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A preliminary molecular phylogeny of the genus *Pholcus* in Iran, with notes on taxonomy

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Abstract
Iran is a large country with diverse and unique climate and ecology; therefore, it is expected to discover an exceptional fauna with high species diversity by carefully examining the unknown areas. A few taxonomic studies have been so far conducted on the genus *Pholcus* in Iran. Taxonomic and preliminary phylogenetic evaluation of widespread species of the genus *Pholcus* from Iran is considered in the present study, based on specimens collected from northern and southwestern parts of the country. A molecular study was undertaken on some representatives of species of the *Pholcus phalangioides* species-group (cellar spiders) using newly designed primers with 350 bp of partial fragments of mtDNA gene, cytochrome oxidase subunit 1 (COI). These preliminary molecular data in line with morphological identifications using characters related to the copulatory organs presented a total of five distinct clades of *Pholcus* that four clades were contributed with formerly identified species and one represented a distinct lineage unknown for science.

Key words: *Species delimitation; DNA-based; CO1; morphology; Iran; Pholcus.*

INTRODUCTION

Pholcidae C. L. Koch, 1850 is one of the most species-rich spider families in the world (World Spider Catalog, 2021). This family, known as daddy long leg spiders, is distributed in a wide variety of habitats including urban areas, dark and humid places (e.g., caves, under rocks and in trees hole) (Huber, 2005). The genus *Pholcus* Walckenaer, 1805 with 329 species is the largest genus in this family (World Spider Catalog, 2021). Some of the species of *Pholcus* are either cosmopolitan or synanthropic species (e.g., *Pholcus phalangioides* (Fuesslin, 1775), *Ph. alticeps* Spassky, 1932 (Huber, 2013).

Iran is a country that its biodiversity is not well explored. In addition, due to climate diversity and topology (Amiri & Eslamian, 2010), a significant cryptic and undiscovered biodiversity is expected for the region. In general, study about spiders are limited in Iran, so that most study have been recently conducted on this widly distributed group (Zamani et al., 2021; Mirshamsi,2005). To date, 13 species of *Pholcus* have been reported from Iran, of which twelve were defined as new species or new records by Senglet (1974, 2008). Another species, recorded by Roewer (1955) as *Pholcus opilionoides* (Schrank, 1781), was probably a misidentification (Zamani et al., 2021). Huber (2011) partially revised the Iranian species and classified the listed species into four species-groups. The majority of the list consist of closely related species placed in the *Pholcus phalangioides* species-group. This group [except for the two widely distributed species *Ph. phalangioides* and *Ph. alticeps*] is largely restricted to Iran, and includes: *Ph.*

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armeniacus Senglet, 1974, *Ph. caspius* Senglet, 2008, *Ph. elymaeus* Senglet, 2008, *Ph. hyrcanus* Senglet, 1974, *Ph. hystaspus* Senglet, 2008, *Ph. medicus* Senglet, 1974 and, *Ph. persicus* Senglet, 1974.

Molecular data, in particular mtDNA markers has been applied from a couple of decades ago to in taxonomy of spiders and other arachnids (Barrett & Hebert, 2005). The barcode region of mtDNA, cytochrome oxidase 1 (COI), are being widely used in molecular identification, in combination with morphological data, to reconstruct the phylogeny of Pholcidae (Astrin et al., 2006; Dimitrov et al., 2013; Huber et al., 2018; Wang et al., 2018).

Some of the species of the genus *Pholcus*, recently described by Senglet (1974, 2008) from Iran, were based on a few specimens that showed subtle difference in their genital structure and its appendages such as procursus. Senglet (1974) did not access to a grand collection of the *Pholcus* from Iran and his finding were mainly based on the collection he gathered through his extrusion in 1973 from Iran. Therefore, it can be concluded that some species might be wrongly described or recorded from the region and a taxonomic revision using both, molecular and morphological method is demanded to discover the real taxonomy and phylogenetic relationship of the genus in the region. By applying a novel set of primers, we used the barcoding region (CO1) to get a preliminary phylogenetic perspective and DNA-based species delimitation of the Iranian representative of the genus. In addition. Morphological characters were examined to find out if the molecular finding are in line with morphological differences. In addition, intraspecific variability of the cosmopolitan species *Ph. phalangioides* was also considered.

MATERIAL AND METHODS

Morphological study

Specimen were collected from various habitats, including caves in northern and southwestern Iran, and were preserved in ethanol. Some specimens from Georgia were also added to the study. Two to four legs of each specimen were kept in absolute ethanol for molecular studies. The vouchers and tissue samples were deposited in the Zoological Museum, University of Isfahan (ZMUI). The taxonomic characters (male palp and procursus) of vouchers were examined using a stereomicroscope equipped with drawing tube. The morphological identification was based on Huber (2011).

Molecular analyses for species delimitation

Specimens used in the molecular analyses are listed in Table 1. Twenty specimens of five morphospecies were used for molecular examination. DNA was extracted from legs using Animal DNA Isolation Kit (DENA ZIST). Partial fragment of the gene cytochrome oxidase subunit 1 (CO1) was amplified using a couple of primer pairs; a newly designed for this study: Hcophol (5-AGTAAARTARGCDCGWGTRTCTACA-3) and Lcophol (5-GATATAGCTTTTCCTCGWATRAATA-3), and also C1j1718 (5-GGAGGATTTGGAAATTGATTAGTTCC-3) and C1N2191 (5-CCTGGWARAATYARAATATAHACTTC-3) (Simon et al., 1994). For PCR reaction in a total volume of 25 µl, 12 µl Red master mix (DENA ZIST), 5 µl non-diluted DNA, 1_1.25 µl primers and 5_6 µl dH_2O were used. The PCR program consist of one cycle with 36 repeats as follows: 45s denaturation at 95˚C, 60s annealing at 51ºC and 60s extension at 72ºC. The PCR products outsourced to Macrogen, South Korea and partial fragment of CO1 with readable sequences (i.e. 350 bp), was amplified (see Table 1).

Some sequences were retrieved from the GenBank (Table 1) for achievement of a better phylogenetic conclusion. Sequences were aligned and corrected manually using BioEdit ver7.2.5 (Hall, 1999). The Bayesian inference (BI) and maximum likelihood (ML) analyses were conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and RaxmlGUI 1.3 (Silvestro & Michalak, 2012), respectively. The best evolutionary model was estimated (K81uf+I), according to the Akaike's criterion with JMODELTEST 2.1.4, (Darriba et al., 2012). The BI was conducted with four Markov Chain Monte Carlo (MCMC) were run for = 10 000 000 generations, printing trees every 1000 generations. The first 25 percent of the total trees were excluded (burn-in phase) from the analysis. The ML trees were inferred from 1,000 bootstrap iterations. *Crossopriza* sp. was used as out-group for tree reconstruction.

The haplotype network for *Ph. phalangioides* was constructed using Pop ART ver1.7 (Leigh & Bryant, 2015) to show intraspecific variability the species.

TABLE1. Collected specimens used for DNA sequencing with locality, coordinates, GenBank accession number and collection number.

Species	Number	Locality	Geographic coordinates	Gen Bank Accession number	Voucher number
Ph. phalangioides	1♀	Iran, Mazandran Province- Noor	36.574º N 52.026° E	OQ872153	SD26
Ph. phalangioides	3♀	Iran, Mazandran Province- Nashtarood	36.749° N 51.015° E	OQ872153	SD82
Ph. phalangioides	1♀	Iran, Golestan Province- Alang dareh	36.791° N 54.451º E	OQ872153	SD71
Ph. alticeps	2♂, 1♀	Iran, Golestan Province- Tangerah	37.398º N 55.780º E	OQ872157	SD54
Ph. alticeps	1♀	Iran, Golestan Province- Alang dareh	36.791° N 54.451º E	OQ872157	SD72
Ph. alticeps	1♀	Iran, Golestan Province- Naharkhoran	36.681º N 52.459° E	OQ872157	SD73
Ph. alticeps	1♂, 3♀	Iran, Khorasan Razavi Province- Neyshabore	36.243° N 58.968° E	OQ872158	SD81
Ph. alticeps	1♂, 1♀	Georgia- Oni	42.577° N 43.434° E	OQ872157	SD86
Ph. persicus	1♂, 1♀	Iran, Kohgilluye va Boyer-Ahmad Province- Ankaboot cave	30.723° N 50.845º E	OQ872154	SD29
Ph. persicus	1♀	Iran, Kohgilluye va Boyer-Ahmad Province- Patave cave	30.957º N 51.264° E	OQ872159	SD90
Ph. cf medicus	1♀	Iran, Lorestan Province- Alashtar, Bozorg cave	33.943º N 48.315º E	OQ872155	SD33
Pholcus sp.	1♀	Iran, Fars Province- Palangan cave	35.090º N 46.602º E	OQ872156	SD40
Ph. phalangioides	-	GERMANY, Bonn, ZFMK, basement x. 2003 (J.J. Astrin)	-	DQ667926	pb05-G100
Ph. phalangioides	-	GERMANY, Bonn, ZFMK, basement ii. 2002 (B.A. Huber)	-	DQ667923	pb05-G55
Ph. phalangioides	-	China	-	MH382632	PH006
Ph. phalangioides		USA		EF537067	G526
Ph. phalangioides	-	Burgos, Spain	-	EU215671	CCRUB phalB
Ph. phalangioides	-	China	-	KY467210	PPH1
Ph. phalangioides	-	China	-	KY467211	PHH2
Ph. phalangioides	-	New Zealand	-	KF477299	Isolste9795
Ph. phalangioides	-	SPAIN, Teulada, Moraira, indoors xii. 2003 (J.J. Astrin)	-	DQ667921	pb05-J271
Ph. phalangioides	-	China	-	KY467212	PPH3
Ph. phalangioides	-	Germany	-	KY269460	ZFMK
Ph. phalangioides	-	Near Barcelona, Spain	-	EU215670	CCRUB phalC
Ph. phalangioides	-	USA, SC, Pickens Co., Clemson University iii. 2001 (W. Reeves)	-	DQ667922	pb05-G52
Ph. phalangioides	-	BRAZIL, Est. Alto da Serra xii. 2003 (B.A. Huber)	-	DQ667919	pb05-B26
Ph. phalangioides	-	South Korea	-	JN817071	LEGO5-2
Ph. phalangioides	-	MADEIRA/PORTUGAL, São Vicente, Laranjal ii. 2003 (J.J. Astrin)	-	DQ667924	pb05-J17
Ph. phalangioides	-	MADEIRA/PORTUGAL, São Vicente, Laranjal ii. 2003 (J.J. Astrin)	-	DQ667925	pb05-J17
Ph. alticeps	-	Germany	-	KY269003	ZFMK
Crossopriza lyoni	-	Maharashtra, India	-	KT383729	-

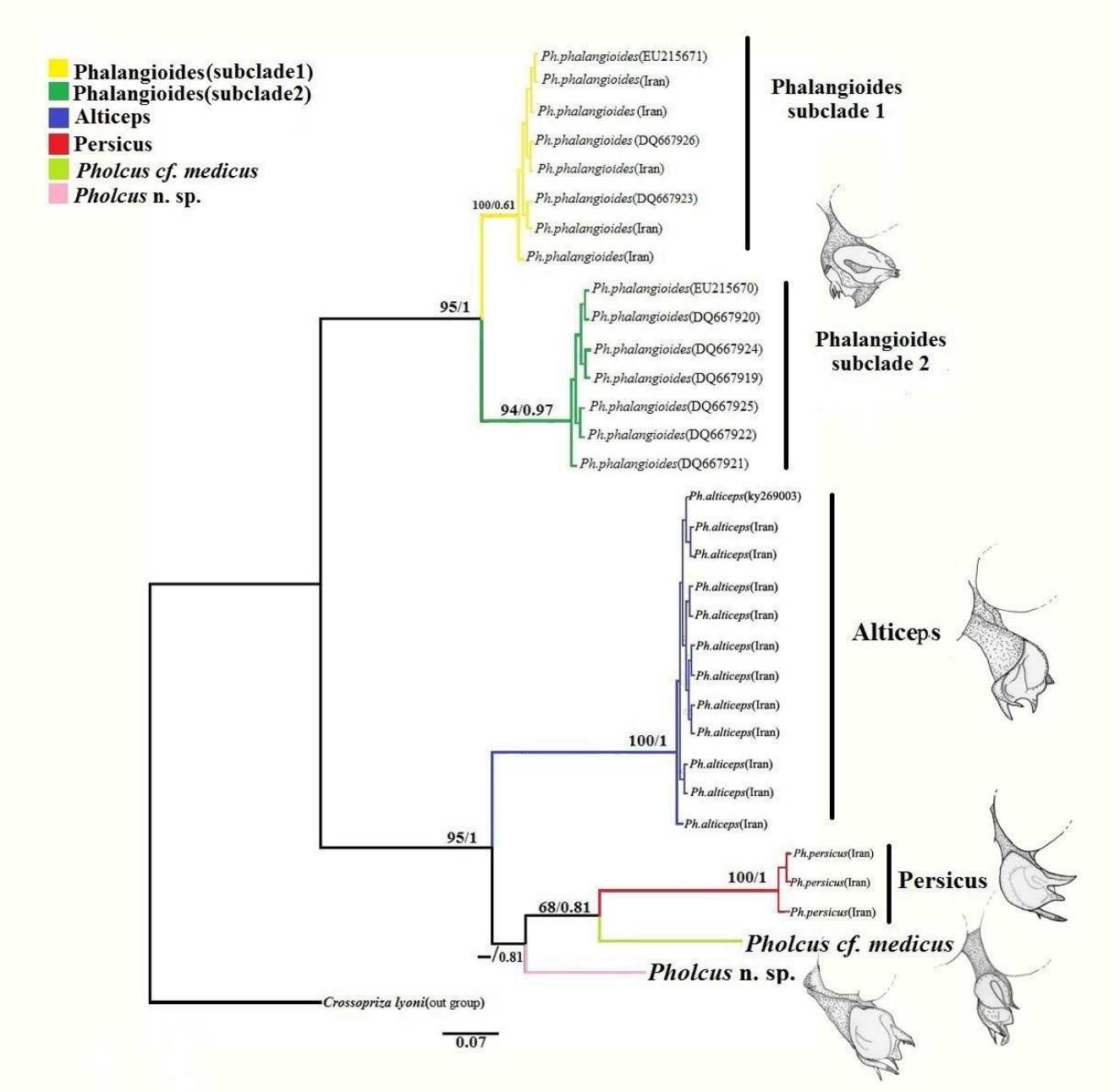
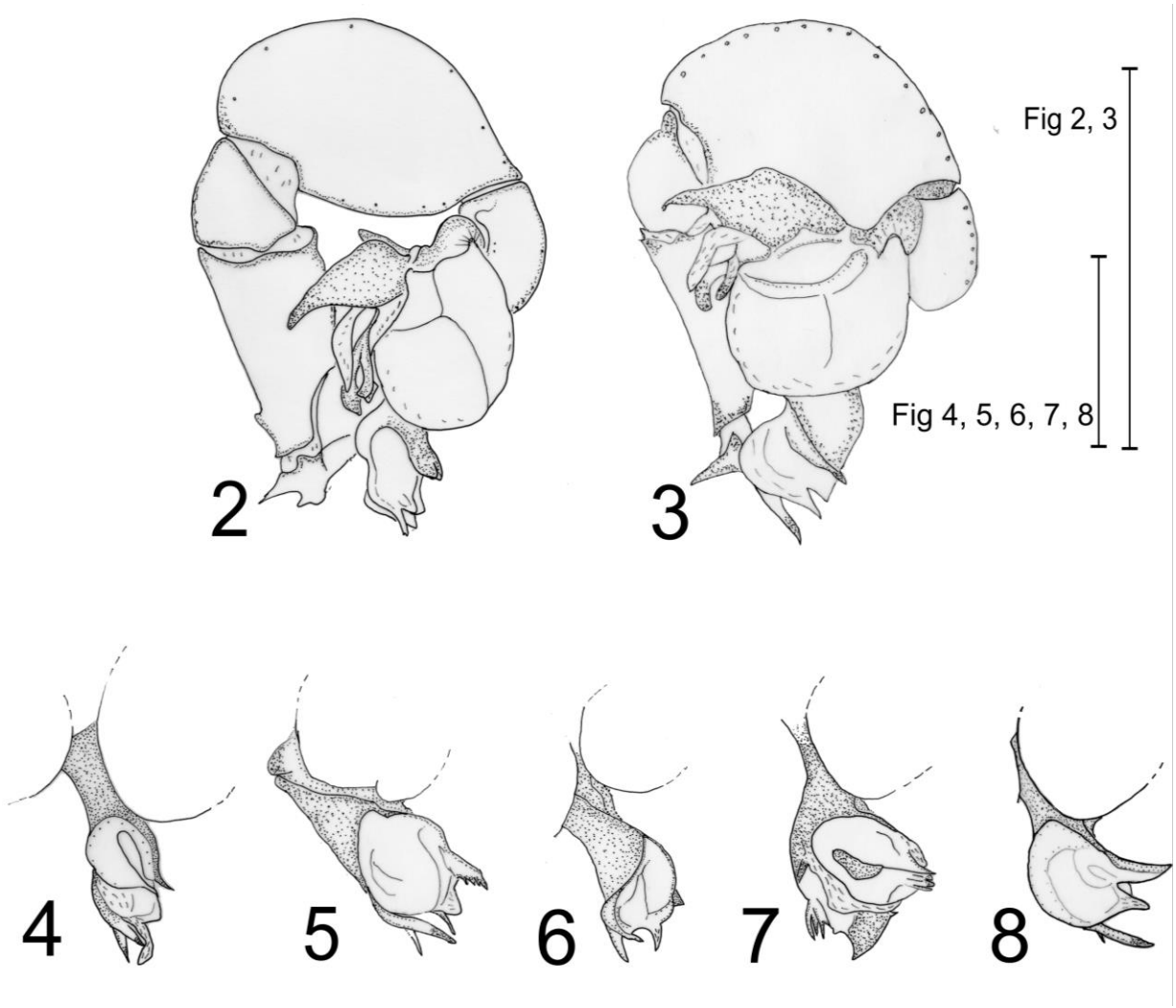


FIGURE 1. Phylogenetic reconstruction of the genus *Pholcus*, based on two mtDNA. Node values show bootstrap values and posterior probabilities from Bayesian and maximum likelihood analyses, respectively.

RESULTS

A phylogenetic tree assigned all specimens into five different clades (Figure 1) including: phalangioides, alticeps, persicus, pholcus sp., and medicus. The phalangioides clade is well supported statistically and holds a sister clade position to the other ingroups. This clade includes all specimens identified as *Ph. phalangioides* and consists of two subclades: subclade 1 contains specimens from Iran and Europe and subclade 2 consists of specimens from Europe, North and South America.



FIGURES 2-8. Prolateral view of the left male palps (2, 3) and prodorsal view of the procursus (4–8) of the *Pholcus* species from Iran. 2. *Pholcus* cf. *medicus*; 3. *Pholcus persicus*; 4. *Pholcus* cf. *medicus*; 5. *Pholcus* sp.; 6. *Pholcus alticeps*; 7. *Pholcus phalangioides*; 8. *Pholcus persicus*. Scale bars = 1 mm and 0.5 mm.

The second group includes four clades, of which the clade *alticeps* (Iran and Georgia) is well supported and the rest of distinctly separated clades belong to three endemic species from southwest of Iran including *Pholcus* cf. *medicus*, *Pholcus* sp. and *Ph. persicus*. In contrast to *Ph. phalangioides*, and *Ph. alticeps,* the other three species were collected from caves. *Pholcus* cf. *medicus* is relatively well supported group (since the value for posterior probability is low) as a sister to *persicus* clade which is a well-supported clade with noticeable intraspecific diversity. *Pholcus* sp. is a genetically distinct clade and holds a basal position to the clade including *Pholcus* cf. *medicus* and *Ph. persicus* with low supporting values which prompts for wider sampling. The molecular data corresponds finely with morphological identifications using characters of copulatory organs. Examining through different details of the male palp lead us to plot the prolateralo-dorsal view of procursus to identify different species. Five different morphospecies were identified and assigned to four previously known and probably one unknown *Pholcus* species for science (see Figure 2_8).

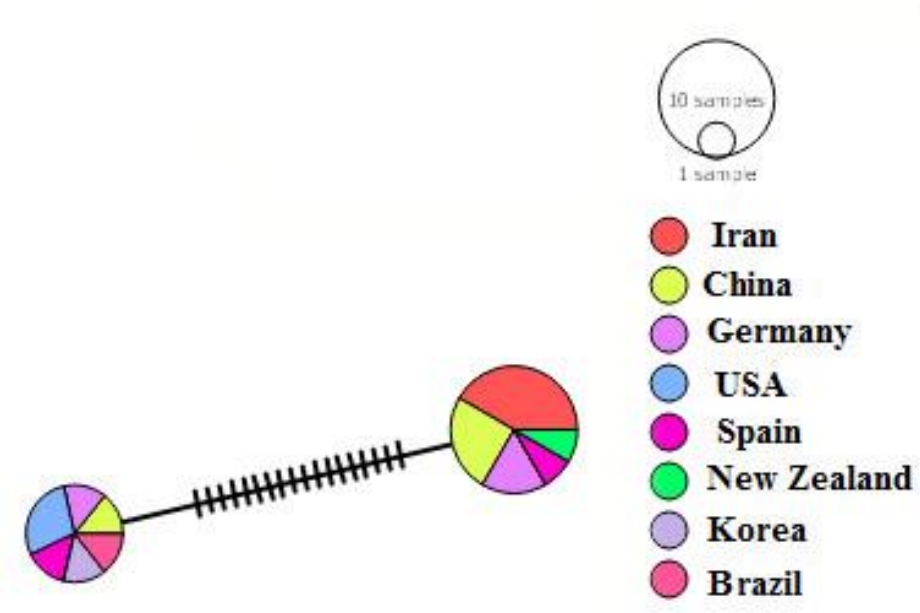


FIGURE 9. Haplotype networks of *Pholcus phalangioides*. Each line represents one substitution, and crossed lines indicating unsampled haplotypes. The circle size is associated with number of individuals in each haplotype.

A statistical parsimony network used to show intraspecific variation in *Ph. phalangioides*. The network (Figure 9) belongs to the cosmopolitan species; *Ph. phalangioides*, was defined by the existence of two main haplotypes with clear genetic partitioning between them. The distance between these two groups of *Ph. phalangioides* was recovered as 4.6%, more than the intraspecific p-distance recovered for other *Pholcus* species, (*Ph. pingtung* from Taiwan ranges between 2.9 and 3.1%; see Huber & Dimitrov, 2014).

DISSCUSSION

Despite the fact that *Pholcus* is the most specious genus of Pholcidae, studies on its taxonomy and phylogeny, particularly in the Middle East region, are limited. Additionally, the pholcid barcode sequence library is totally missing from Iran and other neighbor regions of the Middle East (Huber, per. comm.). As an example, in the main phylogenetic studies on the genus *Pholcus* (Dimitrov et al., 2013; Huber et al., 2018), just a single member of *Pholcus phalangioides* species group was included.

This study is a preliminary attempt to provide the first molecular data for *Pholcus phalangioides* species-group from Iran. Importantly, its molecular findings are in agreement with morphological studies on male characters. Phylogenetic results along with the pattern of haplotype network of *Ph. phalangioides* is probably due to synanthropic lifestyle of the species and its human-mediated dispersal (Astrin et al., 2006; Huber, 2011; Huber et al., 2018). However, a thorough study on the group with the aid of more molecular markers from an extensive sampling in its distribution range is necessary in the future studies.

Our probably undescribed species is collected from the southeastern region of the Zagros Mountains in Fars Province. Further examination and wider sampling is demanded to show the distribution and describe the real morphological and molecular diversity of the undescribed lineage. According to studies, west of Iran is known as a section of the Irano-Anatolian biodiversity hotspot as the Zagros Mountains is considered as a 20th global hotspot sector (Mittermeier *et al*., 2005; Kazemi et al., 2020). Such high degree of endemism and diversity is associated with heterogeneity of the environment, the complexity of the topography and the range of height of the mountain ranges (Frisch, 2006; Noroozi et al., 2008; Gholamifard, 2011; Esmaeili et al., 2016 & Noroozi et al., 2018). Some of the *Pholcus* species described by Senglet (1974, 2008), are morphologically extremely similar to each other. He ddditionally relied on a single specimen for new species' description (e.g. Senglet, 1973). Therefore, an

integrative analysis of the genus, for example by including molecular data, may discover the real diversity and additionally shed light on the evolutionary history and speciation processes of the genus in the Persian plateau.

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