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Borrelia duttonii-like spirochetes parasitize Meriones persicus in East Azerbaijan Province of Iran

Running title: *B. duttonii*-like spirochetes parasitize *M. persicus*

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Abstract

Borrelia persica and *B. microti*/microti-like borreliae have been established as causative agents of relapsing fever in Iran. However, the epidemiology of previously described tickborne relapsing fever (TBRF) species *Borrelia balthazardi* and *Borrelia latyschewii* (*latychevi*) has remained elusive for many years. We investigated *Borrelia* infection in various rodents and small mammals in the TBRF endemic East Azerbaijan Province, northwestern Iran, where *B. perisca* and *B. balthazardi* might coexist. Among trapped rodents (n=210), a 16S real-time PCR detected *Borrelia* DNA in 11 *Meriones persicus*. Multilocus sequence analysis (MLSA) using six different loci, including four coding regions (*flaB*, *glpQ, groEL, p66*) and two non-coding (*rrs*, IGS) followed by phylogeny revealed considerable sequence identity between the borreliae detected, *B. microti,* and East African *Borrelia duttonii*, and *Borrelia recurrentis*. Our results indicate that *B. microti* and microti-like borreliae, including the specimens characterized previously in the south of Iran and the present study, are different ecotypes of *B. duttonii*, i.e., a single species/entity or descendants of a recent common ancestor. Our findings also suggest that the species we had long coined as *B. balthazardi* and the microti-like spirochetes detected herein might be the same.

Keywords: Borrelia microti; Borrelia duttonii; Meriones persicus; East Azerbaijan Province; Iran

1. Introduction

Tickborne relapsing fever (TBRF) is an established endemic disease in Iran (Karimi, 1981). The disease occurs in most parts of the country, i.e., 20 out of 31 provinces (Masoumi Asl et al., 2009). The spirochete *Borrelia persica* is the primary cause of TBRF in rural areas of the west and northwest of the country and foothills of the Alborz Mountains where the argasid *Ornithodoros tholozani* ticks infest stables and abandoned houses (Karimi, 1981; Masoumi Asl et al., 2009; Aghaei et al., 2014). In south Iran, burrow-dwelling *Borrelia*-infected *Ornithodoros erraticus* ticks prevail in the absence of *O. tholozani* (Janbakhsh and Ardelan, 1977). In this area, *in situ* molecular analysis of the spirochetes in relapsing fever patients revealed a close resemblance with *B. microti* and East African *B. duttonii* and *B. recurrentis* (Naddaf et al., 2015, 2017). In central, western, and eastern regions, *B. microti*-infected soft ticks coexist with *O. tholozani* (Masoumi Asl et al., 2009; Naddaf et al., 2012). *Borrelia microti* and microti-like spirochetes in the south of Iran are assumed to represent adapted ecotypes of *B. duttonii* (Naddaf et al., 2012, 2015, 2017). Two other soft ticks relapsing fever spirochetes, *Borrelia balthazardi* and *Borrelia latyschewii* (*latychevi*), have also been reported in Iran (Karimi, 1981; Karimi et al., 1976, 1979; Piazak et al., 2000), but no reports of human infection with these species have been documented since 1976.

Various rodents and other small mammals serve as reservoir hosts for *Onithodoros* ticks and the associated spirochetes (Crowder et al., 2016; Talagrand-Reboul et al., 2018). In West Africa, burrow-dwelling O*rnithodoros* ticks are the primary vectors of TBRFs, and causative spirochetes are maintained in enzootic cycles involving ticks and various rodents and small mammals (Vial

et al., 2006; Trape et al., 2013). The same scenario probably applies to some areas in Iran, with *Borrelia*-infected burrow-dwelling ticks contributing to TBRF.

Here, we investigated *Borrelia* infection in various rodents and small mammals in Iran's East Azerbaijan (Āzarbāijān-e Sharqi) Province where *B. perisca* and *B. baltazardi* might coexist and deployed multilocus sequence analysis (MLSA) using *flaB*, *glpQ*, *groEL*, *p66*, *rrs*, and the intergenic spacer (IGS) loci to characterize the spirochetes.

2. Material and methods

2.1. Study area

East Azerbaijan is the largest and most populated province in the northwest of Iran, bordered by three provinces, Ardabil in the east, Zanjan in the south, and West Azerbaijan in the south and west; it also shares borders with Armenia and the Republic of Azerbaijan in the north. East Azerbaijan covers 45,491 Km^2 and comprises 21 counties, with Tabriz as the capital. This province has a population of ~4 million (3,909,652), of which 30% live in rural areas; people speak Turkish with Azari Dialect (Statistical Centre of Iran, Census 2016). The province is the intersection of two main mountain ranges of Iran, i.e., Zagros and Alborz, and according to the Köppen–Geiger classification, fall within the region B, subdivisions BSk (cold semi-arid climate) (Kottek et al., 2006; Raziei, 2016).

2.2. Rodent trapping

Rodents were captured during two periods, from 23 Oct. to 6 Nov. 2017, and from 8-22 Jun. 2018. During the first period, the animals were entrapped in 15 localities in Maragheh, Azarshahr, Bonab, and Ajabshir counties, and during the second one in 35 localities in Ahar, Kaleybar, Horand, and Sarab counties (**Fig. 1**). The live traps, baited with dates, cucumber, or a cheese-based snack, were installed close to the rodents' burrows entrance in the afternoon and

collected the following day early morning. Trapped rodents were checked onsite for ectoparasites, and then along with background information, including the locality name, plant coverage, date of collection, and coordinates of the spot, were transferred to the pre-planned locations in each of the sampling locations to be dissected. The animals were compared with the vouchers deposited at the Mohammad Hanifi's Health Museum (MHHM), Research Centre for Emerging and Reemerging infectious diseases, Akanlu, Pasteur Institute of Iran, identified based on the morphological features available in the literature (Kryštufek and Vohralík, 2005; Darvish et al., 2014) and photographed. According to the procedure approved by the Ethical Committee of the National Institute for Medical Research (Ethical code: IR NIMAD REC 1396 079), the animals were euthanized, and blood was drawn by cardiac puncture. Smears were prepared from blood and spleens of animals and stained with Giemsa. Sera and spleen samples in alcohol 96% were kept at -20 ºC until used.

2.3. Microscopy

At least 200 Giemsa-stained microscopic fields in blood and spleen smears from animals were examined using the 100× oil immersion objective.

2.4. DNA extraction

DNA extraction from spleen (20-25 mg) and serum (200 µl) samples was performed using a commercial kit (QIAGEN, Hilden, Germany) as recommended by manufacturers and kept at -20 ºC until used.

2.5. Detection of Borrelia DNA

Samples were screened for *Borrelia* DNA by a real-time PCR (qPCR) using the primers and a probe complementary to a 136 bp sequence of the 16S rRNA gene (19) and a genus-specific nested PCR that amplifies a ≈600 bp sequence of *flaB* gene (**Table 1**) using previously published

reagents and conditions (Naddaf et al., 2020; Wodecka 2007). Double distilled water (DDW) served as no template control (NTC) in both assays to ensure reagents were free of DNA contamination. In qPCR, serial dilutions (≈7200, ≈720, ≈72, ≈7.2, and ≈0.72, copy numbers per reactions) of *Borrelia burgdorferi* senso stricto DNA (Amplirun® Borrelia DNA control) were included as positive controls to establish the detection limit.

2.6. BLAST analysis

The amplicons from the second stage of nested PCR were sequenced in both directions with the same primers used in the amplification by the Sanger method in a commercial company (Genomin, Tehran, Iran). The generated sequences were BLASTed (http://blast.ncbi.nlm.nih.gov) against *Borrelia* sequences available in the GenBank database, and similarities were obtained.

2.7. MLSA

Three specimens with the lowest qPCR threshold (C_t), i.e., 28, 30.5, and 34.6, and a positive spleen nested PCR (**Supplementary data 2**) were further analyzed by amplification and sequencing of six different loci using the primers designed by others (**Table 1**) and protocols described elsewhere (Toledo et al., 2010). We also analyzed a previously characterized *B. microti* isolate (Naddaf et al., 2012) alongside the identified specimens. This isolate has been maintained via continual passages in outbred white laboratory mice since the year 2000. When possible, IGS fragments from additional specimens were sequenced to resolve the identified borreliae population structure. The generated sequences were checked for ambiguous sites and edited using CodonCode Aligner software (CodonCode Corp.). The overall mean similarity among sequences was calculated for six loci using the MEGA X in the pair-wise deletion option, Tajima-Nei model (Kumar et al., 2018).

2.8. Phylogeny

Six different loci comprising four coding regions (*flaB*, *glpQ, groEL p66*) and two non-coding (*rrs*, IGS) were generated for the borreliae parasitizing three Persian jirds (*M. persicus*) and *B. microti*. The obtained sequences and similar loci from *B. recurrentis* strain A1*, B. duttonii* strains 1120K3 and Ly, *B. crocidurae* strains Achema and DOU, *B. hispanica* strains CR1 and Sp3, *B. hermsi* strain YOR, *B. parkeri* strain SLO, *B. turicatae* strain 91E135, *B. coriaceae* strain Co53, and *B. anserina* strain Es retrieved from the NCBI database (**Table 2)** were aligned with the Clustal W (Thompson et al., 1994) algorithm implemented in BioEdit 7.0.5 (Hall, 1999). The insertions, deletions, or premature stop codons for protein-coding loci were checked by MEGA X (Kumar et al., 2018). The best fit model of nucleotide substitution was estimated based on Akaike Information Criterion (AIC) for each locus (**Supplementary data 1**) using jModeltest 0.1.1 (Posada, 2008). Phylogenetic ML trees of the *Borrelia* species were constructed using PhyML (Guindon et al., 2010). The concatenated phylogenetic tree was constructed based on the General Time Reversible model (GTR+G+R) using RaxML v.8.2.10 (Stamatakis, 2014), applying six partitions for the dataset with the bootstrap values (ML-BS) obtained from 1000 replicates. Nodal robustness was assessed by bootstrap (BP) analysis, with values ≥70% considered 'good' support. Pure genetic divergence between and within *Borrelia* species was calculated using MEGA X with 1000 bootstraps.

2.9. Nucleotide sequence accession numbers

The generated sequences used for MLST typing were deposited in the GenBank database under accession numbers MW79533-5 for *flaB,* MW79536-9 for *glpQ*, MW795340-2 for *groEL*, MW795343-6 for *P66,* MW737426-9 for *rrs*, and MW767941-5 for IGS.

3. Results

3.1. Rodent species

From eight different counties in the East Azerbaijan Province, we captured 210 small mammals belonging to orders Rodentia (n= 204), Eulipotyphla (n= 5), and Carnivora (n=1) (**Fig. 2, Supplementary data 2**).

3.2. Microscopy

The microscopy detected a few spirochetes in only one spleen smear belonging to a qPCR-positive animal (Mp423). The rest of the specimens were negative for spirochetes as determined by microscopy.

3.3. qPCR and nested PCR

qPCR detected *Borrelia* DNA in the spleen (n=11) and sera (n=6) of *M. persicus* rodents captured in Maraghe and Bonab counties (**Supplementary data 2**). The mean C_t values ± SD for the reactions containing ≈7200, ≈720, ≈72, ≈7.2, and ≈0.72, copy numbers were 29.3±0.8, 32.6±0.8, 36.6±0.9, 40.5±0.4, and 42.2±0.1, respectively; no measurable C_t value was obtained with the NTCs and the reactions containing ≤ 0.072 copy numbers. The *flaB* PCR amplified a 600 bp band in eight spleen samples (**Supplementary data 2**). The rest of the *M. persicus* specimens and the other species were negative for *Borrelia* DNA.

3.4. BLAST analysis

The two 588-bp and 598-bp *flaB* sequences from borreliae in two Persian Jirds (Mp423 and Mp430) exhibited 99.30%-99.83% identity with the East Africa species, *B. recurrentis* A1, *B. duttonii* Ly, and *B. duttonii* 1120K3 (acc. nos. CP000993, CP000976, and GU357617), 99.49%-99.50% with B. *microti* Abyek (acc. No. JF708951), and 98.33%-98.64% with *B. crocidurae* Achema (acc. No. CP003426).

3.5. Phylogenetic analysis

The mean similarities among sequences in the six borreliae loci ranged from 99.43% to 99.95%, with the lowest in IGS and the highest in *rrs* (**Table 3**). Phylogenetic trees constructed for single and concatenated sequences revealed a close relationship between the borreliae parasitizing Persian jirds (*M. persicus*) in East Azerbaijan Province of Iran, East African *B. duttonii*, and *B. recurrentis,* and the Iranian *B. microti*. This clustering also encompassed microti-like relapsing fever agents of southern Iran, where relevant sequences, i.e., *glpQ* and IGS, were available. These relationships were supported by high BP (99) in the concatenated tree, as well as in *glpQ* (BP=91), *p66* (BP=99), and IGS (BP=76) trees. Nevertheless, BP values were low for *flaB* (BP=50), *groEL* (<50), and *rrs* (<50) loci (**Fig 3**). Genetic divergence between all *B. microti*/microti-like borreliae identified in Iran and the East African *B. recurrentis*/*B. duttonii* complex ranged from 0.1 to 2.2 in *rrs* and IGS, respectively. These values were similar to the genetic divergence within *B. recurrentis*/*B. duttonii* complex by *flaB*, and IGS loci ranging from 0.1 to 2.2, respectively. For the *rrs* gene, the within-group divergence of *B. recurrentis*/*B. duttonii* complex (0.2) was higher than the divergence between *B. recurrentis*/*B. duttonii* vs. *B. microti*/microti-like (0.1) (**Supplementary data 3**). Among the loci studied, the IGS sequences of two samples from Iran (EAz_Mp423 and EAz_Mp424) shared a 14-bp *deletion* with *B. hispanica* strains Sp3 and CR1 (Acc. Nos. GU350716 and GU350715) and *B. duttonii* 1120K3 (Acc. No. GU350721).

4. Discussion

Here, we characterized the borreliae that infect *M. persicus* rodents in the TBRF endemic East Azerbaijan Province, northwest of Iran.

In Iran, the occurrence of four TBRF borreliae, *B. persica*, *B. microti*, *B. balthazardi*, and *B. latyschewii* has remained controversial for many years. Robust molecular evidence has established *B. persica* and *B. microti*/microti-like borreliae as TBRF causative agents in Iran (Ras et al., 1996; Rafinejad et al., 2011; Shirani et al., 2016; Naddaf et al., 2011, 2012, 2015, 2017). *Borrelia microti* (*Spirochaeta recurrentis*) was first detected in the blood of voles (*Microtus* sp.) in Hesark County, Tehran, in 1941. Unlike *B. persica*, this spirochete did not produce a significant spirochetemia in the adult guinea pigs and was assumed a different species (Rafyi 1947). This species, maintained by *Ornithodoros erraticus* soft ticks, also parasitize other rodent species, including *M. persicus, Tatera indica, Nesokia indica, Mus musculus,* and *Cricetulus migratorius* (Rafyi and Maghami 1949). Molecular characterization of *B. microti* isolates originated from *O. erraticus* ticks exhibited the highest identity with the East Africa borreliae *B. duttonii* and *B. recurrentis* (Naddaf et al., 2012). Later, *in situ* molecular analysis revealed microti-like spirochetes in relapsing fever patients in the south of Iran (Naddaf et al., 2015, 2017).

No genetic data are available to support *B. balthazardi* and *B. latyschewii* as distinct entities. *Borrelia latyschewii* records are limited to infected *O. tartakovskii* ticks in Khorasan Province (now Khorasan Razavi), east of the country (Baltazard, 1952; Baltazard et al., 1955; Piazak et al., 2000). However, *B. balthazardi* was described as a relapsing fever causative agent in febrile patients presenting thrombocytopenic purpura in East Azerbaijan Province during the late 1960s to mid-1970s. Agar gel diffusion and complement fixation assays ruled out Crimean-Congo hemorrhagic fever (CCHF), while simultaneous microscopical examination of the Giemsa-stained blood smears and subsequent dark field microscopy revealed spirochetes in one patient (Karimi et al., 1976). The spirochete identity as a novel species separate from *B. persica*

was achieved via differential *in vivo* pathogenicity and the number of periplasmic flagella (Karimi, 1981; Karimi et al., 1979). Unfortunately, *B. microti* and *B. latyschewii* were not included in the analysis as their geographical distribution range was assumed to be limited to the local areas out of East Azerbaijan Province.

In this study, MLSA revealed a high degree of identity and clustering between the newly detected borreliae and *B. duttonii*, *B. recurrentis*, *B. microti,* and the microti-like relapsing fever agent reported from the south of Iran. In phylogeny, *Borrelia* sequences generated in this study and other sequences from Iran grouped in the same clade alongside East African borreliae with high BP support for most loci, except *rrs* and *groEL* (**Fig. 3)**. These two genes also showed the lowest genetic divergence among *Borrelia* species, presuming more conservative markers.

Despite several attempts, we obtained no definitive sequences for *flaB* and groEL from the Mp496 specimen due to overlapped bases; however, this specimen grouped with Mp423 and Mp430 in the concatenated tree in the absence of these sequences. *Borrelia persica* was not included in our phylogenetic analysis, as the sequences were not available for all loci. Neither had we access to a strain to amplify and sequence the loci. In a phylogenetic analysis using available loci (*flaB*, *glpQ*, and *rrs*), this species appeared intermediate between African and American species sharing a distant ancestor with former ones (**Supplementary data 4**), similar to previous studies (Ras et al. 1996; Elbir et al. 2014; Naddaf et al. 2012, 2017).

Genetic divergence of *B. recurrentis*/*B.duttonii* complex vs. *B. microti*/microti-like borreliae group completely coincided with genetic divergence within East African *B. recurrentis*/*B. duttonii* complex (**Supplementary data 3**). These results suggest that *B. microti*/microti-like borreliae from Iran are seemingly different ecotypes of a single species/entity or descendants of a recent common ancestor. Also, considering the close affinity of these borreliae with those from

East Africa and the low genetic divergence (0.1-2.2%) (**Supplementary data 3**), one might assign these strains as *B. duttonii* ecotypes. Hence, the species we had long coined as *B. balthazardi* in the East Azerbaijan Province may be the same as a microti-like spirochete maintained in cycles involving rodents and *Ornithodoros* ticks. Unlike microti-like and most other relapsing fever borreliae, no vertebrate except humans serves as reservoir hosts for *B. duttonii* and louse adapted *B. recurrentis* (Cutler, 2010). Complete genomic sequencing suggests that *B. recurrentis* is a decaying genome that evolved from either *B. duttonii* or a common ancestral strain (Lescot et al., 2008; Cutler et al., 2008). *Ornithodoros moubata* ticks that vector *B. duttonii* in East Africa have undergone domestication with a predilection for infesting traditional style 'Tembe' human dwellings (Cutler, 2010).

Our findings and a recent report of a *B. microti* infection in an Italian woman returning from Kyrgyzstan and Tajikistan (Mancini et al., 2020) suggest that *B. microti*/microti-like borreliae might have a broader geographical range in Asia than previously assumed. The presence of these borreliae might be obscured by other *Borrelia* species, e.g., *B. persica* in the west and central Iran, or the lack of access to reliable identification and genetic delineation tools. Accurate diagnosis of relapsing fever agents mostly becomes possible following infected travelers' return to their privileged homelands (Colin de Verdiere et al., 2011; Stete et al., 2018; Mancini et al., 2020).

In West, North, and Central Africa, an exhaustive study on the epidemiology of the TBRF revealed nine different burrow-dwelling *Ornithodoros* species, of which six species contained *Borrelia* DNAs representing *B. crocidurae*, *B. merionesi,* and *B. hispanica*. *Borrelia crocidurae* was maintained only by *O. sonari*, while *B. merionesi* and *B. hispanica* were harbored by two and three tick species, respectively (Trape et al., 2013). In Iran and possibly other regions in the

West and Middle Asia, *B. microti*/microti-like spirochetes seem to perpetuate in cycles involving rodents and *Ornithodoros* ticks. Unfortunately, the data on burrow-dwelling *Ornithodoros* ticks in Iran and neighboring countries are minimal. *Ornithodoros erraticus* and *O. tartakowskii* that vector *B. microti* and *B. latyschewii* are the only known burrow-dwelling species in Iran by morphology (Karimi, 1981).

In the present study, among 210 blood smears, we detected spirochetes in only one spleen smear as infections in most natural reservoir species are often relatively occult and benign (Smith, 2005; Larsson et al., 2006a). Relapsing fever borreliae might be neurotropic (Barbour and Hayes, 1986; Cadavid and Barbour, 1998), and invading the central nervous system (CNS) is characteristic of some species like *B. duttonii* and *B. turicatae* (Larsson et al. 2006b; Sethi et al. 2006). Hence, herein, we might have missed some borreliae infections in the animals by overlooking spirochetes in the brain.

In our study, among 210 animals, qPCR and nested PCR detected *Borrelia* DNA in 11 (~5.24 %) and eight (~3. 81%) specimens. The variation in the positivity rate might be due to the higher sensitivity of the qPCR, which can detect DNA amounts equivalent to as low as one-thousandth of a single *B. burgdorferi* cell (Ornstein and Barbour 2006). Our previous study also showed the same discrepancy in the sensitivity of these assays (Naddaf et al. 2020). None of the qPCR negative specimens yielded amplicon in the nested PCR assay.

Apart from soft tick relapsing fever borreliae, the spirochetes maintained by hard ticks might also contribute to relapsing fever in some areas of Iran. Recently, in the north of the country, *Borrelia miyamotoi* and other genetically related relapsing fever spirochetes were detected in *Ixodes ricinus* and *Rhipicephalus* ticks, respectively (Naddaf et al., 2020).

A limitation in our study was the lack of investigating the soft ticks that maintain and transmit this borrelia species in the area. Molecular characterization of burrow-dwelling *Ornithodoros* species and their associated borreliae in extended geographical areas, including East Azerbaijan Province, would disclose more of the identity of relapsing fever borreliae and their possible role in human infections in Iran and Asia.

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Declaration of Competing Interest

Ahmad Ghasemi and Saied Reza Naddaf equally contributed to this work as the first author. The authors declare no issue to be conceived as a conflict of interest related to this article.

References

Aghaei, A., Ghazinezhad, B. Naddaf, S.R., 2014. Detection of *Borrelia* DNA in *Ornithodoros tholozani* ticks and their Eggs. J. Med. Microbiol. Infec. Dis. 2**,** 118-120.

Baltazard, M., 1952. [*Ornithodorus tartakovskyi* Olenev 1931 and *Borrelia* (*Spirochaeta*) *latychevi* Sofiev 1941]. Ann. Parasitol. Hum. Comp. 27**,** 311-328.

Baltazard, M., Pournaki, R., Bahmanyar, M. Chamsa, M., 1955. [*Ornithodorus tartakovskyi* Olenev 1931 and *Borrelia* (*Spirochaeta*) *latychevii* Sofiev 1941; supplementary note]. Ann. Parasitol. Hum. Comp. 30**,** 225-242.

Barbour, A.G. Hayes, S.F., 1986. Biology of *Borrelia* species. Microbiol Rev. 50**,** 381-400.

Bunikis, J., Garpmo, U., Tsao, J., Berglund, J., Fish, D. Barbour, A.G., 2004. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology (Reading). 150**,** 1741-1755.

Cadavid, D. Barbour, A.G., 1998. Neuroborreliosis during relapsing fever: review of the clinical manifestations, pathology, and treatment of infections in humans and experimental animals. Clin. Infect. Dis. 26**,** 151-164. https://doi.org/10.1086/516276.

Colin De Verdiere, N., Hamane, S., Assous, M.V., Sertour, N., Ferquel, E. Cornet, M., 2011. Tickborne relapsing fever caused by *Borrelia persica*, Uzbekistan and Tajikistan. Emerg Infect Dis. 17**,** 1325-1327. https://doi.org/10.3201/eid1707.101894

Crowder, C.D., Ghalyanchi Langeroudi, A., Shojaee Estabragh, A., Lewis, E.R.G., Marcsisin, R.A. Barbour, A.G., 2016. Pathogen and host response dynamics in a mouse model of *Borrelia hermsii* relapsing fever. Vet. Sci. 3. https://doi.org/10.3390/vetsci3030019.

Cutler, S.J., 2010. Relapsing fever--a forgotten disease revealed. J. Appl. Microbiol. 108**,** 1115-1122. https://doi.org/1111/j.1365-2672.2009.04598.x.

Cutler, S.J., Scott, J.C. Wright, D.J.M., 2008. Phylogenic relationship between *Borrelia recurrentis* and *Borrelia duttonii*. Int. J. Med. Microbiol. 298 (S1)**,** 193-202.

Darvish, J., Mohammadi, Z., Mahmoudi, A. Siahsarvie, R., 2014. Faunistic and taxonomic study of Rodents from northwestern Iran. Iran. J. Anim. Biosyst. 10, 119–136. https://doi.org/10.22067/ijab.v10i2.44285.

Elbir, H., Gimenez, G., Robert, C., Bergström, S., Cutler, S., Raoult, D. Drancourt, M., 2012. Complete genome sequence of *Borrelia crocidurae*. J Bacteriol. 194**,** 3723-3724. https://doi.org/10.1128/jb.00118-12.

Elbir, H., Abi-Rached, L., Pontarotti, P., Yoosuf, N. Drancourt, M., 2014. African relapsing fever *Borreliae* genomospecies revealed by comparative genomics. Front. Public Health. 2, (43). https://doi.org/10.3389/fpubh.2014.00043.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59**,** 307-321. https://doi.org/10.1093/sysbio/syq010.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT Nucl. Acids Symp. Ser. 41**,** 95-98.

Janbakhsh, B. Ardelan, A., 1977. The nature of sporadic cases of relapsing fever in Kazeroun area, southern Iran. Bull. Soc. Pathol. Exot. Filiales. 70**,** 587-589.

Karimi, U., 1981. Relapsing fever and its epidemiology (in Farsi), Pasteur Institute of Iran (PII).

Karimi, Y., Hannoun, C., Ardouin, P. Ameli, M., 1976. Sur le purpura he'morragique observe' dans l'Azerbaidjan-Est de l'Iran. Me'd Mal. Infec. 6**,** 399-404.

Karimi, Y., Hovind-Hougen, K., Birch-Andersen, A. Asmar, M., 1979. *Borrelia persica* and *B. baltazardi* sp. nov.: experimental pathogenicity for some animals and comparison of the ultrastructure. Ann. Microbiol. (Paris). 130B**,** 157-168.

Kottek, M., Grieser, J., Beck, C., Rudolf, B. Rubel, F., 2006. World map of the Köppen-Geiger climate classification updated. Meteorol. Z. 15**,** 259-263. https://doi:1127/0941-2948/2006/0130.

Kryštufek, B., Vohralík, V., 2005. Mammals of Turkey and Cyprus: rodentia i: sciuridae, Dipodidae, Gliridae, Arvicolinae. Zgodovinsko društvo za južno Primorsko.

Kumar, S., Stecher, G., Li, M., Knyaz, C. Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35**,** 1547-1549. https://doi: 10.1093/molbev/msy096.

Larsson, C., Andersson, M., Guo, B.P., Nordstrand, A., Hagerstrand, I., Carlsson, S. Bergstrom, S., 2006a. Complications of pregnancy and transplacental transmission of relapsing-fever borreliosis. J. Infect. Dis. 194**,** 1367-1374. https://doi.org/10.1086/508425.

Larsson, C., Andersson, M., Pelkonen, J., Guo, B.P., Nordstrand, A. Bergström, S., 2006b. Persistent brain infection and disease reactivation in relapsing fever borreliosis. Microbes Infect. 8, 2213-2219. https://doi: 10.1016/j.micinf.2006.04.007.

Lescot, M., Audic, S., Robert, C., Nguyen, T.T., Blanc, G., Cutler, S.J., Wincker, P., Couloux, A., Claverie, J.M., Raoult, D. Drancourt, M., 2008. The genome of *Borrelia recurrentis*, the agent of

deadly louse-borne relapsing fever, is a degraded subset of tick-borne *Borrelia duttonii*. PLoS Genet. 4**,** e1000185. https://doi.org 10.1371/journal.pgen.1000185.

Mancini, F., Innocenti, P., Baumgartner, M., Binazzi, R., Troi, C., Pagani, E. Ciervo, A., 2020. *Borrelia microti* infection in an Italian woman returning from Kyrgyzstan and Tajikistan. Travel. Med. Infect. Dis. 35**,** 101448. https://doi: 10.1016/j.tmaid.2019.07.004.

Masoumi Asl, H., Goya, M.M., Vatandoost, H., Zahraei, S.M., Mafi, M., Asmar, M., Piazak, N. Aghighi, Z., 2009. The epidemiology of tick-borne relapsing fever in Iran during 1997-2006. Travel. Med. Infect. Dis. 7**,** 160-164. https://doi.org/10.1016/j.tmaid.2009.01.009.

Naddaf, S.R., Kishdehi, M. Siavashi, M., 2011. Comparison of PCR-based diagnosis with centrifuged-based enrichment method for detection of *Borrelia persica* in animal blood samples. Iran J Arthropod Borne Dis. 5, 7-12.

Naddaf, S.R., Ghazinezhad, B., Bahramali, G. Cutler, S.J., 2012. Phylogenetic analysis of the spirochete *Borrelia microti*, a potential agent of relapsing fever in Iran. J. Clin. Microbiol. 50**,** 2873-2876. https://doi.org/10.1128/JCM.00801-12.

Naddaf, S.R., Ghazinezhad, B., Sedaghat, M.M., Asl, H.M. Cutler, S.J., 2015. Tickborne relapsing fever in southern Iran, 2011-2013. Emerg. Infect. Dis. 21**,** 1078-1080. https://doi.org/10.3201/eid2106.141715.

Naddaf, S.R., Ghazinezhad, B., Kazemirad, E. Cutler, S.J., 2017. Relapsing fever causative agent in Southern Iran is a closely related species to East African borreliae. Ticks Tick Borne Dis. 8**,** 882-886. https://doi.org/10.1016/j.ttbdis.2017.07.006.

Naddaf, S.R., Mahmoudi, A., Ghasemi, A., Rohani, M., Mohammadi, A., Ziapour, S.P., Nemati, A.H. Mostafavi, E., 2020. Infection of hard ticks in the Caspian Sea littoral of Iran with Lyme

borreliosis and relapsing fever borreliae. Ticks Tick Borne Dis. 11**,** 101500. https://doi: 10.1016/j.ttbdis.2020.101500.

Ornstein, K. Barbour, A.G., 2006. A reverse transcriptase-polymerase chain reaction assay of *Borrelia burgdorferi* 16S rRNA for highly sensitive quantification of pathogen load in a vector. Vector Borne Zoonotic Dis. 6**,** 103-112. https://doi.org/10.1089/vbz.2006.6.103.

Piazak, N., Seyyed Rashti, M.A. Assmar, M., 2000. Distribution of *Ornithodoros tartakowskyi* and its infection rate with *Borrelia latyschevii* in Serakhs area, Khorasan Province (in Farsi). Iranian J. Public Health. 1-4**,** 103-108.

Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol Biol Evol. 25**,** 1253-1256. https://doi.org/10.1093/molbev/msn083.

Rafinejad, J., Choubdar, N., Oshaghi, M., Piazak, N., Satvat, T., Mohtarami, F. Barmaki, A., 2011. Detection of *Borrelia persica* infection in *Ornithodoros tholozani* using PCR targeting rrs gene and xenodiagnosis. Iran J Public Health. 40**,** 138-145.

Rafyi, A., 1947. [*Spirochaeta microti* n. sp., vole parasite (*Microtus* sp.) in Iran]. Bull Soc Pathol Exot Filiales. 40, 149-151.

Rafyi, A. Maghami, G.R., 1949. Recherches sun l'infection experimentale des rongeurs par *Borrelia microti*. Bull Soc Pathol Exot. 12, 215-221.

Ras, N.M., Lascola, B., Postic, D., Cutler, S.J., Rodhain, F., Baranton, G. Raoult, D., 1996. Phylogenesis of relapsing fever *Borrelia* spp. Int. J. Syst. Bacteriol. 46**,** 859-865. https://doi.org/10.1099/00207713-46-4-859.

Raziei, T., 2016. Köppen-Geiger climate classification of Iran and investigating its changes during 20th century. J. Earth Space Phys. 43**,** 419–439.

Sethi, N., Sondey, M., Bai, Y., Kim, K.S. Cadavid, D., 2006. Interaction of a neurotropic strain of Borrelia turicatae with the cerebral microcirculation system. Infect Immun. 74, 6408-6418. https://doi.org/10.1128/IAI.00538-06.

Shirani, D., Rakhshanpoor, A., Cutler, S.J., Ghazinezhad, B. Naddaf, S.R., 2016. A case of canine borreliosis in Iran caused by *Borrelia persica*. Ticks Tick Borne Dis. 7**,** 424-426. https://doi.org/ 10.1016/j.ttbdis.2015.12.020.

Smith, R.P. 2005. Tick-borne diseases of humans. Emerg. Infect. Dis. 11, 1808–1809. https://doi.org/ 10.3201/eid1111.051160.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 30**,** 1312-1313. https://doi.org/10.1093/bioinformatics/btu033.

Statistical Centre of Iran, Census 2016. https://www.amar.org.ir/english/Populationand-Housing-Censuses (accessed 31 Mar. 2021).

Stete, K., Rieg, S., Margos, G., Häcker, G., Wagner, D., Kern, W.V. Fingerle, V., 2018. Case report and genetic sequence analysis of candidatus *Borrelia kalaharica*, Southern Africa. Emerg. Infect. Dis. 24**,** 1659-1664. https://doi.org/10.3201/eid2409.171381.

Talagrand-Reboul, E., Boyer, P.H., Bergstrom, S., Vial, L. Boulanger, N., 2018. Relapsing Fevers: Neglected tick-borne diseases. Front. Cell. Infect. Microbiol. 8**,** 98. https://doi.org/10.3389/fcimb.2018.00098.

Thompson, J.D., Higgins, D.G. Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap

penalties and weight matrix choice. Nucleic Acids Res. 22**,** 4673-4680. https://doi.org/10.1093/nar/22.22.4673.

Toledo, A., Anda, P., Escudero, R., Larsson, C., Bergstrom, S. Benach, J.L., 2010. Phylogenetic analysis of a virulent *Borrelia species* isolated from patients with relapsing fever. J Clin. Microbiol. 48**,** 2484-2489. https://doi.org/10.1128/JCM.00541-10.

Trape, J.F., Diatta, G., Arnathau, C., Bitam, I., Sarih, M., Belghyti, D., Bouattour, A., Elguero, E., Vial, L., Mane, Y., Balde, C., Prugnolle, F., Chauvancy, G., Mahe, G., Granjon, L., Duplantier, J.M., Durand, P. Renaud, F., 2013. The epidemiology and geographic distribution of relapsing fever borreliosis in West and North Africa, with a review of the *Ornithodoros erraticus* complex (Acari: Ixodida). PLoS One. 8**,** e78473. https://doi.org/10.1371/journal.pone.0078473.

Vial, L., Diatta, G., Tall, A., Ba El, H., Bouganali, H., Durand, P., Sokhna, C., Rogier, C., Renaud, F. Trape, J.F., 2006. Incidence of tick-borne relapsing fever in west Africa: longitudinal study. Lancet. 368**,** 37-43. https://doi.org/10.1016/S0140-6736(06)68968-X.

Wodecka, B., 2007. [Significance of red deer (*Cervus elaphus*) in the ecology of *Borrelia burgdorferi* sensu lato]. Wiad. Parazytol. 53**,** 231-237.

Figures caption:

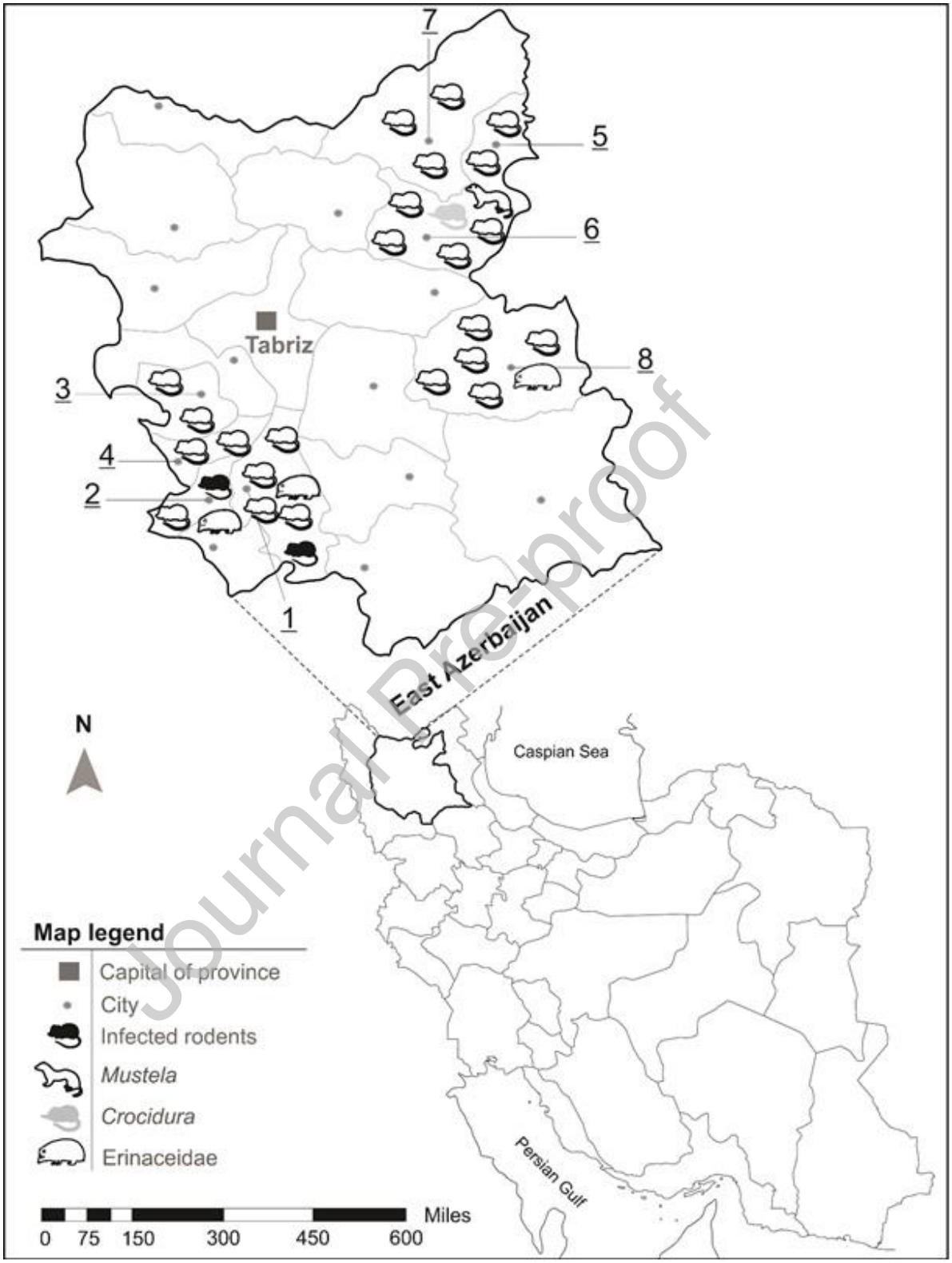

Fig 1. Map of Iran showing the East Azerbaijan Province and sampling localities, 1, Maraghe; 2, Bonab; 3, Azarshahr; 4, Ajabshir; 5, Horand; 6, Ahar; 7, Kaleybar; 8, Sarab. The symbols represent different animals, and the black ones indicate the *Borrelia*-infected specimens.

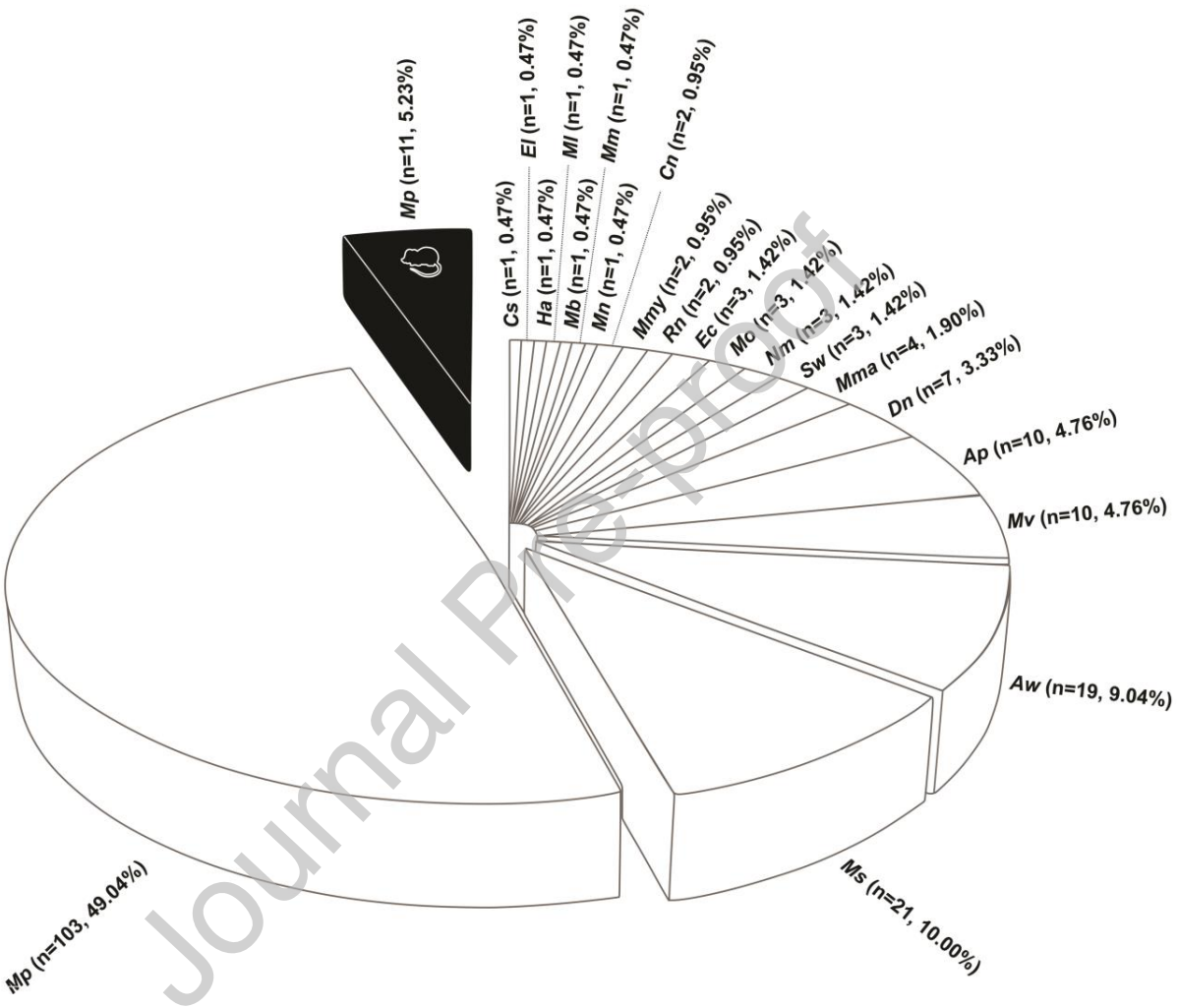

Fig. 2: The pie graph demonstrating the number (N) and percentages of the small mammal species captured in this study. Abbreviations: ***Mp*** (*Meriones persicus*), ***Cs*** (*Crocidura suaveolens*), ***El*** (*Ellobius lutescens*), ***Ha*** (*Hemiechinus auritus*), ***Ml*** (*Meriones libycus*), ***Mb*** (*Mesocricetus brandti*), ***Mm*** (*Mus musculus*), ***Mn*** (*Mustela nivalis*), ***Cn*** (*Chionomys nivalis*), ***Mmy*** (*Microtus mystacinus*), ***Rn*** (*Rattus norvegicus*), ***Ec*** (*Erinaceus concolor*), ***Mo*** (*Microtus*

obscurus), ***Nm*** (*Nothocricetulus migratorius*), ***Sw*** (*Scarturus williamsi*), ***Mma*** (*Mus macedonicus*), ***Dn*** (*Dryomys nitedula*), ***Ap*** (*Arvicola persicus*), ***Mv*** (*Meriones vinogradovi*), ***Ap*** (*Apodemus witherbyi*), ***Ms*** (*Microtus socialis*). The black slice shows the percentage of qPCR *Borrelia*-positive *M. persicus*.

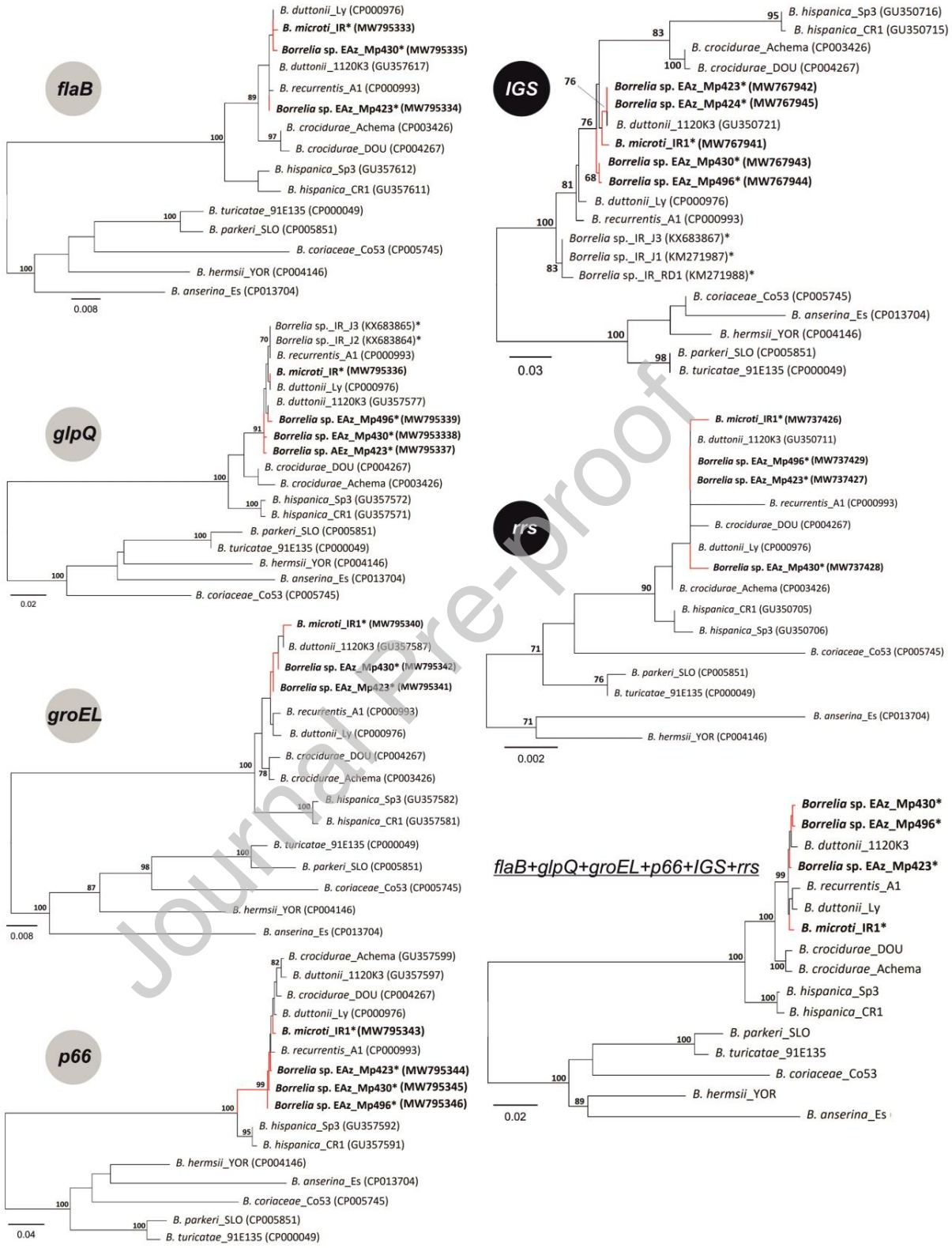

Fig. 3: Maximum Likelihood phylogenetic relationships of the analyzed *Borrelia* in the present study based on the four coding loci, *flaB*, *glpQ*, *groEL*, *p66* (gray circles), two non-coding loci, *IGS* and *rss* (black circles), and the concatenated dataset from the 7387-bp-long sequences (*flaB+glpQ+groEL+p66+IGS+rss*) of 16 *Borrelia* strains. Only BP values ≥70 are indicated. *groEL* and *flaB* genes were not obtained for isolate EAz-496. The asterisks (*) show the Iranian sequences, and the bold asterisks (*) show sequences generated in this study.

Table 1: The primers and the probe used for identification and characterization of *Borrelia* specimens.

Target Gene	Primers/probe	Sequence (5' to 3')	Amplicon size (bp)	Reference
16S rRNA	primers	16SBOR-Fw: GGTCAAGACTGACGCTGAGTCA 16SBOR-Rev: GGCGGCCACTTAACACGTTAG	136	(Ornstein and Barbour, 2006)
	probe	16SBOR-P :Fam-TCTACGCTGTAAACGATGCACACTTGGTG-BHQ-1		
flaB	outer	132f: 5′-TGGTATGGGAGTTTCTGG-3′ 905r: 5′-TCTGTCATTGTAGCATCTTT-3′	774	(Wodecka, 2007)
	inner	220f: 5′-CAGACAACAGAGGGAAAT-3′ 823r: 5′-TCAAGTCTATTTTGGAAAG CACC-3’	604	
rrs	fragment 1	GTTTGATCCTGGCTTAGAAC TTACAATCTTTCGACCTTCTT	417	(Toledo et al., 2010)
rrs	fragment 2	CACACTGGAACTGAGATACGG TTCGCCTCTGGTATTCTTCCT	419	(Toledo et al., 2010)
rrs	fragment 3	TGCGTAAAATACCACAGCTCA TGAGTCCCCATCTTTACATGC	547	(Toledo et al., 2010)
rrs	fragment 4	TACCAGGGCTTGACATATACA GAGGTGATCCAGCCACACTTT	561	(Toledo et al., 2010)
IGS	outer	GTATGTTTAGTGAGGGGGGTG GGATCATAGCTCAGGTGGTTAG	807	(Bunikis et al., 2004)
IGS	inner	AGGGGGGTGAAGTCGTAACAAG GTCTGATAAACCTGAGGTCGGA	765	(Bunikis et al., 2004)
flaB	fragment 1	TCATAAATCATAATACGTCAG AATGTCCATGAAGCTTGTGA	514	(Toledo et al., 2010)
flaB	fragment 2	CTGAAGAGCTTGGAATGCAAC AGGTACTTGATTTGCTTGTGC	538	(Toledo et al., 2010)
glpQ	fragment 1	CATTAATTATAGCTCACAGAG AACAAGCATTATCAATTTTCC	599	(Toledo et al., 2010)
glpQ	fragment 2	TATGGCATAAACAACAAGGTA AATCTGTAAATAGACCATCTA	453	(Toledo et al., 2010)
p66	fragment 1	TTTAGATTTGATATGGATGA GATATGTGTCCAAGTATAGA	899	(Toledo et al., 2010)
p66	fragment 2	TTCCTCAATAACATATGGTCT ACACTTCCATTTTGATCTTT	945	(Toledo et al., 2010)
groEL	fragment 1	TGGCTAAGGACATATATTTTA ATCTTTGCCAACTCTGTCCAT	512	(Toledo et al., 2010)
groEL	fragment 2	TTCTGCAAATAATGATACTTC AACATTCTCAAGAGTAAGTCC	487	(Toledo et al., 2010)
groEL	fragment 3	ATTGCTATACTTACTGGAGG TAAATAGAACTCTCAAATCCA	524	(Toledo et al., 2010)

groEL	fragment 4	AAGGTTTTGAGATTGTGAAGA TTACATCATTCCCATTCCAG	323	(Toledo et al., 2010)

Table 2: Details of sequences used in phylogenetic analysis.

***Borrelia* species**	**Host/isolation source**	**Location**	**GenBank Acc. No.**	**Reference**	**Gene**
B. duttonii Ly	Human CFS	Tanzania	CP000976	(Lescot et al., 2008)	complete genome
B. duttonii 1120K3	*O. moubata*	Congo	GU357617	(Toledo et al., 2010)	*flaB*
			GU357577	(Toledo et al., 2010)	*glpQ*
			GU357587	(Toledo et al., 2010)	*groEL*
			GU350721	(Toledo et al., 2010)	IGS
			GU357597	(Toledo et al., 2010)	*P66*
			GU350711	(Toledo et al., 2010)	*rrs*
B. recurrentis A1	Human blood	Ethiopia	CP000993	(Lescot et al., 2008)	complete genome
B. crocidurae Achema	*O. sonrai*	Mauritania	CP003426	(Elbir et al., 2012)	complete genome
B. crocidurae DOU	*O. sonrai*	Mali	CP004267	Unpublished	complete genome
B. hispanica Sp3	*O. erraticus*	Spain	GU357612	(Toledo et al., 2010)	*flaB*
			GU357572	(Toledo et al., 2010)	*glpQ*
			GU357582	(Toledo et al., 2010)	*groEL*
			GU350716	(Toledo et al., 2010)	IGS
			GU357592	(Toledo et al., 2010)	*P66*
			GU350706	(Toledo et al., 2010)	*rrs*
B. hispanica CR1	ND	ND	GU357611	(Toledo et al., 2010)	*flaB*
			GU357571	(Toledo et al., 2010)	*glpQ*
			GU357581	(Toledo et al., 2010)	*groEL*

			GU350715	(Toledo et al., 2010)	IGS
			GU357591	(Toledo et al., 2010)	*P66*
			GU350705	(Toledo et al., 2010)	*rrs*
B. turicatae 91E135	*O. turicata*	USA	CP000049	Unpublished	complete genome
B. parkeri SLO	*O. parkeri*	USA	CP005851	Unpublished	complete genome
B. coriaceae Co53	*O. coriaceus*	USA	CP005745	Unpublished	complete genome
B. hermsii YOR	Human blood	USA	CP004146	Unpublished	complete genome
B. anserina Es	Chicken	USA	CP013704	Unpublished	complete genome
†*Borrelia* sp. IR-J2	Human blood	Iran	KX683864	(Naddaf et al., 2017)	*glpQ*
†*Borrelia* sp. IR-J3	Human blood	Iran	KX683865	(Naddaf et al., 2017)	*glpQ*
†*Borrelia* sp. IR-J1	Human blood	Iran	kM271987	(Naddaf et al., 2015)	IGS
†*Borrelia* sp. IR-RdD1	Human blood	Iran	KM271988	(Naddaf et al., 2015)	IGS
†*Borrelia* sp. IR-J3	Human blood	Iran	KX683867	(Naddaf et al., 2017)	IGS

ND, not defined; †, microti-like borreliae.

Table 3: The Mean similarities among the six loci generated in this study.

Locus	No. sequences	Length bp		similarity
flaB	2	975		99.78%
glpQ	3	878		99.48%
groEL	2	1593		99.87%
P66	3	1618		99.75%
rss	3	1479		99.95%
IGS	4	581		99.43%