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Hedieh Jafari

Razi Vaccine and Serum
Research Institute, Agricultural
Research, Education and
Extension Organization
(AREEO), Ahvaz, Iran

Fatemeh Saalabi

Razi Vaccine and Serum
Research Institute, Agricultural
Research, Education and
Extension Organization
(AREEO), Ahvaz, Iran

Abbas Jelodar

Department of Basic Sciences,
Shahid Chamran University of
Ahvaz, Iran

Shahrokh Navidpour

Razi Vaccine and Serum
Research Institute, Agricultural
Research, Education and
Extension Organization
(AREEO), Karaj, Iran

Elham Jahanifard

Department of Medical
Entomology, Ahvaz Jundishapur
University of Medical Sciences,
Ahvaz, Iran

Alireza Forouzan

Razi Vaccine and Serum
Research Institute, Agricultural
Research, Education and
Extension Organization
(AREEO), Ahvaz, Iran

Behzad Masihpour

Razi Vaccine and Serum
Research Institute, Agricultural
Research, Education and
Extension Organization
(AREEO), Karaj, Iran

Correspondence

Hedieh Jafari

Razi Vaccine and Serum
Research Institute, Agricultural
Research, Education and
Extension Organization
(AREEO), Ahvaz, Iran

Phylogenetic study on *Orthochirus iranus* by using morphological and molecular methods (Scorpiones: Buthidae)

Hedieh Jafari, Fatemeh Saalabi, Abbas Jelodar, Shahrokh Navidpour, Elham Jahanifard, Alireza Forouzan and Behzad Masihpour

Abstract

Scorpion samples of *Orthochirus iranus* were collected from Khuzestan province in the southwest of Iran in the summer of 2017. The purpose of this study was morphometric analysis of male and female of *orthochirus iranus* and phylogenetic relationship of this species with other scorpion of buthidae family. The most important character in males and females, along with the position and teeth of the pectin, was the fourth segment of the metazoma, which was taller and wider in males, while the length of the female was higher than males ($P < 0.05$). The 16SrRNA gene fragment of *Orthochirus iranus* formed a cluster relative with a good bootstrap score. These samples isolated are closely related, as they clustered together as a Cluster. Comparison with the sequences showed that the highest level of identity was scored with *Leiurus quinquestriatus* (78%), *Androctonus australis* (75%), *Buthus draa* (75%).

Keywords: *Orthochirus iranus*, Morphology, Phylogeny, Buthidae

Introduction

Scorpions are venomous arthropods in the class Arachnida, order Scorpiones and considered to be living fossils [9, 12]. Scorpions have different characteristics from other arthropods such as feeding behavior, longevity and biology of reproduction [17]. Over 2,000 species of scorpions have been described and these colonize a worldwide range of habitats [9, 12, 19]. For this reason, scorpions are one of the most useful models in different fields of biology, physiology and evolutionary ecology [12, 17]. Each method of taxonomy that will be studied according to the principle of evolution must have the features that each species of family studies be closer to their ancestral features closer.

Iran is one of the most diverse fauna scorpions in the West Asia region. This high level of species diversity with congenial habitat in this area is one of the richest fauna to study this arthropod [1]. Khuzestan is one of the largest provinces with diverse humidity (10 to 90%), temperature (0 to 60 °C) and ecological niches for arthropods in Iran. Scorpion Buthidae with more than ten species and one subspecies is important family of scorpion of Khuzestan province [14]. The genus *Orthochirus* sp. has been known in recent years, although given novelty of this species are in need of review. Scorpions of genus *Orthochirus* include eight species and study on correlation between morphological characteristics and phylogeny of this genus has been limited [8, 13].

Previous studies on identification of scorpion and determine relationships between them based on analyzing of morphological characters such as Trichobothrium pattern, chelicerae, Carapace, pedipalp, femur, mesosomal, metasomal and these studies may limited to identify the genus and species [4, 7]. Morphological and morphometric studies alone do not represent relationships within species and to identify sibling species phylogeny parameters are required [10]. Molecular and Phylogenetic relationships based on morphological characters studies can be helpful and solved for the challenges [10].

In this study, *Orthochirus iranus* collected from different part of Khuzestan province and examples of species based on morphology, morphometric and molecular studies gene sequences of the 16S ribosomal RNA (16srRNA).

2. Materials and Methods

2.1. Sample collection

Scorpion samples of *Orthochirus* sp. were collected from Khuzestan province in the southeast of Iran, Lowland deserts with sandy substrate prevails montane with dominant rocky substrates (Gamboe 31° 22' 54" N, 48° 32' 8"E, shadegan 30° 39' 52"N, 48° 35' 03"E, Albaji 31° 32' 05"N, 48° 33' 22"E) in the summer of 2017. All specimens were collected by UV light at night. They were transported in glass jars contains ethanol 70% and glycerin (95:5 ml) to the Razi Reference Laboratory of scorpion researches, Ahvaz-Iran. For DNA extraction, samples were kept in 95% ethanol and frozen at -20 °C.

2.2. Morphological measurements

Morphological measurements were taken using digital calipers. The identification of male and female was by method of Hjelle 1990 [7]. Morphometric study was performed on 16 male and 14 female of *O. iranus*. 26 preserved specimens were measured per species and average measurements were calculated. In this study, measured on the basis of morphological traits and trichobothrium pattern based on Vachan (1974) was performed [19]. Morphological characteristics, including color, pedipalp, prosoma, metasoma, and trichobuthria pattern and the makeup were done using a stereomicroscope.

2.3. Abbreviations of morphometric ratios

Ca_L/AW: carapace length to anterior width; Ca_AW/PW: carapace anterior width to posterior width;;Ch_L/W: pedipalp chela length to width; Met-I_L/W: metasomal segment I length to width; Met-I_L/H: metasomal segment I length to height; Met-II_L/W: metasomal segment II length to width; Met-II_L/H: metasomal segment II length to height; Met-III_L/W: metasomal segment III length to width; Met-III_L/H: metasomal segment III length to height; Met-IV_L/W: metasomal segment IV length to width; Met-IV_L/H: metasomal segment IV length to height; Met-V_L/W: metasomal segment V length to width; Met-V_L/H: metasomal segment V length to height.

2.4. Genomic DNA extraction

Genomic DNA was extracted from the grounded scorpion tissue (0.5-1.0 g) and was extracted using DNA extraction kit (CinnaGen, Iran) according to the manufacturer's instruction. DNA concentration and purity determined by calculating the absorbance ratio A260/280.

2.5. PCR amplification and sequencing

PCR amplification was carried out on aliquots of the *Orthochirus* genomic DNA as template with initial denaturation for 3 min at 95 °C, followed by 35 cycles of 1 min at 94° C, 45 s at 55 °C, and 1 min at 72 °C and, finally, 5 min of incubation at 72° C in a final reaction volume of 25 μ l containing 50 mM KCl, 10mM Tris-HCl (pH 8.3), 20 pmol of each primers, deoxynucleotides (each at 220 μ M), 1.5 mM MgCl₂, 100 ng genomic DNA, and 1 U *Taq* polymerase. The primers were 16S-F 5'- AGAG-TGACGGGCAATATGTG and 16S-R 5'- CAGCGGCTGCGGTTATAC. The Amplified PCR products were electrophoresed through a 1% agarose

gel, and stained with DNA Safe Stain (Sinaclon, Iran) before detection by UV transillumination. The amplified DNA fragments were extracted from agarose gel prior to perform DNA sequencing according to the dideoxy termination method using an automated Applied Biosystems 373 DNA sequencer. The sequence was determined for both strands by using overlapping fragments.

2.6. DNA analysis

The DNA sequences comparisons and putative functions of the DNA fragments were done using the blastn and blastx algorithms programs in the National Center for Biotechnology Information (NCBI) GenBank database. Similar sequences are downloaded from the NCBI database. The phylogenetic analysis was performed using the neighbor-joining method with 1000 replicates of bootstrapping using the MEG7 software. A 16S sequence of *M Aedes albopictus* was used as outgroup.

2.7. Statistical analyses

In the morphometric study, a t-test was used to compare forewing length of male and female in the *Orthochirus iranus* full data set.

3. Results

3.1. Morphometric analysis

The mesosoma and metasoma was black, the chela of pedipalp and tibiae of legs was pale yellow to yellowish brown. Mesosoma and carapace was densely granulated and intraocular area was smooth pectinal teeth number 17 or 18 in the females and 19 to 21 in the males. The IV and V segments of metasoma were ventrally punctate and had two dorsolateral carinae and two ventrolateral carinae. The segments I to III bear weakly punctate and granulated. In males, spaces of punctae for the IV segment were granulated but in females were smooth. In posterior half of V segment dorsal surface bears a median row of granules. The telson had hair and punctate, lacks granules. The femur of pedipalps had four granulose. The patella and chela had smooth carinae. The movable fingers had 8 or 9 rows of granules in internal surface. Tarsomere I of first to third legs usually with bristlecombs composed of only 5 or 6 bristles, which may be irregular and not always form bristlecombs.

Trichobothrium *d*2 of pedipalp femur in some specimen absent and some of them fully developed on dorsal surface. This character was not related to sexual dimorphism or special region.

Statistical analyzes were performed on 17 morphometric ratios (32 variables) for 30 adult *Orthochirus iranus* specimens in both males (n=16) and females (n=14) (Table 1). Analysis of T-test in both males and females showed that there were not significant differences between the one morphometric traits measured in these groups ($P < 0.05$). As showed in this table, there were two morphometric ratios MTIVL/H and MTVL/W of which 17 morphometric ratios had a significant difference between the male and female. The fourth segment of metasoma in male (1.42 ± 0.025 mm) was longer than female (1.34 ± 0.03 mm). This segment in male (1.16 ± 0.034 mm) was wider than female (1.07 ± 0.017 mm).

Table 1: T- test analysis of morphometric measurements of the females and male of *Orthochirus iranrus*, $P < 0.05$; ns, no significant difference.

Variabe	Mean±SE		
	Male (n=16)	Female (n=14)	
CL/AW	1.61±0.024	1.57±0.026	Ns
CL/PW	0.99±0.022	0.95±0.029	Ns
CAW/PW	0.63±0.021	0.58±0.012	Ns
X/Y	0.99±0.0	0.97±0.048	Ns
MTIL/H	0.91±0.028	0.91±0.041	Ns
MTIIL/H	0.98±0.028	1.00±0.022	Ns
MTIIL/H	1.09±0.045	1.02±0.023	Ns
MTIVL/H	1.42±0.025	1.34±0.03*	$P < 0.05$
MTVL/H	1.65±0.032	1.59±0.029	Ns
MTIL/W	0.72±0.018	0.69±0.027	Ns
MTIIL/W	0.79±0.022	0.79±0.021	Ns
MTIIL/W	0.86±0.035	0.81±0.019	Ns
MTIVL/W	1.08±0.023	1.04±0.050	Ns
MTVL/W	1.16±0.034	1.07±0.017*	$P < 0.05$
Ch_L/W	1.74±0.051	1.65±0.060	Ns
TIL/H	2.92±0.086	2.97±0.073	Ns
TIL/W	2.69±0.08	2.64±0.060	Ns

3.2. Sequence characteristics and Estimation of intraspecific sequence divergence

DNA fragments replicated by primer 16sRNA of *Orthochirus iranrus* were sequenced. The 16sRNA sequences belonging to *Orthochirus sp.* were retrieved from GenBank (JQ423118.1). Estimates of variation among sequences were based on a

fragment length of 464 nucleotides (Table 2). Average inter-species genetic distance between *Orthochirus iranrus* and *Orthochirus sp.* showed that two species *Orthochirus sp.* divergence (6%) of each other, or genetically closer together. The highest genetic divergence was found between two species *Orthochirus iranrus* and *Hottentotta gentili* (23%).

Oi	1	TATACTAAAAGAGTGAAGAACTAAAATTAAAAATGTTAAAGAAAAGAGAGTGCAATCACGAGTTTATTTTTACGGTAA
Osp	1	TATACTAAAAGAGTAAAGAACTTTGGATTTTAAAATGCTAAAAAGGAGAAAGCAATTTACGAGTTTATTTTTACGGTAA
Bd	1	TATACCGTAAAAGTGAAGAAATTTTCAGTTTACAATTTGGAATAATTTT---GGGAGAAAGTTAAAATTT---TTGGAAAGGTAA
Bp	1	TATACCGTAAAAGTGAAGAAATTTTCAGTTTACAATTTGGAATAATTTT---GGGAGAAAGTTAAAATTT---TTGGAAAGGTAA
Aa	1	---ACTGTAAGGGTAAAGAAATTTAATTTTTTAATTAAGAAGTTGAAG---AAAGGAAAGGAAAAATTTTCTTTTAAGGTAA
Aam	1	TATACTGTAAGGGTAAAGAAATTTAATTTTTTAATTTGAAGTTGAA---AAAGGAAAGGAAAAATTTTCTTTTAAGGTAG
Am	1	---ACCGTAAAGGTAAAGAAATTTAGTTTTAATTTATGAGTTTAT---AAATGAAAAGAAAAATTTTCTTTTAAGGTAA
Ag	1	---ACCGTAAAGGTAAAGAAATTTTGGTTTTAATTTGAAGAAATTT---AAAGAAAAGAAAATTTTCTTTTAAGGTAA
Lq	1	TATACCGTAAAGATGAAGAACTTTTGGTTTTATTTTGAAGAAA---GTTTGAATTTTCTTTTATTTTGAAGGTAA
Hg	1	TATACTAAAAGTGAAGAAATCTTAGTAATAAATAATAAAACTTT---TATTGCTTGGAAAAATTTCTTTACGAGGTGA
Oi	81	AAT---CITTAATAAATCTTTTTTTCAATTTTATTT---TTTTGAAAGCTTAAAAATGAAACTAGGATTAGATACCCATTAT
Osp	80	AATCTTTAATAAATATTTTTTTCAATTTTATTT---TTTTTGAAGTTTAAAAATGAACTAGGATTAGATACCCATTAT
Bd	77	AAT---TTTGAATAAATAATCTTTTCAGATTATAAATTTTAAAAAATTCAAAAGTAAAAGTAACTAGGATTAGATACCCATTAT
Bp	77	AAT---TTTGAATAAATAATCTTTTCAGATTATAAATTTTAAAAAATTCAAAAGTAAAAGTAACTAGGATTAGATACCCATTAT
Aa	76	AAT---TGAAGAAGATAATCTTTTCAATTTTATAG---CITTTGAAATTCAAAAGTAAAAGTAACTAGGATTAGATACCCATTAT
Aam	78	AAT---TGAAGAAGATAATCTTTTCAATTTTATAG---CITTTGAAATTCAAAAGTAAAAGTAACTAGGATTAGATACCCATTAT
Am	75	AAT---TGAAGAAGATAATCTTTTCAATTTTATAG---CITTTGAAATTTTACGAGTGA---ACTAGGATTAGATACCCATTAT
Ag	75	AAT---TGAAGAAGATAATCTTTTCATATTTAAAA---CITTTGAAAGTTTAAAGATGAAACTAGGATTAGATACCCATTAT
Lq	76	AATNTTAAATATAAATGTTTTTTTNTTTATCCTNT---CITTTGAAAGCTCAAAGTAAAATAGGATTAGATACCCATTAT
Hg	78	AATTTTAAAGAATTTCTGAAACTCATTATTAAT---TTTTAAAAAGCTTCTATTAAGTAACTAGGATTAGATACCCATTAT
Oi	158	TAAATAAATGTAACAGAATTAGGTAGTATTAGATGCTTTGAAACTTAAAGAATTTGGCGGTATTTTAATCTAATTAGAGGA
Osp	159	TAAATAAAGTAAAGCAATTAGGTAGTATTAGATGCTTTGAAACTTAAAGAATTTGGCGGTATTTTAATCTAATTAGAGGA
Bd	156	TATAGAATAAAGAGAAATTAGGTAGTAAATGGATGCTTTGAAACTTAAAGAATTTGGCGGTGTTTTAATCTAATCAGAGGA
Bp	156	TATAGAATAAAGAGCAATTAGGTAGTAAATGGATGCTTTGAAACTTAAAGAATTTGGCGGTGTTTTAATCTAATCAGAGGA
Aa	153	TATTGACATAAAGCAAAATTAGGTAGTAGTAGATGCTTTGAAACTTAAAGAATTTGGCGGTGTTTTAATCTTACCAGAGGA
Aam	155	TATTGACATAAAGCAAAATTAGGTAGTAGTAGATGCTTTGAAACTTAAAGAATTTGGCGGTGTTTTAATCTTACCAGAGGA
Am	152	TATTAAAAAATAAATAATTAGGTAGTATTGCAAGCTTTGAAACTTAAAGAATTTGGCGGTATTTTAATCTTATCAGAGGA
Ag	152	TATTAAAAAATAAATAATTAGGTAGTATTGCAAGCTTTGAAACTTAAAGAATTTGGCGGTGTTTTAATCTTATCAGAGGA
Lq	154	TAGAGAAAGAAAAAATAATAGGTAGTAAATAGAAGCTTTGAAACTTAAAGAATTTGGCGGTATTTTAATCTTACCAGAGGA
Hg	155	TAAAGAGAGTAAAAAATAATAGGTAGTAAATAGTGTCTTTGAAACTTAAAGAATTTGGCGGTGTTTTAATCTTATTAGAGGA
Oi	238	ACTTGTGTTTTTAATTCGAAATTAAGTCTGTTAATCTTACTTAAATTTCT---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Osp	239	ACTTGTGTTTTTATTCGAAATTAAGTCTGTTAATCTTACTTAAATTTCT---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Bd	236	ACTTGTGTTTTTAAGTAAATTAAGTCTGTTTATCTTACTTAAATTTAA---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Bp	236	ACTTGTGTTTTTAAGTAAATTAAGTCTGTTTATCTTACTTAAATTTAA---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Aa	233	ACTTGCCTTATAACTCGAAATTAAGTCTGTTAATCTTACTTAAATTTA---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Aam	235	ACTTGCCTTATAACTCGAAATTAAGTCTGTTAATCTTACTTAAATTTA---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Am	232	ACTTGTATTGTAAGTAAATTAAGTCTGTTAATCTTACTTAAATTTG---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Ag	232	ACTTGCCTTATAAATCGAAATTAAGTCTGTTTATCTTACTTAAATTTA---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Lq	234	ACTTGTGTTTTTAATTCGAAATTAAGTCTGTTAATCTTACTTAAATTTA---GTTTGTATATCGTCGTAA---AAATAAACTTTA
Hg	235	ACTTGTATTTTAATTCGAAATTAAGTCTGTTAATCTTACTTAAATTTA---GTTTGTATATCGTCGTAA---AAATAAACTTTA

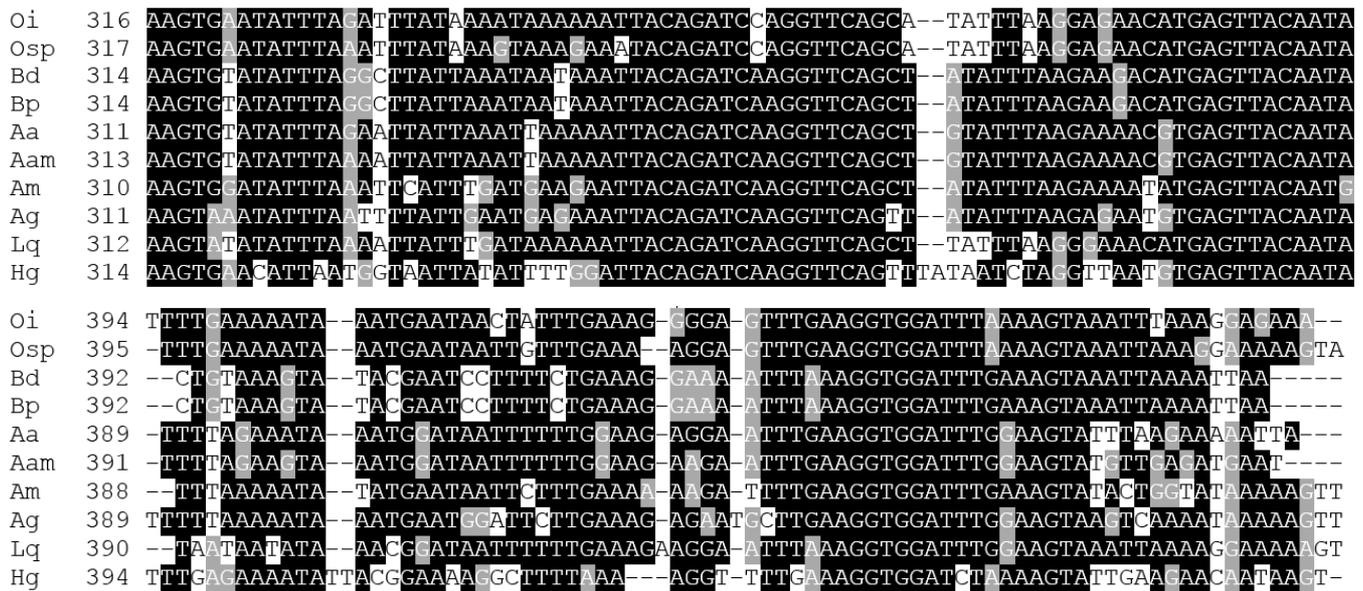


Fig 1: BLAST alignment between *Orthochirus iranus*(oi), *orthochirus sp.* (oi) *Leiurus quinquestriatus*(Lq, *Androctonus australis* (Aa), *Buthus draa* (Bd), *Buthus paris*(Bp), *Androctonus amoreuxi*(Aam) and *Hottentotta gentili*(Hg)

Comparison with the sequences contained in the GenBank database revealed similarity with the several fragments described in several scorpions (38-78%). The highest level of identity was scored with *Leiurus quinquestriatus* (78%),

Androctonus australis (75%), *Buthus draa* (75%). A sequence analysis also shows identity (91%) *orthochirus sp.* from Morocco (Figure-1).

Table 2: The average K2P genetic distance of *Orthochirus iranus* with *orthochirus sp.* (oi), *Buthus draa* (Bd) *Leiurus quinquestriatus* (Lq), *Androctonus australis* (Aa), *Androctonus amoreuxi*(Aam), *Androctonus mauritanicus* (Am), *Androctonus gonneti* (Ag) *Buthus occitanus* (Bo), *Buthus paris*(Bp), *Buthus mariefranceae* (Bm), *Buthus atlantis* (Ba), *Buthus ibericus* (Bi), *Buthus boumalenii* (Bbo), *Buthus boumalenii* (Bbo), *Hottentotta gentili* (Hg)), *Mesobuthus mauritanicus*(Mm), *Mesobuthus gibosus* (Mg), *Odontobuthus doriae* (Od), *Aedes albopictus* (Aae) for the 16SrRNA gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Oi																			
2. Osp	0.06																		
3. Bd	0.13	0.15																	
4. Lq	0.15	0.15	0.12																
5. Aa	0.15	0.17	0.13	0.13															
6. Aam	0.15	0.15	0.12	0.12	0.01														
7. Am	0.15	0.13	0.14	0.13	0.17	0.15													
8. Ag	0.17	0.16	0.14	0.14	0.15	0.13	0.13												
9. Bo	0.11	0.12	0.08	0.13	0.12	0.11	0.15	0.10											
10. Bb	0.15	0.15	0.07	0.12	0.15	0.13	0.12	0.12	0.09										
11. Bm	0.15	0.15	0.07	0.12	0.14	0.13	0.14	0.15	0.12	0.09									
12. Ba	0.16	0.17	0.09	0.12	0.12	0.11	0.11	0.11	0.09	0.10	0.11								
13. Bi	0.12	0.14	0.10	0.15	0.13	0.12	0.15	0.11	0.06	0.11	0.13	0.09							
14. Bbo	0.16	0.17	0.09	0.14	0.13	0.13	0.16	0.14	0.09	0.10	0.12	0.13	0.12						
15. Od	0.18	0.18	0.16	0.13	0.16	0.16	0.17	0.17	0.16	0.19	0.16	0.17	0.18	0.16					
16. Hg	0.23	0.22	0.21	0.22	0.22	0.21	0.28	0.27	0.23	0.24	0.26	0.24	0.22	0.25	0.29				
17. Mm	0.17	0.18	0.17	0.20	0.13	0.13	0.15	0.21	0.18	0.18	0.18	0.17	0.19	0.20	0.17	0.23			
18. Mg	0.21	0.19	0.21	0.18	0.19	0.19	0.20	0.21	0.22	0.20	0.21	0.17	0.24	0.22	0.18	0.22	0.20		
19. Aae	0.29	0.31	0.35	0.40	0.41	0.40	0.38	0.33	0.32	0.35	0.39	0.36	0.34	0.37	0.41	0.42	0.41	0.44	

3.3. Phylogenetic Analysis

Phylogenetic tree constructed based on neighbor-joining analysis of 16S rRNA gene revealed that *Orthochirus iranus* with *Orthochirus sp.* (JQ423118.1), *Leiurus quinquestriatus* (JQ423131.1), *Buthus occitanus* (EO523755.1), *Androctonus australis* (KJ538348.1), *Buthus draa* (KF548099.1), *Buthus paris* (KF548098.1), *Androctonus amoreuxi* (JQ423120.1) and *Hottentotta gentili* (JQ423119.1), *Mesobuthus gibosus* (AJ716204.2), *Mesobuthus mauritanicus* (KJ538244.1), *Buthus ibericus* (KF824923.1), *Buthus boumalenii*

(KF824912.1), *Odontobuthus doriae* (KX388482.1), *Buthus mariefranceae* (KF824928.1) the outgroup species and *Aedes albopictus* outgroup family. The tree obtained by simultaneous analysis of MEGA7 (Fig. 2) was preferred. In the phylogenetic tree, the two main clades were visible. The samples of the species *Orthochirus iranus* can be related to a clade but quite apart, they were in a subfolder which is supported by Bootstrap 90%. The Nucleotide sequence, were mostly congruent morphologically, with no differences limited to supported groups.

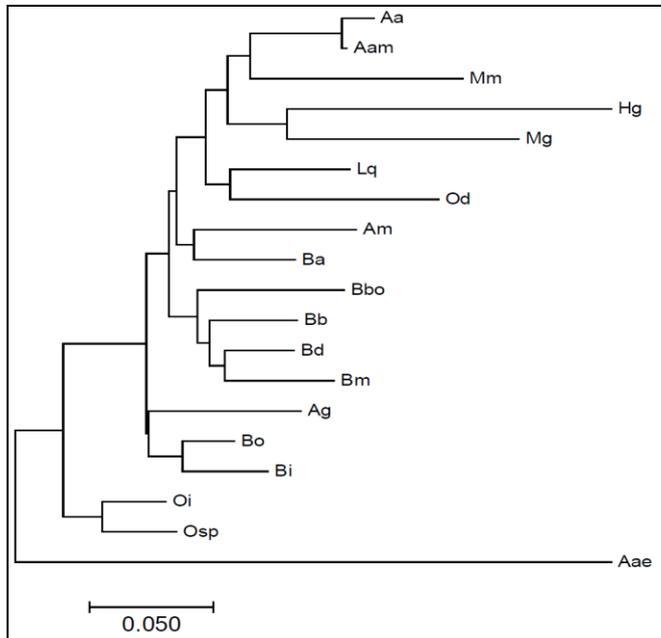


Fig 2: The neighbor-joining tree was conducted in MEGA7.

Orthochirus iranus with *orthochirus sp.* (oi), *Leiurus quinquestriatus* (Lq), *Buthus occitanus* (Bo), *Androctonus australis* (Aa), *Buthus draa* (Bd), *Buthus paris* (Bp), *Androctonus amoreuxi* (Aam) and *Hottentotta gentili* (Hg) *Mesobuthus gibosus* (Mg), *Mesobuthus mauritanicus* (Mm), *Buthus ibericus* (Bi), *Buthus boumalenii* (Bb), *Odontobuthus doriae* (Od), *Buthus mariefranceae* (Bm)

4. Discussion

The family Buthidae is the most diverse family of scorpions in Iran^[1]. In this family, *Orthochirus sp.* is one of the genus which eight described species. Whereas several ecological morphotypes exist in Iran, among the listed *Orthochirus sp.*, are the most geographically widespread and must be considered as habitat generalist species^[13, 20, 21]. *Orthochirus irannus* was known from Creek hills of the west and southwest of Iran and on the edge of the Zagros Mountains^[8, 13, 14].

Morphological study showed that the genus *Orthochirus sp.* was significant due to reduced telson and shape of carapace^[3, 19, 21], in this study, measurements of different parts of *Orthochirus irannus* showed that the size of the telson is very small in relation to body length. Because of the morphological characteristics differences in *Orthochirus sp.* created a separate subfamily Orthochirinae for this genus^[8]. So far, the speculation about the classification of the Scorpions was based on morphology^[7, 12]. Trichobothrium pattern was one of the morphological characteristics that important in identification of scorpions^[16], but in the *Orthochirus irannus*, trichobothrium pattern was not a constant factor for species determination. Trichobothrium *d2* of pedipalp femur in some specimen absent and some of them fully developed on dorsal surface. This character was not related to special region or sexual dimorphism^[13]. Due to the complexity of the taxonomic position of *Orthochirus iranus* in this study, the morphometry and phylogenic of this scorpion were studied. According to the result of measurements with regard to sex of *Orthochirus irannus* showed that the size of the female was larger than the male ($P < 0.05$). The fourth segment of metasoma in male was longer and wider than female ($P < 0.05$). Statistical analysis of morphological index had showed considerable morphological conservation for *Orthochirus irannus* exception forth segment of metasoma MTIVL/H and MTIVL/W in male larger than females ($P < 0.05$).

Molecular phylogeny methods could overcome these problems by combining DNA sequencing information in different specimens and allowing the derivation of the entire gene tree to be determined^[2, 5, 15]. Although morphological diversity has created challenges in their phylogeny study, phylogeny hypotheses and many classification schemes have been suggested to them. The preliminary phylogeny of Buthidae based on the 16S rRNA gene showed that genus *Anomalobuthus* and *Orthochirus sp* genus are phylogenetically close. Highly derived positions of *Orthochirus sp.* will require creation of subfamilies to accommodate monophyly for other clades of Buthidae^[2, 11]. However, considerable morphological conservation was apparent for, *Orthochirus irannus*, statistical analysis of morphological features was consistent with the possible phylogenetic. The 16SrRNA gene fragment of *Orthochirus iranus* formed a cluster relative to the only scorpion of *Orthochirus innesi* from Morocco. The highest and lowest genetic distances with *Orthochirus iranus* were found with the *Hottentotta gentili* and *Buthus draa* respectively.

Phylogenic analysis on genera of buthidae based on the mitochondrial cytochrome oxidase showed that *Orthochirus bicolor* had maximum similarity with *Androctonus mauretanicus* and *Odontobuthus bidentatus* and this study showed that *Orthochirus sp.* was nested within the larger clade of buthidae comprising genera^[18].

The phylogenetic analysis and sequence divergence data presented here based on 16SrRNA sequence data indicate trichobothrium pattern was not an appropriate indicator for taxonomic classification for *Orthochirus iranus* and molecular analysis of species with the determination of morphological characteristics was more appropriate in determining the taxonomic position of species.

5. Conclusion

The present study concludes that metasoma and fourth segment in *Orthochirus iranus* had a key role in identifying species and forth segment of metasoma MTIVL/H and MTIVL/W in male larger than females ($P < 0.05$). Phylogenetic analysis by 16srRNA showed that highest level of identity was scored with *Leiurus quinquestriatus*, *Androctonus australis*, *Buthus draa*. The gene fragment of *O. iranus* formed a cluster relative to the only scorpion of *Orthochirus sp.* from morocco, with a good bootstrap score, *Orthochirus iranus* isolated from the Khuzestan province of Iran are closely related, as they clustered together as Cluster B. The genetic distances of *O. iranus* ranged from 6 to 21% and the lowest interspecific distance was between *O. iranus* and *Osp* (6%). Although the sample size is not large enough to draw a final conclusion, but the percentage of sequence divergence was high enough for interspecific comparisons to provide separation of species.

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7. References

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