, and *O. persa* isolates from the GenBank database. These findings underscore the importance of integrating molecular tools with morphology for accurate scorpion taxonomy.

Keywords: Buthidae, Genetic Variation, Mitochondrial DNA, Phylogeny.

1. Introduction

Orthochirus Karsch, 1892 is a genus of scorpions within the family Buthidae. The genus was originally established by Karsch (1) as a replacement for the preoccupied name *Orthodactylus* Karsch, 1881. Since its inception, the taxonomic status of *Orthochirus* has been subject to considerable debate. Kraepelin (2) initially regarded it as a mere synonym of *Butheolus*, but it was later reinstated as a valid genus by Simon (3).

The genus *Orthochirus* comprises small-sized scorpions with a broad distribution across North Africa, the Middle East, Arabian Peninsula, Central Asia, and India (4). To date, 53 species within this genus have been described (5), of which three species, *O. fomichevi*, *O. mesopotamicus*, and *O. iraqus*, have been recorded in Iraq (6). According to Kachel et al. (7), each *Orthochirus* species in Iraq exhibits a restricted geographic range: *O. fomichevi* inhabits the northern region and the foothills of the Zagros Mountains; *O. iraqus* is distributed across the central and western plains; and *O. mesopotamicus* occurs in the southern humid and plain areas of the country. This spatial segregation underscores the species-specific habitat preferences and limited distributional overlap within Iraq.

O. fomichevi was first described as a new species based on morphological characteristics by Kovařík et al. (6). Prior to this, it had been misidentified as *O. zagrosensis*; however, *O. fomichevi* can be differentiated by the presence of granules on the dorsal surface of metasomal segment V, a feature absents in *O. zagrosensis* (6). The known distribution of *O. fomichevi* includes the Kurdistan region of Iraq and parts of Turkey. This initial misidentification underscores the limitations of relying exclusively on morphological traits for accurate species delimitation within the genus *Orthochirus*, highlighting the need for integrative taxonomic approaches.

DNA barcoding has emerged as a widely adopted molecular approach to overcome challenges in species identification, particularly for taxa exhibiting high morphological similarity or compromised specimen integrity. This technique primarily targets the 5′ region of the mitochondrial cytochrome c oxidase subunit I (COI) gene, which exhibits sufficient interspecific variability to serve as a standardized genetic marker for species-level discrimination, commonly referred to as the "barcode of life" (8, 9). The effectiveness of DNA barcoding is further enhanced when integrated with detailed morphological analyses (10), providing a robust framework for accurate taxonomic resolution across diverse animal groups.

Although the mitochondrial COI gene sequence of *O. fomichevi* is available in the GenBank database, no published studies have yet addressed its phylogenetic placement within the genus. This gap highlights the necessity for comprehensive molecular analyses to elucidate the evolutionary relationships of *O. fomichevi*. Accordingly, this descriptive, cross-sectional study integrates morphological identification with mitochondrial COI gene analysis to infer the molecular phylogeny of *O. fomichevi*, thereby contributing to a deeper understanding of its taxonomic and evolutionary context within *Orthochirus*.

2. Materials and methods

2.1. Study area

The study area is located within Duhok province in the Kurdistan region of Iraq, a predominantly mountainous region bordering Turkey and Syria (Figure 1). Geographically, it lies between latitudes 36°40'E and 37°20'N and longitudes 43°20'E and 44°10'E. Duhok province is administratively divided into seven districts: Zakho, Sumel, Duhok, Amedi, Shekhan, Akre, and Bardarash. The overall study was conducted from June 2024 to March 2025. Ethical considerations were strictly adhered to throughout the study to ensure compliance with relevant guidelines and regulations.

2.2. Taxon sampling

O. fomichevi specimens were collected nocturnally from June to October 2024 using a high-intensity ultraviolet (UV) lamp, capitalizing on the species' nocturnal behavior and natural fluorescence under UV light, which facilitates detection. Collected specimens were divided for preservation based on subsequent analyses: some were fixed in 95% ethanol and stored at -20°C

for molecular studies, while others were preserved in 80% ethanol and maintained at room temperature for morphological examination. The geographical distribution of *O. fomichevi* was mapped using ArcGIS Pro 3.0.1 software (Esri, USA).

2.3. Species identification and imaging

All specimens were identified morphologically using the taxonomic key provided by Kovařík et al. (6). Morphological identifications were verified by an experienced scorpion taxonomist. Morphological terminology follows the standards established by Sissom (11). High-resolution images of whole specimens were captured with a Canon EOS 7D camera. Image stacking was performed using Helicon Focus software (HeliconSoft, Ukraine) to enhance depth of field. The focus stacking methodology was adapted from the Canon-Cognisys system, as recommended by Brecko et al. (12).

2.4. DNA extraction

Genomic DNA was extracted from approximately 20 mg of metasomal muscle tissue using the AddPrep Genomic DNA Extraction Kit (Addbio, Korea). Prior to extraction, muscle tissue was washed in physiological saline solution (PiONEER, Iraq) for 15–20 minutes to remove residual ethanol and subsequently dried on filter paper. The dried tissue was transferred to a 1.5 ml Eppendorf tube and homogenized using a propylene micropestle (Shenzhen Baimai Life Science, China). The subsequent extraction steps were conducted according to the manufacturer's protocol. The isolated DNA was stored at -20°C until further analysis.

2.5. Mitochondrial COI gene fragment amplification

Polymerase chain reaction (PCR) amplification of the barcode region of the mitochondrial COI gene was performed using a TC1000-G-Pro Thermal Cycler (DLAB, China). The reaction employed the universal primers LCO1490 (5'-GGTCAACAAATCATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') as described by Folmer et al. (13). PCR reactions were conducted in a total volume of 20 µL, containing 0.5 µL (10 pmol) of each primer, 10 µL of Addstart Taq Master Mix (2× concentration; Addbio, Korea), 2 µL of template DNA (~20 ng/µL), and 7 µL of water. The thermal cycling conditions are detailed in Table 1. Amplification success was verified by electrophoresis on a 1.5% agarose gel. PCR products were subsequently sent to Macrogen Inc. (Seoul, Korea) for Sanger sequencing.

Table 1. Steps and conditions of thermal cycling for PCR.

Steps	Temperatures	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1 cycle
Denaturation	94 °C	30 seconds	35 cycles
Annealing	48 °C	30 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	7 minutes	1 cycle

2.6. DNA sequence processing

Sanger sequencing trace files were manually checked and trimmed by SeqTrace 0.9.0 software (14) to generate consensus sequences, which were then subjected to BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against GenBank records to confirm DNA sequence identities and to search for closely related sequences for phylogenetic construction. The study sequences were deposited in GenBank under the accession numbers listed in Table 2.

2.7. Phylogenetic analysis

All sequences were aligned using the ClustalW algorithm implemented in MEGA 11 software (15). Phylogenetic relationships were inferred using the maximum likelihood (ML) method, with branch support assessed via 1,000 bootstrap replicates. The optimal substitution model was selected based on Bayesian Information Criterion (BIC) scores (16). The resulting ML tree generated in MEGA 11 was exported in Newick format and subsequently visualized and edited using FigTree 1.4.4 software to produce a high-quality phylogenetic tree.

Mean genetic distances within and between *O. fomichevi* and closely related sequences (excluding the outgroup) were calculated using the Kimura 2-parameter model (K2P) (17) to estimate evolutionary divergence among the sequences.

3. Results

3.1. Taxonomy

3.1.1. Family Buthidae C. L. Koch, 1837

3.1.1.1. Genus *Orthochirus* Karsch, 1892

3.1.1.1.1. *Orthochirus fomichevi* Kovařík, Yağmur, Fet & Hussen, 2019

Type locality and type depository: Iraq, **Sulaymaniyah Province**, Chaqzhi Khwaroo; František Kovařík, private collection, Prague, Czech Republic (FKCP).

Materials examined: Iraq, **Duhok province**, **Zakho district**, Shahida, 37°08'01.1"N 42°40'24.0"E, 506 m above sea level (ASL), 2♂2♀; Khrababk, 37°08'30.6"N 42°45'02.1"E, 526 m ASL, 1♂3♀; Salka, 37°06'50.8"N 42°39'05.7"E, 573 m ASL, 2♂3♀; Betas, 37°04'02.6"N 42°42'06.1"E, 675 m ASL, 1♂; Dubanik, 37°02'30.2"N 42°50'19.9"E, 709 m ASL., 4♂3♀; **Sumel district**, Sumel, 36°50'31.3"N 42°51'52.8"E, 446 m ASL, 6♂2♀; Esmahil Ava, 36°57'25.3"N 42°38'12.9"E, 504 m ASL, 13♂11♀; **Duhok district**, Nizarke, 36°50'20.7"N 43°04'03.5"E, 659 m ASL, 6♂7♀; **Shekhan district**, Atrush, 36°51'07.7"N 43°21'54.3"E, 1014 m ASL, 7♂8♀.

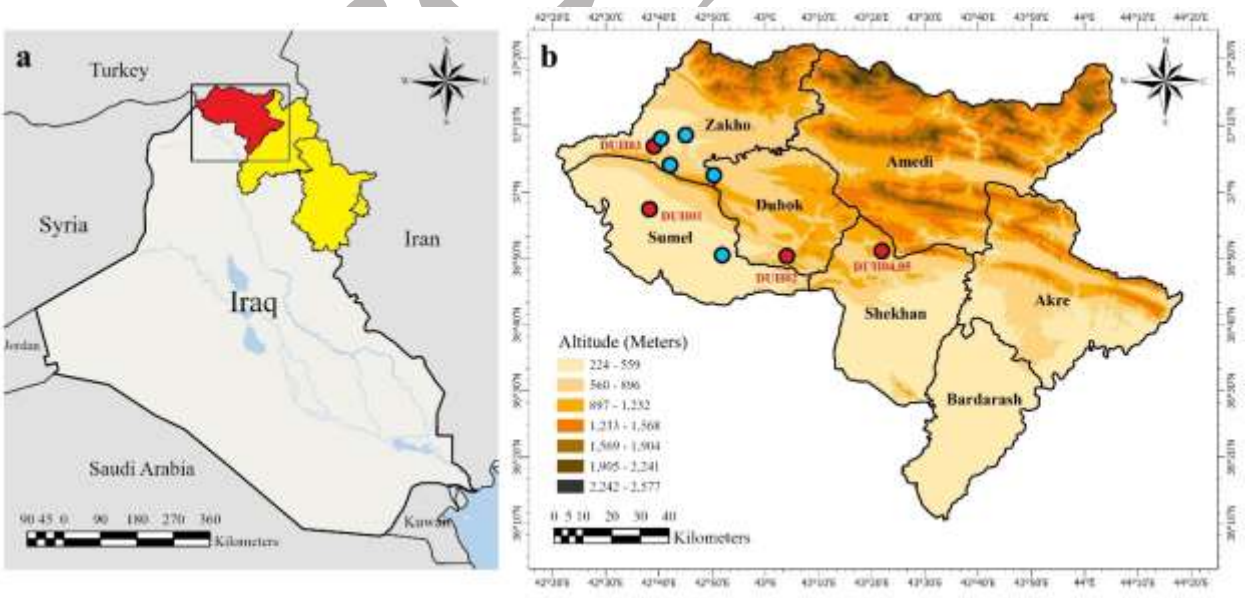

Figure 1. Geographical distribution of *O. fomichevi* plotted on the topography of Duhok province. (a) Map of Iraq showing the Kurdistan region (red and yellow polygons), with Duhok province shown in red. (b) Topographic map of Duhok province: blue and red circles indicate collection sites of the isolates used in morphological and molecular studies, respectively.

Diagnosis

The general body coloration is predominantly black, with the exception of the chelae fingers and the distal segments of the legs, which are yellow (Figure 2a-d). Adult body length ranges from 30.12 to 39.91 mm in males and 30.37 to 45.34 mm in females; females are generally larger than males. The trichobothrium d_2 on the femur is typically absent. The movable finger of the chela bears 8–9 rows of denticles, including both internal and external denticles, as well as four subterminal denticles. The number of pectinal teeth ranges from 21 to 23 in males and 17 to 20 in females. Mesosomal sternite VII presents a granulate surface characterized by prominent granulate carinae (Figure 2e). Metasomal segment I bears granules, whereas segments II and III lack granules on the ventral and lateral surfaces, displaying a punctate and bumpy texture (Figure 2f). Metasomal segment V is distinguished by dense granulation along the dorsal mesial surface (Figure 2g). Telson is elongated and smooth, lacks granules. The vesicle is long, with lateral and ventral punctation. Aculeus is long and curved.

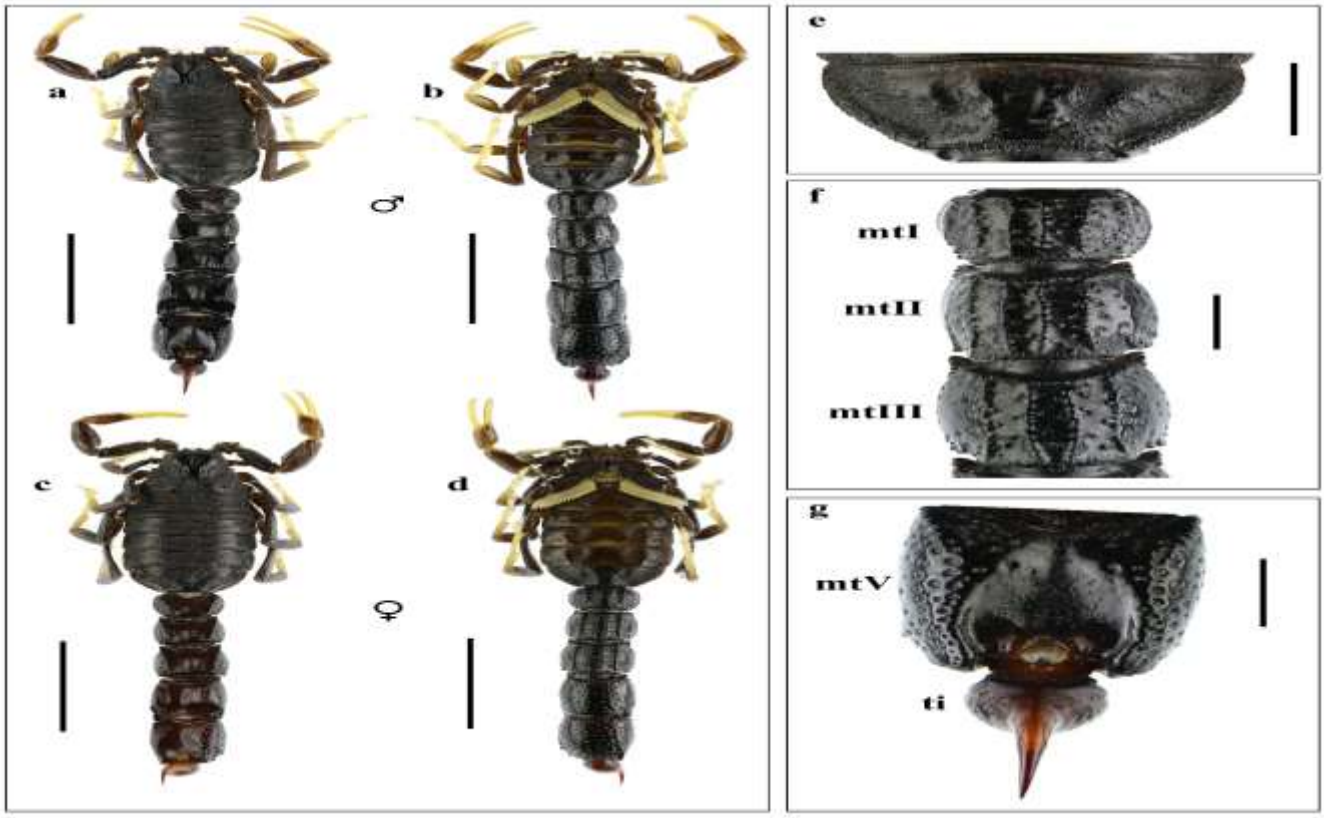

Figure 2. *O. fomichevi*. Showing dorsal (a, c) and ventral (b, d) aspects of male (a, b) and female (c, d). Detailed images: (e) Sternite VII; (f) Ventral aspect of metasomal segments I-III; (g) Dorsal aspect of metasomal segment V and telson. Abbreviations: (mtI-V) Metasomal segments I-V, (ti) Telson. Scales 10 mm (a-c), 2 mm (e-g).

3.2. Phylogenetic analysis

The barcode region of the COI gene was successfully sequenced from five *O. fomichevi* specimens (Table 2) collected from four distinct localities. These sequences were supplemented with 12 additional COI sequences of various *Orthochirus* species

retrieved from the GenBank database. Additionally, a sequence of *Hottentotta tamulus* was included as an outgroup for phylogenetic analyses (Table 3).

Table 2. Details of *O. fomichevi* isolates from Duhok province used in molecular study, including their corresponding COI gene data.

	Locality data			COI gene data	
Isolates	**Localities**	**Latitude**	**Longitude**	**Accession numbers**	**Length (base pairs)**
DUH01	Esmahil Ava	36°57'25.3"N	42°38'12.9"E	PV219459	641
DUH02	Nizarke	36°50'20.7"N	43°04'03.5"E	PV219460	641
DUH03	Salka	37°06'50.8"N	42°39'05.7"E	PV219461	639
DUH04	Atrush	36°51'07.7"N	43°21'54.3"E	PV219462	639
DUH05	Atrush	36°51'07.7"N	43°21'54.3"E	PV219463	639

Table 3. Accession numbers of *Orthochirus* species and outgroup sequences retrieved from GenBank.

Species	**Localities**	**Accession numbers**
O. fomichevi	Iraq	MT232527
O. innesi	Morocco	ON255632
O. innesi	Egypt	MZ669861
O. innesi	-	JQ514244
O. persa	Iran	PP574852
O. persa	Iran	PP574854
O. persa	Iran	PP574853
O. bicolor	India	KT716038
O. bicolor	India	MF422331
O. glabrifrons	Oman	ON255631
O. afghanus	Afghanistan	ON255630
O. vachoni	Somaliland	ON255629
H. tamulus	Outgroup	KT716031

A phylogenetic tree for *O. fomichevi* was constructed using the ML method, with the TN93+G model (18), selected as the best-fit model by MEGA software. The ML tree with the highest log likelihood value of -1,738.62 was selected for visualization. In this tree (Figure 3), sequences of *O. fomichevi* formed a highly-supported clade with *O. fomichevi* from Iraq, as evidenced by a high bootstrap support value of 98. However, the interspecific relationships with other *Orthochirus* species could not be well resolved due to low bootstrap values. In contrast, different *O. innesi* isolates did not form a clade with each other; instead, each formed a separate clade, which indicates that they belong to different taxa. Additionally, *O. afghanus* did not form a separate clade; instead, it grouped with *O. persa* in a highly-supported clade (bootstrap value = 98), which indicates that they belong to the same taxon.

An examination of evolutionary divergence, calculated using the K2P model (Table 4), showed that the mean intraspecific divergence among members of *O. fomichevi* was low (0.54%), indicating a high level of genetic similarity within the species. Additionally, *O. fomichevi* exhibited a close genetic affinity with *O. innesi* from Morocco, with a mean interspecific divergence of 6.56%. In contrast, *O. fomichevi* was more genetically divergent from *O. vachoni*, with a divergence of 12.68%, compared to other *Orthochirus* species. Moreover, *O. innesi* isolates ON255632, JQ514244, and MZ669861 were highly divergent from each other, with divergence values ranging from 7.45 to 10.91%. Additionally, *O. afghanus* was very close to *O. persa* (divergence value = 2.37%).

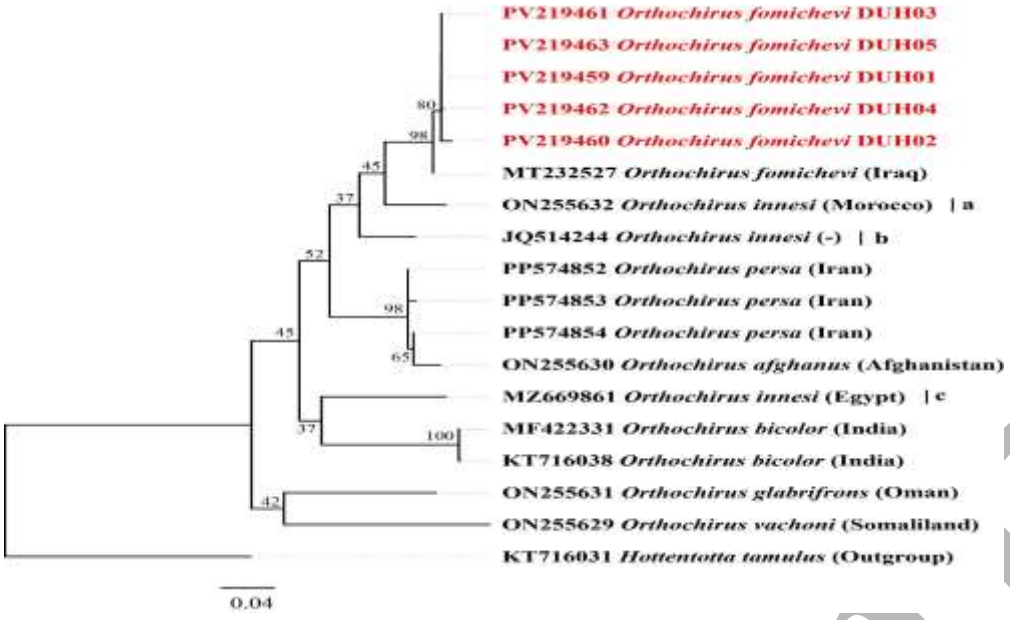

Figure 3. Maximum likelihood phylogenetic tree of *O. fomichevi* inferred from the COI gene. Study sequences are shown in red. Numbers above and below branches indicate bootstrap support values. The scale bar reflects the number of substitutions per nucleotide site. (a), (b), and (c) represent the isolates mentioned in Table 4.

Table 4. Estimates of net evolutionary divergence in the COI sequences within and between *Orthochirus* species. Shown here are the mean genetic distances calculated using the K2P model. The values in bold represent intraspecific divergence. (a), (b) and (c) represent the isolates mentioned in Figure 3.

	Species	1	2	3	4	5	7	8	8	9
1	*O. fomichevi*	**0.0054**								
2	*O. innesi* (a)	0.0656	**N/A**							
3	*O. innesi* (b)	0.0740	0.0745	**N/A**						
4	*O. innesi* (c)	0.1012	0.1091	0.0989	**N/A**					
5	*O. persa*	0.0933	0.0911	0.0863	0.1024	**0.0067**				
6	*O. glabrifrons*	0.1119	0.1315	0.1286	0.1451	0.1238	**N/A**			
7	*O. afghanus*	0.1122	0.1006	0.0902	0.1173	0.0237	0.1249	**N/A**		
8	*O. bicolor*	0.1159	0.1363	0.1084	0.1132	0.1131	0.1180	0.1115	**0.0020**	
9	*O. vachoni*	0.1268	0.1312	0.1325	0.1454	0.1457	0.1251	0.1491	0.1528	**N/A**

4. Discussion

In the present study, eighty-one specimens of *O. fomichevi* were collected from multiple locations across Duhok province. These scorpions were predominantly encountered in open plain habitats characterized by sparse vegetation. Morphological analysis consistently confirmed species identification based on diagnostic traits, while molecular analysis of the COI gene revealed that *O. fomichevi* forms a strongly supported clade (bootstrap = 98) distinct from other *Orthochirus* species. The intraspecific divergence was low (0.54%), indicating high genetic homogeneity, while interspecific divergence with other species ranged from 6.56% to 12.68%. Notably, sequences labeled as *O. innesi, O. persa,* and *O. afghanus* in GenBank showed evidence of misidentification.

Prior records have documented *O. fomichevi* in the provinces of Duhok, Erbil, and Sulaymaniyah within the Kurdistan region of Iraq, as well as in Hakkari province from Turkey (6,19,20). Morphologically, *O. fomichevi* can be distinguished from other *Orthochirus* species by several diagnostic features, including a densely granulated mesosomal sternite VII with well-developed granulate carinae; a metasomal segment V exhibiting dense granulation along the dorsal mesial surface; and metasomal segments II and III that are smooth, punctate, and bumpy ventrally and laterally, lacking granules (6). All examined specimens in this study exhibited these defining morphological characteristics.

To complement morphological identification, phylogenetic analysis based on the mitochondrial COI gene was performed. The resulting phylogenetic tree demonstrated that the specimens collected in this study and previously sequenced isolates of *O. fomichevi* form a strongly supported clade (bootstrap value = 98), distinct from other *Orthochirus* species. Estimates of genetic divergence corroborate these findings; the mean intraspecific divergence within *O. fomichevi* was 0.54%, reflecting high genetic similarity among conspecific individuals. In contrast, mean interspecific divergence between *O. fomichevi* and other *Orthochirus* species ranged from 6.56% to 12.68%, with *O. innesi* from Morocco (ON255632) exhibiting the lowest divergence, thereby representing the closest relative to *O. fomichevi*.

These results are consistent with previous molecular studies on the genus *Orthochirus*, such as Jafari et al. (21), who analyzed COI and 16S rRNA gene sequences from *O. iranus*, *O. farzanpayi*, *O. zagrosensis*, and *O. stockwelli* populations in Iran. Their findings similarly reported low intraspecific divergence alongside relatively higher interspecific divergence, supporting the phylogenetic patterns observed in the present study. However, they did not deposit their data in GenBank; that's why we did not include them in our analysis. Similarly, Barahoei (22) also reported low intraspecific divergence, particularly in *O. persa*. The *Orthochirus* fauna of Iraq includes two other species: *O. mesopotamicus* and *O. iraqus* (7). In this study, we were unable to infer their phylogenetic relationships due to the unavailability of their COI data in GenBank.

Additionally, the three isolates of *O. innesi* (ON255632, JQ514244, and MZ669861) retrieved from GenBank did not group together; instead, each formed a separate clade. The genetic divergence analysis further supported these findings, in which the divergence between them was relatively high (7.45–10.91%). The phylogenetic relationships between the isolates JQ514244 (unknown) and MZ669861 (Egypt) were previously inferred by Mohammed-Geba et al. (23), in which the two isolates grouped under the same clade. However, their study was based on a wide range of divergent species from different genera, and no *Orthochirus* species other than the ones mentioned; therefore, they were grouped together. The type locality of *O. innesi* is Cairo, Egypt (3); therefore, we conclude that the isolate MZ669861 represents the true *O. innesi*, whereas the isolates ON255632 and JQ514244 belong to two misidentified specimens which could not be assigned due to limited COI data in GenBank.

Furthermore, *O. afghanus* from Afghanistan failed to form a distinct clade; instead, it formed a highly supported clade (bootstrap value = 98) with the *O. persa* isolates from Iran. This indicates that they belong to the same species. The interspecific divergence further supports these findings, in which the divergence value between them was very low (2.37%) to be considered two separate species. The localities of the isolates of *O. afghanus* (ON255631) and *O. persa* (PP574852, PP574854, and PP574853) analyzed in this study are close to each other (24, 22); therefore, it was not possible to conclude whether they belong to *O. afghanus* or *O. persa*. Generally, species misidentification is very common in the case of scorpions, even in dedicated literature (25), which has led to many incorrect placements of scorpions in the GenBank database (26).

A limitation of this study was the inability to comprehensively investigate certain districts due to the unstable political situation, their distance from our research center, and the logistical challenges associated with sample collection in these areas. Additionally, the limited availability of COI sequence data for *Orthochirus* species in GenBank restricted comparative molecular analyses.

In conclusion, this study provides robust evidence for the accurate identification and phylogenetic placement of *O. fomichevi* within the genus *Orthochirus*, confirmed by consistent morphological traits and a strongly supported phylogenetic clade with low intraspecific genetic divergence. The unexpected divergence and placements of isolates labeled *O. innesi*, *O. persa*, and *O. afghanus* highlight the need for thorough taxonomic revision and expanded genetic sampling. To address challenges from misidentifications in public databases, we recommend integrating morphological and molecular tools in scorpion taxonomy

and expanding molecular datasets to include additional *Orthochirus* species, which will improve database accuracy and enhance biodiversity knowledge in the region.

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Author's Contribution

Study concept and design: H.S.K, F.R.A
Acquisition of data: F.R.A
Analysis and interpretation of data: F.R.A
Drafting of the manuscript: F.R.A
Critical revision of the manuscript for important intellectual content: H.S.K
Statistical analysis: F.R.A
Study supervision: H.S.K

Ethics

The study protocol was approved by the Animal Ethics Committee of College of Science, University of zakho, under the code: AEC-031.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Data Availability

The COI sequence data are available in GenBank (https://www.ncbi.nlm.nih.gov/) under the accession numbers PV219459, PV219460, PV219461, PV219462, and PV219463.

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