Available online at scholarcommons.usf.edu/ijis

International Journal of Speleology
Official Journal of Union Internationale de Spéléologie

Molecular phylogeny of cave dwelling *Eremogryllodes* crickets (Orthoptera, Myrmecophilidae) across Zagros Mountains and Southern Iran

Mohadeseh S. Tahami ¹, Mina Hojat-Ansari ², Anna Namyatova ³,
and Saber Sadeghi ^{1*}

¹Entomology Research Laboratory, Biology Department, Adabiat St., Shiraz University, 7146713565, Shiraz, Iran

²Gastroenterohepatology Research Center, Shiraz University of Medical Sciences, Khalili St., Shiraz, Iran

³Chromas Research Resource Center, Saint Petersburg State University, Oranienbaumskoye sh. 2, 198504, Saint Petersburg, Russia

Abstract: Recently, several new species and subspecies from the genus *Eremogryllodes* Chopard, 1929 (Insecta: Orthoptera: Myrmecophilidae) inhabiting caves of Iran, have been described based on morphology. The high variation of genitalia structure along with high similarity of external morphology between populations hamper the precise species identification. Thus, molecular approaches are critical to determine the taxo-nomic positions of species/subspecies of this genus. Here we provide the molecular phylogeny, based on the 16S rRNA mitochondrial gene, of recently described species of *Eremogryllodes* along with some uni-identified specimens from the same region. The results support the monophyly of the family Myrmecophilidae. The topology of the 16S rRNA tree did not completely support the five morpho-species. The three main recovered clades mainly grouped specimens by their geographical locations. Our study suggests the possibility of more than one species in one cave and the presence of cryptic species among cave dwelling crickets, based on the 16S rRNA marker.

Keywords: Iran, Orthoptera, cave crickets, *Eremogryllodes*, 16S rRNA, phylogeny

Received 17 October 2020; Revised 9 June 2021; Accepted 9 June 2021

Citation: Tahami, M.S., Hojat-Ansari, M., Namyatova, A., Sadeghi S., 2021. Molecular phylogeny of cave dwelling *Eremogryllodes* crickets (Orthoptera, Myrmecophilidae) across Zagros Mountains and Southern Iran. International Journal of Speleology, 50(2), 213-221.
<https://doi.org/10.5038/1827-806X.50.2.2360>

INTRODUCTION

Myrmecophilidae (Insecta: Orthoptera), or ant-loving crickets, together with the mole crickets, Gryllotalpidae, are located in the superfamily Gryllotalpoidea. This family is divided into two subfamilies, Myrmecophilinae and Bothriophylacinae. *Eremogryllodes* is the genus inside Bothriophylacinae, tribe Bothriophylacini, with 11 described species (Chopard 1948, 1968; Gorochoy 1979, 1980; Cigliano et al., 2020). The taxonomic position of *Eremogryllodes* has been under discussion for a long time. It was placed inside the family Gryllidae, between Gryllomorphinae and Phalangopsinae (Chopard, 1929), at first, but later its position was considered between Myrmecophilinae and Mogoplistinae (still inside Gryllidae) (Chopard, 1934), based on superficial similarities. Later on, the genus was placed in Mogoplistinae until it was eventually resurrected in Myrmecophilidae: Bothriophylacinae: Bothriophylacini (Tahami et al., 2017). Its latest taxonomical position was based

on similarities of specialized features such as the structure of the epiphallus and more developed plates of ecto- and endoparameral apodemes, with the other genus of this tribe, *Bothriophylax* (Tahami et al., 2017).

Eremogryllodes is a poorly-known genus of Myrmecophilidae and it is only reported from North Africa, southwest and Central Asia. Until recently, no substantial review of *Eremogryllodes* and its subfamily, Bothriophylacinae was available (Tahami et al., 2017). Members of this genus mostly inhabit caves and burrows of rodents and reptiles. They have a miniature body with milky to white colour that perfectly camouflages the crickets on the cave walls. These morphological characteristics along with its cryptic lifestyle hamper the collection of the specimens which may be why the genus has not received considerable attention by taxonomists and the true geographic range of *Eremogryllodes* remains unclear. Recently, six new species and three subspecies of genus *Eremogryllodes* from caves

*ssadeghi@shirazu.ac.ir

of Iran were described (Tahami et al., 2017, 2018). All recorded populations have been collected inside caves, e.g., cave walls, arch, and crevices, near the entrance to the furthest middle zone of the cave, sometimes referred as parahypogean zone (Prous et al., 2004; Tahami et al., 2016). There are no data on the life cycle of *Eremogrylodes* species and other biological aspects of their life however, we assume that the populations of Iranian *Eremogrylodes* are very restricted to their natural habitats, as we didn't encounter individuals outside the cave environment during our sampling. The close opposition and the lack of hybrid zone between cave dwelling populations has been extensively discussed in Hubell and Norton (1978). Even when leaving caves at nights, crickets' spatial distribution is mainly limited to feeding or mating in close distance to their habitat (Hubell & Norton, 1978; Carchini et al., 1995). These characteristics specific to cavernicolous crickets can hinder gene exchange between populations. However, more studies need to be carried out to confirm this assumption for cave-dwelling *Eremogrylodes* taxa.

Organisms living in caves with similar environmental factors, such as darkness, high humidity, stable temperature range throughout the year, and poor food resources, undergo convergent evolution (Culver et al., 1995; Culver & Pipan, 2009a). This phenomenon is also resonated in cave dwelling populations of *Eremogrylodes* meaning that they exhibit highly similar external morphology to each other. This feature along with their high variation of genitalic features within and between populations have made any attempt of accurate species delineation difficult. Therefore, molecular studies are essential to more reliably delineate species.

Until now, only the genus *Myrmecophilus* has been included in all phylogenetic studies of Ensifera,

as the representative of family Myrmecophilidae (Zhou et al., 2010; Song et al., 2015; Chintauan-Marquier et al., 2016; Zhou et al., 2017). In the current research, we had the opportunity to include sequences of *Eremogrylodes* populations for the first time from caves of Iran. We aim to test the relationship between the two subfamilies of Myrmecophilidae; *Myrmecophilus* as the representative of Myrmecophilinae, and *Eremogrylodes* as the representative of Bothriophylacinae. We base our current research on species described in Tahami et al. (2017, 2018), collected by the first author (MT) in the Iranian Zagros Mountains and a part of southern Iran using 16S mitochondrial gene. The mitochondrial 16S rRNA gene is considered to be informative for closely related cricket taxa (Taylan et al., 2013; Taylan & Şirin, 2016). Therefore, the current study has three main aims: (1) to test whether the described morpho-species correspond to the molecular evidence, (2) to test for the presence of cryptic species and (3) to test the monophyly of Myrmecophilidae.

MATERIAL AND METHODS

Sample collection

A vast and extensive cave survey was carried out throughout the entire Zagros ranges and some provinces in the south of Iran. Specimens were collected by fine brush, hand, and aspirator and were preserved in absolute ethanol for molecular studies. In total, 56 caves were investigated and *Eremogrylodes* specimens were collected from 32 caves. A distribution map of the caves is given in Fig. 1. We aimed to amplify 16S mitochondrial rRNA gene region for representatives of five out of the six species described in Tahami et al. (2017, 2018): *Eremogrylodes dilutus*, *E. iranicus*, *E. persicus*, *E. bifurcatus* and *E.*

Fig. 1. Distribution map of the cave localities for sampled *Eremogrylodes* specimens, the map includes Zagros Mountain ranges and two provinces in the south of Iran (Yazd and Kerman provinces).

spinulatus. Furthermore, we included 20 immature specimens from Sarjosh, Zakariya, Ashkan, Gakal, Abu Nasr, Rayan, Yaran, and Chah Doozakhi caves. Although the attempt of specimen collection for those caves was made more than once and at different times, we were not able to find mature males. In some cases, we captured males that seemed to be mature judging from their size, but abdomens were empty of any mating apparatus. The developmental mechanisms of this morphological peculiarities is not clear however, it is possible that in these populations, the sub-adult stage is rather long (longer than one year) as is seen

in cave-dwelling Dolichopoda species (Di Russo, 1987; Carchini et al., 1994). A total of 48 specimens of *Eremogrylodes* were subjected to 16S rRNA studies. To check the monophyly of the genus *Eremogrylodes* and its family, Myrmecophilidae, we included sequences of Myrmecophilidae (*Myrmecophilus*), Gryllotalpidae (*Grylloyalpa*), Mogolistidae (*Ornebius*), and Gryllidae (*Gryllus*) along with *Melnopus sanguinipes* (Acrididae) as a distant outgroup available in Genbank (www.ncbi.nlm.nih.gov). Table 1 and [Supplementary Table S1](#) provide the accession numbers of all sequences included in the analysis.

Table 1. Profile of *Eremogrylodes* samples used in this study with their Genbank accession numbers for 16S rRNA.

No.	Voucher Code	Accession Code-16S	Morphological species attribution	Cave	Locality
1	M1994	MH266701	<i>Eremogrylodes dilutus</i>	Balezar	Fars Prov.
2	M1627	MH266702	<i>Eremogrylodes dilutus</i>	Ab Konardoan	Khuzestan Prov.
3	M1965	MH266703	<i>Eremogrylodes dilutus</i>	Ab Konardoan	Khuzestan Prov.
4	M1990	MH266704	<i>Eremogrylodes dilutus</i>	Ab Konardoan	Khuzestan Prov.
5	M1628	MH266705	<i>Eremogrylodes dilutus</i>	Deh Sheykh	Kohgiluyeh & Boyer-Ahmad Prov.
6	M19105	MH266706	<i>Eremogrylodes dilutus</i>	Deh Sheykh	Kohgiluyeh & Boyer-Ahmad Prov.
7	M1995	MH266707	<i>Eremogrylodes dilutus</i>	Deh Sheykh	Kohgiluyeh & Boyer-Ahmad Prov.
8	M1724	MH266708	<i>Eremogrylodes dilutus</i>	Dasht-e Shir	Khuzestan Prov.
9	M19101	MH266709	<i>Eremogrylodes dilutus</i>	Ker Palang	Khuzestan Prov.
10	M1624	MH266710	<i>Eremogrylodes dilutus</i>	Pebdeh	Khuzestan Prov.
11	M1629	MH266711	<i>Eremogrylodes dilutus</i>	Sarab	Ilam Prov.
12	M1562	MH266712	<i>Eremogrylodes dilutus</i>	Momtaz	Fars Prov.
13	M1561	MH266713	<i>Eremogrylodes iranicus</i>	Khane Khoda	Yazd Prov.
14	M1744	MH266714	<i>Eremogrylodes iranicus</i>	Khane Khoda	Yazd Prov.
15	M1720	MH266715	<i>Eremogrylodes iranicus</i>	Ab Kamouneh	Fars Prov.
16	M1625	MH266716	<i>Eremogrylodes iranicus</i>	Malousjan	Fars Prov.
17	M1997	MH266717	<i>Eremogrylodes iranicus</i>	Malousjan	Fars Prov.
18	M1938	MH266718	<i>Eremogrylodes bifurcatus</i>	Gelim Goosh	Kermanshah Prov.
19	M1979	MH266719	<i>Eremogrylodes bifurcatus</i>	Botkhane	Kermanshah Prov.
20	M1982	MH266720	<i>Eremogrylodes bifurcatus</i>	Pirmorad	Lorestan Prov.
21	M1943	MH266721	<i>Eremogrylodes bifurcatus</i>	Kerend	Kermanshah Prov.
22	M1626	MH266722	<i>Eremogrylodes persicus</i>	Tadovan	Fars Prov.
23	M02	MH266724	<i>Eremogrylodes sp.</i>	Zakariya	Fars Prov.
24	M1680	MH266725	<i>Eremogrylodes sp.</i>	Zakariya	Fars Prov.
25	M1987	MH266732	<i>Eremogrylodes sp.</i>	Zakariya	Fars Prov.
26	M19104	MH266729	<i>Eremogrylodes sp.</i>	Zakariya	Fars Prov.
27	M1681	MH266726	<i>Eremogrylodes sp.</i>	Ashkan	Fars Prov.
28	M1985	MH266731	<i>Eremogrylodes sp.</i>	Ashkan	Fars Prov.
29	M1950	MH266730	<i>Eremogrylodes sp.</i>	Rayan	Fars Prov.
30	M1991	MH266733	<i>Eremogrylodes spinulatus</i>	Chah Doozakhi	Fars Prov.
31	M1998	MH266736	<i>Eremogrylodes spinulatus</i>	Chah Doozakhi	Fars Prov.
32	M19100	MH266728	<i>Eremogrylodes spinulatus</i>	Chah Doozakhi	Fars Prov.
33	M1996	MH266735	<i>Eremogrylodes sp.</i>	Gakal	Kohgiluyeh & Boyer-Ahmad Prov.
34	M1999	MH266737	<i>Eremogrylodes sp.</i>	Gakal	Kohgiluyeh & Boyer-Ahmad Prov.
35	M1980	MH347906	<i>Eremogrylodes iranicus</i>	Sahlak	Fars Prov.
36	M1972	MH347907	<i>Eremogrylodes iranicus</i>	Sahlak	Fars Prov.
37	M1947	MH347908	<i>Eremogrylodes bifurcatus</i>	Tang-e_Lor	Lorestan Prov.
38	M1948	MH347909	<i>Eremogrylodes bifurcatus</i>	Tang-e_Lor	Lorestan Prov.
39	M1719	MH266723	<i>Eremogrylodes persicus lari</i>	Khan	Fars Prov.
40	M1958	MH347910	<i>Eremogrylodes persicus lari</i>	Khan	Fars Prov.
41	M1722	MH347911	<i>Eremogrylodes dilutus</i>	Shekam_Kooseh	Khuzestan Prov.

42	M1945	MH347912	<i>Eremogryllodes dilutus</i>	Palangan	Fars Prov.
43	M1934	MH347913	<i>Eremogryllodes dilutus</i>	Palangan	Fars Prov.
44	M1957	MH347914	<i>Eremogryllodes persicus torangae</i>	Torang	Kerman Prov.
45	M1952	MH347915	<i>Eremogryllodes sp.</i>	Yaran	Kerman Prov.
46	M1954	MH347916	<i>Eremogryllodes sp.</i>	Sarjoshier	Khuzestan Prov.
47	M1955	MH347917	<i>Eremogryllodes sp.</i>	Sarjoshier	Khuzestan Prov.
48	M1992	MH266734	<i>Eremogryllodes sp.</i>	Abu Nasr	Fars Prov.

DNA extraction and sequencing technique

DNA was extracted from the hind leg using a local animal extraction kit (Dena Zist Asia, Mashhad, Iran), and Qiagen DNeasy DNA Blood & Tissue Kit (QUIAGEN). For amplification, we used PCR protocol according to Robillard and Desutter-Grandcolas (2006) with some modifications of annealing temperature and cycle duration (Table 2). We used primers specifically designed for *Myrmecophilus* 16S rRNA fragment (Robillard and Desutter-Grandcolas 2006). Generally, a 388–431 base pairs (bps) fragment of the mitochondrial 16S rRNA was amplified through the polymerase chain reaction (PCR) by Bioer, XP cyler (BIOER), and Bio-RAD MjMini thermal cyler (Bio-RAD). Amplification was performed in the 25- μ l volume containing 0.5–1 μ l of each primer (100 mM), 3–5 μ l DNA template of each

sample, and 12.5 μ l of 2X PCR Master Mix, Genetbio, Cat. no. G-2000 (including dNTP, MgCl₂, PCR buffer and Taq DNA polymerase). An initial duration or equal 5–10 min at 94°C was applied. A positive control was added in each PCR run to check for possible errors. Double-stranded amplified DNAs were checked for the presence and quality of the desired gene segment using 1% agarose gel electrophoresis. PCR products were initially sent for single-strand sequencing with the forward primer, however, the reverse strands were also sequenced to clarify the observed ambiguous sites in some samples. PCR purification and sequencing were carried out in MacroGen (Korea), Research Resource Center Molecular and Cell Technologies (St. Petersburg State University, Russia) and Dr. Faghihi genetic lab (Bayan Gen Pars Institute, Shiraz- Iran).

Table 2. PCR protocol and primers used for 16S rRNA marker.

Primer ¹	Sequence (5'–3')	Denaturation	Annealing	Cycles	Final Elongation
16SAG	CGCCTGTTTATCAAAAACATGT	30 s at 94°C	40 s at 55°C	36–40	7 min at 72°C
16SBG	AGATCACGTAAGAATTTAATGGTC				

¹Robillard and Desutter-Grandcolas (2006)

Alignment and phylogenetic analysis

Sequences were edited using BioEdit 7.2.5 software (Hall et al., 2011), and aligned using Clustal W algorithm, as implemented in software packages Molecular Evolutionary Genetics Analysis (MEGA) v6 (Tamura et al., 2011) and then manually checked. In some cases, sequences with a high amount of nucleotide ambiguity and/or if the sequences did not blast with orthopteran mitochondrial genes, they were considered errors and removed from further analyses. Two separate phylogenetic trees were reconstructed by Maximum likelihood (ML) and Bayesian inference (BI) methods using CIPRES Science Gateway (Miller et al., 2010). For Bayesian analysis, we first applied the model test implemented in PAUP* version 4.0b10 (Swofford, 2002) and MrModeltest2 (Nylander, 2004) software. The model for 16S matrix revealed as GTR+I+G, out of 24 models of nucleotide substitution. The alignment was converted to the Nexus format using the Mesquite software (Maddison, 2018) to run the Bayesian analysis. Bayesian analysis was done through the CIPRES Science Gateway, using MrBayes on XSEDE (3.2.6) interface, with 50000000 generations for the total of 66 16S sequences. The Maximum Likelihood analysis was performed 10 times with different random seeds each time, using RAxML-HPC v.8 on XSEDE (8.2.10) package with the maximum bootstrap number of 1000 replicates. The corresponding outputs were visualized using FigTree 1.4.3 (Drummond & Rambaut, 2007). To have a better

visualization of how samples are phylogenetically clustered with regard to their geographical location, we projected the phylogenetic tree of *Eremogryllodes* on the geographic map of cave localities obtained from DIVA-GIS 7.5.0 (<http://www.diva-gis.org/gdata>) in CorelDRAW 2019, version 21.1.0.643.

Genetic diversity

Factors of genetic diversity including nucleotide diversity (π), number of haplotypes (h), and genetic distance (F_{st}) were calculated using the program DnaSP V.5.0 (Librado & Rozas, 2009).

RESULTS

Forty-eight sequences of 16S rRNA from *Eremogryllodes* voucher specimens were successfully amplified and aligned. Samples information are given in Table 2. Eighteen additional 16S rRNA sequences from families discussed in the introduction (*Myrmecophilus*, *Gryllus*, *Gryllotalpa*, *Ornebius*) plus *Melanoplus sanguinipes*, as an outgroup, were extracted from Genbank ([Supplementary Table S1](#)). These families are included because of the controversies over the real position of *Eremogryllodes* during past decades (refer to introduction). In the present study, the final number of sites (excluding fixed gaps/missing data) for 16S is 360 bps of which 212 are invariable, 148 are variable (polymorphic) and 115 are parsimony informative.

Phylogeny

The general topology of the Bayesian (BI) tree corresponds to the maximum likelihood (ML) tree for our 16S rRNA marker (Fig. 2 and [Supplementary Fig. S1](#)) hence, we chose to focus only on the BI tree. Among all included taxa in this study, *Eremogryllodes* specimens are clustered with those of *Myrmecophilus*. Within the *Eremogryllodes* lineage, three well-supported clades are recovered (Fig. 2). Clade A includes only *E. bifurcatus* and is separated from the two other clades with high bootstrap support (BS) of 100-88 (BI-ML), the only exception for this species is specimens of Tang-e Lor cave that are located within

Clade B. Specimens of *E. dilutus* are mainly clustered into clade B, but *E. dilutus* from Palangan and Dasht-e Shir caves are placed in clade C. Clade B has high support of 99-82 (BI-ML), and also contains unidentified specimens from Sarjosh, Zakariya, Ashkan, and Gakal caves. Clade C is a mix of *E. iranicus*, *E. persicus*, and *E. spinulatus*, plus *E. dilutes* from the two mentioned caves and *E. bifurcatus* from Tang-e Lor cave. Other unidentified specimens from Sahlak, Yaran, Chah Doozakh and Sarjosh caves, are also placed in clade C. Among these, almost all specimens of *E. iranicus* are grouped in one sub-clade except the one from Sahlak cave.

Fig. 2. Phylogeny of four families of crickets, Myrmecophilidae, Gryllotalpidae, Mogoplistidae, and Gryllidae, based on 16S rRNA marker, obtained by Bayesian inference. Support values are given at each node. The tree is rooted on *Melanoplus sanguinipes*. Species name, cave name, and accession number are provided at the tip of the tree.

According to Figure 3, Clade A covers Northern Zagros, clade B mostly covers the central zone, and clade C mostly covers southern Iran (including Southern Zagros, and regions outside the Zagros ranges in Yazd and Kerman Prov.). There is an overlap

between the distributional region of clades B and C; specimens from Ashkan, Balezar and Momtaz caves and one specimen from Zakariya cave (Fars Prov.) are grouped in clade B while all the rest of caves from Fars Province and southern regions in Iran (Kerman and

Yazd Prov.) belong to clade C. Likewise the geographic distribution range of specimens belonging to clade C are extended northward e.g., specimens from Dasht-e Shir, Sarjoshir, and Tang-e Lor caves.

Genetic diversity

Nucleotide Diversity (ND) was calculated at 0.122 (π), number of haplotypes 38 (H), and haplotype diversity 0.985 (HD). The genetic distance (F_{st}) between the five included morphological species was calculated at 0.416, and between the three phylogenetic clades recovered in this study was calculated at 0.716.

DISCUSSION

Taxonomic status of Myrmecophilidae

In the present study, the 16S rRNA marker strongly supports the monophyly of Myrmecophilidae. In recent phylogenetic studies of Ensifera, Myrmecophilidae showed a close affinity with Gryllotalpidae (Zhou et al., 2010; Chintauan-Marquier et al., 2015). This phylogenetic relationship is not recovered in the

current study. However, we should keep in mind that only *Myrmecophilus* used to be included as the representative of the family Myrmecophilidae. It is also possible that this topology is due to an incomplete data set and including all cricket taxa may change the current phylogenetic position of Myrmecophilidae.

Phylogeny of *Eremogrillodes*

The phylogenetic analysis revealed that all *Eremogrillodes* samples are clustered together with 99 percent of bootstrap support in BI tree. The supporting values for each clades and sub-clades were also high (BS = 100 in most cases). In our BI tree, three main clades are recovered which could only partially separate the five morpho-species from each other. Most of the samples attributed to *E. bifurcates*, morphologically, are clustered into clade A, excluding only Tang-e Lor cave. Likewise, clade B comprises most of the members of *E. dilutus* from various caves except for Palangan and Dasht-e Shir caves. Members of *E. iranicus*, *E. persicus* and *E. spinulatus* could not be recovered as separate species as they are grouped in clade C.

Fig. 3. Phylogenetic tree of *Eremogrillodes* projected on the geographic map of the sample locations showing the distribution of molecular species. Species name, cave name, and accession number are provided at the tip of the tree.

Our phylogenetic tree suggests that molecular species tend to be clustered geographically, as close caves are mostly clustered within one clade (Fig. 3), albeit exceptions for clades B and C are noticeable with clade B having an extension southward and clade C northward. Clade A showed a clear separated distribution zone but the geographical lines between clades B and C was not clear.

The sample from Dasht-e Shir cave has been proposed as a subspecies of *Eremogrillodes dilutus* (*E. d. bakhtiyari*) (Tahami et al., 2017). Based on our molecular result, it can even be considered as distant as a new species. Morphological variations of the body colour pattern, distal part of the genital plate, and the differences in genitalia, described in Tahami et al. (2017) also support this idea. However, we would need more sequences from the putative *E. d. bakhtiyari* to be able to make a strong statement

over its genetic distance and taxonomy. The separated specimens of Palangan and Tang-e Lor caves, *E. dilutus* and *E. bifurcates*, respectively, can represent cryptic species as no discriminating morphological characteristics could be found to exclude them from their conspecifics. This scenario is highly likely as convergent evolution resulting from exposure to similar selection pressures can produce cryptic species, therefore, two genetically different species are given one name due to their morphological similarities (Wiens et al., 2003; Pfenninger & Schwenk, 2007). Cryptic species has been reported for *Myrmecophilus*, which lives inside ant nests, and for cave populations of the cricket family, Rhaphadiphoridae (Taylor et al., 2007; Komatsu et al., 2008).

Overall, the observed variation within populations of cave-dwelling cricket species across different localities is not a strange phenomenon. This might simply be

due to microhabitat variation and different ecological factors that affect the genetic diversification (Fujisawa, et al., 2015). Incomplete lineage sorting of the mitochondrial gene is another factor that might affect the phylogenetic placement of different specimens (Funk & Omland, 2003). Introgression could also be considered as a reason; however, it is less likely, as caves are known to be isolated calcrete aquifers under the ground, which is equivalent to oceanic islands, the 'subterranean island (archipelago) hypothesis' (Cooper et al., 2007; Juan et al., 2010), and isolated habitats above the ground with the surface act like an ocean of different environmental variables, sunlight, and predators (Culver & Pipan, 2009b), all of which prevent free movement of subterranean species between caves, thus reducing the gene flow. This is also supported by the high level of genetic distance (F_{st}) in both cases (between species: 0.416, between lineages: 0.746) which indicates almost no gene flow. Previous studies on the genetic structure of *Dolichopoda* have indicated relatively high genetic divergence and low gene flow even between conspecific populations (Allegrucci et al., 1987, 1997). The other noticeable result is the placement of the two individuals of *E. persicus lari* from Khan cave in two different clades. A similar situation can be seen for unidentified specimens of Zakariya and Sarjoshir caves. The phylogenetic separation of specimens from one cave locality may be due to the fact that genetic diversity within populations of cave-dwelling crickets are generally higher than normal (Taylor et al., 2007). The relatively high HD for 16S (0.95) indicates that multiple haplotypes can exist in one cave population but then will be placed within a genetically divergent group in the phylogenetic tree (Taylor et al., 2007). On the other hand, it is also probable that the separated specimen from Khan cave is a cryptic species, as there were no significant differences between the two specimens of Khan cave. The occurrence of substantial genetic diversity inside the cave populations, and the presence of more than one species inside one cave (i.e., cryptic species) has been observed in many cases (Buhay & Crandall, 2005; Finlay et al., 2006; Lejeune & Chevaldonné, 2006; Taylor et al., 2007; Komatsu et al., 2008; Guzik et al., 2009). Finally, with regard to our collection of Iranian cave dwelling *Eremogrylodes* which show a wide range of variable features in their morphology and genitalia from cave to cave, morphological traits might not be a robust indicator for the species delineation in this case. One reason is the molecular clustering of *E. iranicus*, *E. persicus*, and *E. spinulatus* together. Moreover, populations of *E. dilutus* from the caves Pebdeh, Deh Sheykh, Balezar, Ab Konardoun, and Ker Palang, although show substantial variations in the coloration, hind tibiae armament, distal part of the genital plate, and the genital structures such as distal part of rachis, endoparameral sclerites, and ectoparameres, are clustered together with the holotype (type locality: Momtaz cave) in clade A. On the other hand, members of Palangan cave are left outside the clade A while no noticeable variation could be diagnosed between those specimens and

the holotype. However, we should not exclude the fact that having only a fraction of the mitochondrial gene marker (incomplete lineage sorting) and the possibility of human error in DNA amplification process and sequencing error could have affected our final results.

CONCLUSION

In the current study, specimens of genus *Eremogrylodes* have been subjected to molecular studies for the first time. As a result, the monophyly of Myrmecophylidae was supported. The 16S rRNA marker could only partially separate the morpho-species, however, with variations inside clades. This emphasizes the need to adding more mitochondrial and nuclear gene markers in order to recover a higher resolution phylogeny. Moreover, it was suggested that the presence of cryptic species in some caves are highly probable. Here, we treat our results preliminary as further molecular studies, more preferably using next generation sequencing approach, are required to shed light on the robust taxonomic status of newly-described species.

ACKNOWLEDGMENTS

We thank the Iranian Caving Club and the local guides for their technical help in caving and specimen collections. We thank Aleksandr Dyomin, Svetlana Galkina, Roozbeh Khafefi, and Golnaz Sayyadzadeh for their kind cooperation in the process of sequencing, alignments, sequence submission, and data analysis. We would like to thank Dr. Saeid Negahban for providing the distribution map of the cave localities by Arc GIS. We are deeply grateful to Alsu Saifitdinova for her kind advice and for providing the opportunity for M.T to work in the Chromas research complex in St. Petersburg, Russia. A part of DNA extraction and amplification was done in the molecular laboratory of Islamic Azad University of Kerman. The Iran National Science Foundation (INSF) is appreciated for partial financial support of the study. Last but not least, we would like to thank unknown reviewers for their careful review and valuable inputs to the original manuscript.

Authorship statement: SS and MT designed and directed the study. MH, and AN collaborated with MT in molecular lab work and data analyzing. MT and SS carried out the caving and data collection as well as writing the paper with input from all authors. All authors contributed to the interpretation of the results and refinement of the final manuscript.

REFERENCES

- Allegrucci, G., Cesaroni, D., Sbordoni, V., 1987. Adaptation and speciation of *Dolichopoda* cave crickets (Orthoptera, Rhaphidophoridae): geographic variation of morphometric indices and allozyme frequencies. *Biological Journal of the Linnean Society*, 31, 151-160. <https://doi.org/10.1111/j.1095-8312.1987.tb01986.x>

- Allegrucci, G., Minasi, M.G., Sbordoni, V., 1997. Patterns of gene flow and genetic structure in cave-dwelling crickets of the Tuscan endemic, *Dolichopoda schiavazzii* (Orthoptera, Rhaphidophoridae). *Heredity*, 78, 665-673. <https://doi.org/10.1038/hdy.1997.106>
- Buhay, J.E., Crandall, K.A., 2005. Subterranean phylogeography of freshwater crayfishes shows extensive gene flow and surprisingly large population sizes. *Molecular Ecology*, 14, 4259-4273. <https://doi.org/10.1111/j.1365-294X.2005.02755.x>
- Carchini, G., Rampini, M., Sbordoni, V., 1994. Life cycle and population ecology of the cave cricket *Dolichopoda geniculata* (Costa) from Valmarino cave (Central Italy). *International Journal of Speleology*, 23, 3-4. <https://doi.org/10.5038/1827-806X.23.3.6>
- Chintauan-Marquier, I.C., Legendre, F., Hugel, S., Robillard, T., Grandcolas, et al., 2016. Laying the foundations of evolutionary and systematic studies in crickets (Insecta, Orthoptera): a multilocus phylogenetic analysis. *Cladistics*, 32, 1-28. <https://doi.org/10.1111/cla.12114>
- Chopard, L., 1929. Note sur les Orthoptères cavernicoles du Tonkin. *Bulletin de la Société de Zoologie de France*, 54, 224-238.
- Chopard, L., 1934. Études zoologiques sur le Sahara Central; Orthoptère. *Bulletin de la Société d'Histoire Naturelle d'Afrique du Nord*, 4, 115-116.
- Chopard, L., 1948. Three new species of Gryllidae (Orthoptera) from Arabia. *Proceedings of the Royal Entomological Society of London. Series B, Taxonomy*, 17(1-2), 1-4. <https://doi.org/10.1111/j.1365-3113.1948.tb00874.x>
- Chopard, L., 1968. Pars 12. Fam. Gryllidae: Subfam. Mogoplistinae, Myrmecophilinae, Scleropterinae, Cachoplistinae, Pteroplistinae, Pentacentrinae, Phalangopsinae, Trigonidiinae, Eneopterinae. Fam. Oecanthidae, Gryllotalpidae. Dr. W. Junk N.V., s Gravenhage, 215-500 p.
- Cigliano, M., Braun, H., Eades, D.C., Otte, D., 2020. Orthoptera species file online, Version, 5.0. <http://orthoptera.speciesfile.org/>
- Cooper, S.J., Bradbury, J.H., Saint, K.M., Leys, R., Austin, A.D., Humphreys, W.F., 2007. Subterranean archipelago in the Australian arid zone: mitochondrial DNA phylogeography of amphipods from central Western Australia. *Molecular Ecology*, 16, 1533-1544. <https://doi.org/10.1111/j.1365-294X.2007.03261.x>
- Culver, D.C., Kane, T.C., Fong, D.W., 1995. *Adaptation and natural selection in caves: the evolution of Gammarus minus*. Harvard University Press, Cambridge, 223 p. <https://doi.org/10.4159/harvard.9780674419070>
- Culver, D.C., Pipan, T., 2009a. *The biology of caves and other subterranean habitats*. Oxford University Press, Oxford, 254 p.
- Culver, D.C., Pipan, T., 2009b. Caves, as islands. In: Gillespie, R., Gillespie, R.G., Clague, D.A. (Eds.), *Encyclopedia of islands*. University of California Press, Berkeley, p. 150-153. <https://doi.org/10.1525/9780520943728-035>
- Di Russo, C., Vellei, A., Carchini, G., Sbordoni, V., 1987. Life cycle and age structure of *Dolichopoda* populations (Orthoptera, Raphidophoridae) from natural and artificial cave habitats. *Italian Journal of Zoology*, 54, 337-340. <https://doi.org/10.1080/11250008709355606>
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, 7, 214. <https://doi.org/10.1186/1471-2148-7-214>
- Finlay, J., Buhay, J., Crandall, K., 2006. Surface to subsurface freshwater connections: phylogeographic and habitat analyses of *Cambarus tenebrosus*, a facultative cave-dwelling crayfish. *Animal Conservation*, 9, 375-387. <https://doi.org/10.1111/j.1469-1795.2006.00046.x>
- Fujisawa, T., Vogler, A.P., Barraclough, T.G., 2015. Ecology has contrasting effects on genetic variation within species versus rates of molecular evolution across species in water beetles. *Proceedings of the Royal Society B*, 282, 2014-2476. <https://doi.org/10.1098/rspb.2014.2476>
- Funk, D.J., Omland, K.E., 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, 34, 397-423. <https://doi.org/10.1146/annurev.ecolsys.34.011802.132421>
- Gorochoy, A.V., 1979. Crickets of the genera *Tartarogryllus* and *Modicogryllus* (Orthoptera, Gryllidae) in Soviet Central Asia. *Entomological Review*, 57, 64-69.
- Gorochoy, A.V., 1980. Crickets of the genera *Eremogryllodes* Chop. and *Myrmecophilus* Berth. (Orthoptera, Gryllidae) and systematic position of the tribe Bothriophylacini. *Entomological Review*, 59, 287-293.
- Guzik, M., Cooper, S., Humphreys, W., Austin, A., 2009. Fine-scale comparative phylogeography of a sympatric sister species triplet of subterranean diving beetles from a single calcrete aquifer in Western Australia. *Molecular Ecology*, 18, 3683-3698. <https://doi.org/10.1111/j.1365-294X.2009.04296.x>
- Hubbell, T.H., Norton, R.M., 1978. The systematics and biology of the cave-crickets of the North American tribe Hadenocini (Orthoptera: Saltatoria: Ensifera: Rhaphidophoridae: Dolichopodinae). *Miscellaneous publications, Museum of Zoology, University of Michigan*, no. 156.
- Hall, T., Biosciences, I., Carlsbad, C., 2011. BioEdit: an important software for molecular biology. *GERF Bulletin of Biosciences*, 2, 60-61.
- Juan, C., Guzik, M.T., Jaume, D., Cooper, S.J., 2010. Evolution in caves: Darwin's 'wrecks of ancient life' in the molecular era. *Molecular Ecology*, 19, 3865-3880. <https://doi.org/10.1111/j.1365-294X.2010.04759.x>
- Komatsu, T., Maruyama, M., Ueda, S., Itino, T., 2008. mtDNA phylogeny of Japanese ant crickets (Orthoptera: Myrmecophilidae): diversification in host specificity and habitat use. *Sociobiology*, 52, 553-565.
- Lejeune, C., Chevaldonné, P., 2006. Brooding crustaceans in a highly fragmented habitat: the genetic structure of Mediterranean marine cave-dwelling mysid populations. *Molecular Ecology*, 15, 4123-4140. <https://doi.org/10.1111/j.1365-294X.2006.03101.x>
- Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451-1452. <https://doi.org/10.1093/bioinformatics/btp187>
- Maddison, W.P., Maddison, D.R., 2018. Mesquite: a modular system for evolutionary analysis. Version 3.51 (<http://www.mesquiteproject.org>)
- Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Gateway Computing Environments Workshop (GCE)*, Institute of Electrical and Electronics Engineers, p. 1-8.
- Nylander, J. A.A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

- Pfenninger, M., Schwenk, K., 2007. Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. *BMC Evolutionary Biology*, 7, 121. <https://doi.org/10.1186/1471-2148-7-121>
- Prous, X., Ferreira, R.L. & Martins, R.P. Ecotone delimitation: epigean-hypogean transition in cave ecosystems. *Austral Ecology* 29, 374–382 (2004). <https://doi.org/10.1111/j.1442-9993.2004.01373.x>
- Robillard, T., Desutter-Grandcolas, L., 2006. Phylogeny of the cricket subfamily Eneopterinae (Orthoptera, Grylloidea, Eneopteridae) based on four molecular loci and morphology. *Molecular Phylogenetics and Evolution*, 40, 643–661. <https://doi.org/10.1016/j.ympev.2005.10.019>
- Song, H., Amedegnato, C., Cigliano, M.M., Grandcolas, L.D., Heads, S.W., Huang Y., Otte D., Whiting, M.F., 2015. 300 million years of diversification: elucidating the patterns of orthopteran evolution based on comprehensive taxon and gene sampling. *Cladistics*, 31, 621–651. <https://doi.org/10.1111/cla.12116>
- Swofford, D.L., 2002 *PAUP*-Phylogenetic Analysis Using Parsimony* (*and other methods). Ver. 4.0b10. Sinauer Associates, Sunderland, Massachusetts. <https://doi.org/10.1111/j.0014-3820.2002.tb00191.x>
- Tahami, M.S., Merkl, O., Sadeghi, S., 2016. *Leptodes* of Iran, with description of six new cavernicolous species (Coleoptera: Tenebrionidae: Pimeliinae: Leptodini). *Annales Zoologici*, 66, 589–606. <https://doi.org/10.3161/00034541ANZ2016.66.4.012>
- Tahami, M.S., Gorochoy, A.V., Sadeghi, S., 2017. Cave and burrow crickets of the subfamily Bothriophylacinae (Orthoptera: Myrmecophilidae) in Iran and adjacent countries. *Zoosystematica Rossica*, 26, 241–275. <https://doi.org/10.31610/zsr/2017.26.2.241>
- Tahami, M.S., Gorochoy, A.V., Sadeghi, S. 2018. Two new species of cave crickets of the genus *Eremogryllodes* (Orthoptera: Myrmecophilidae: Bothriophylacinae) from Iran. *Zoosystematica Rossica*, 27 322–327. <https://doi.org/10.31610/zsr/2018.27.2.322>
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739. <https://doi.org/10.1093/molbev/msr121>
- Taylan, M.S., Di Russo, C., Rampini, M., Ketmaier, V., 2013. Molecular systematics of the genus *Troglophilus* (Rhaphidophoridae, Orthoptera) in Turkey: mitochondrial 16S rDNA evidences. *ZooKeys*, 33–46. <https://doi.org/10.3897/zookeys.257.4133>
- Taylan, M.S., Şirin, D., 2016. Speciation of the genus *Dolichopoda* in Anatolia with reference to the role of ancient central lake system. *Insect Systematics and Evolution*. 473, 267–283. <https://doi.org/10.1163/1876312X-47032143>
- Taylor, S.J., Weckstein, J.D., Takiya, D.M., Krejca, J.K., Murdoch, J.D., Veni, G., et al., 2007. Phylogeography of cave crickets (*Ceuthophilus* spp.) in central Texas: A keystone taxon for the conservation and management of federally listed endangered cave arthropods. *Illinois Natural History Survey Technical Report*, 58, 1–45.
- Wiens, J.J., Chippindale, P.T., Hillis, D.M., 2003. When are phylogenetic analyses misled by convergence? A case study in Texas cave salamanders. *Systematic Biology*, 52, 501–514. <https://doi.org/10.1080/10635150390218222>
- Zhou, Z., Ye, H., Huang, Y., Shi, F., 2010. The phylogeny of Orthoptera inferred from mtDNA and description of *Elimaea cheni* (Tettigoniidae: Phaneropterinae) mitogenome. *Journal of Genetics and Genomics*, 37, 315–324. [https://doi.org/10.1016/S1673-8527\(09\)60049-7](https://doi.org/10.1016/S1673-8527(09)60049-7)
- Zhou, Z., Zhao, L., Liu, N., Guo, H., Guan, B., Di, J., Shi, F., 2017. Towards a higher-level Ensifera phylogeny inferred from mitogenome sequences. *Molecular Phylogenetics and Evolution*, 108, 22–33. <https://doi.org/10.1016/j.ympev.2017.01.014>