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RESEARCH ARTICLE

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Molecular detection and phylogenetic analysis of *Bovicola caprae* in the west and northwest of Iran based on cytochrome oxidase 1 marker

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ABSTRACT

Lice are permanent, obligate ectoparasites for birds and mammals. *Bovicola caprae* causes hypersensitivity, irritation, dermatitis, anemia, lower weight gain, and lower productivity in goats. This study was conducted to investigate *B. caprae* by molecular methods based on the mitochondrial genome in the West and Northwest of Iran. A total of 1017 samples of chewing lice collected from ten cities in five provinces were identified using diagnostic keys. After DNA extraction and PCR, samples were sent for sequencing. Morphological results were consistent with molecular examinations. Nucleotide sequencing of samples isolated from different cities based on mitochondrial genome showed 100% intraspecific similarity. The sequences of *B. caprae* isolated in this study appeared in a branch next to the Canadian and Chinese samples in the phylogenetic tree with more than 90% similarity. The results of mitochondrial gene analysis in the present research showed that this fragment is useful for showing intraspecific similarity and species and genus differentiation of *B. caprae*.

Keywords

COX1, Iran, Lice, Molecular study

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Abbreviations

PCR: Polymerase chain reaction
COX1: Cytochrome oxidase 1
mtDNA: Mitochondrial DNA
MBST: Molecular biological system transfer
µl: Microliter
µm: Micromolar
mM: Millimolar

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Introduction

Lice are obligate ectoparasites of birds and mammals and are classified in the order Phthiraptera. Four phthirapteran suborders are recognized: the chewing louse suborders Amblycera, Ischnocera, and Rhynchophthirina, and the sucking louse suborder Anoplura [1-3]. They are hemi-metabolic insects and spend all stages of their lives on a host [2, 4, 5]. In these four suborders, there are 24 families, 303 genera, and about 5000 species of lice, of which about 4000 species infest birds and about 1000 species infest mammals [6].

B. caprae, the goat-biting louse, has a brownish-red head and thorax, with a yellow abdomen and brown crossbands, and a truncated anterior margin of the head. Their life cycle lasts about 3-4 weeks and they spend all their lives on one host [4, 7], leading to a decrease in the quality and production of wool in goats [8]. Lice infestation is a major problem for small ruminants and causes serious damage to livestock through mortality and reduced productivity, reproduction, fertility, and skin value in the tanning industry [9]. Symptoms of lice infestation in goats include hypersensitivity to the protein in the lice saliva, which results in irritation and alopecia as the main clinical signs. Secondary infections may occur due to excessive scratching [4]. Lice can also cause hypoglycemia, hypoproteinemia, and hypoalbuminemia following the chronic loss of nutrients [10]. Moreover, lice infestation reduces productivity and reproduction in goats because of anemia and miscarriage. Reduced productivity in goats due to decreased weight gain and reproductive disorders is of considerable economic significance [4].

Lice species are conventionally identified based on morphological characteristics. However, accurate identification of species and subspecies of lice based on morphological characteristics is difficult because lice have a large variety and similar morphology. To solve this problem, researchers have used molecular markers, including mitochondrial genes and nuclear ribosomal genes. An appropriate genetic marker is a basic prerequisite for success in many evolutionary studies [11]. mtDNA is a valuable evolutionary tool for diverse structural and evolutionary aspects. These features include easy isolation, high copy number, no recombination, protected sequence and structure in metazoans, and a wide range of mutations in different molecular regions. Certain characteristics of COX1 make it a unique and suitable marker for evolutionary studies. Its size and structure have been preserved in the studied aerobic organisms, and mutation studies have mapped its reaction centers, facilitating the interpretation of sequence differences in gene function [11].

As generally agreed, lice are categorized in four suborders. In contrast, there is no agreement on the phylogenetic relationships of these groups and their classification [12]. Although phylogeny at the suborder level was suggested by Lyal in 1985, it was flawed due to the questionable monophyly of Ischnocera as a suborder [6]. Since then, many studies have addressed phylogeny at the subordinate level in lice. Johnson et al. (2002) analyzed the sequence of three genes (EF1-18S-cox1) of 21 species from four suborders and showed that Ischnocera is monophyletic [13]. In another study, Yoshizawa et al. (2003) analyzed the rrnL, rrnS sequence of 18 species and showed that Ischnocera was paraphyletic, grouping the species of Trichodectidae and Anoplura together [14]. Genetic analysis of insect species provides useful information on the taxonomic relationships, epidemiology, disease transmission, and control. Despite the importance of the economic damage of lice infestation, little genetic evaluation has been performed in this field. Therefore, to fill this gap, the *B. caprae* DNA sequence was analyzed using a cox1 mitochondrial marker for the first time in Iran.

Result

All collected specimens were of the *B. caprae* species. Table 1 shows the total number of samples collected per city. Using primers (HCO2198, LCO1490), cox1 was amplified in ten samples of a 669 bp fragment, and the PCR product was observed on 1.5% agarose gel. After sequencing the purified PCR samples of cox1, the sequences were validated in the BLAST system of NCBI and compared with the reference sequences in the GenBank. Next, they were registered in the GenBank with assigned access numbers (ID: OK135715-OK135724). The sequences were aligned using MEGA software and the ClustalW method. Phylogenetic relationships were investigated by drawing a phylogenetic tree based on the maximum composite likelihood method with a bootstrap test (1000 replications).

The phylogenetic tree showed a high similarity in the sequences of the isolates in this study. All *B. caprae* samples isolated from the studied cities were located in one sub-branch. Samples of *B. caprae* cox1 isolated from Iranian goats in this study were located next to the Chinese *B. caprae* specimen (MF927687.1). The alignment results of all isolates in this study showed that these sequences are completely similar (Figure 1). During the analyses, one of the sequences related to the city of Bahar isolate (OK135715) was compared with other similar sequences in GenBank and was examined bioinformatically (Figure 2). The phyloge-

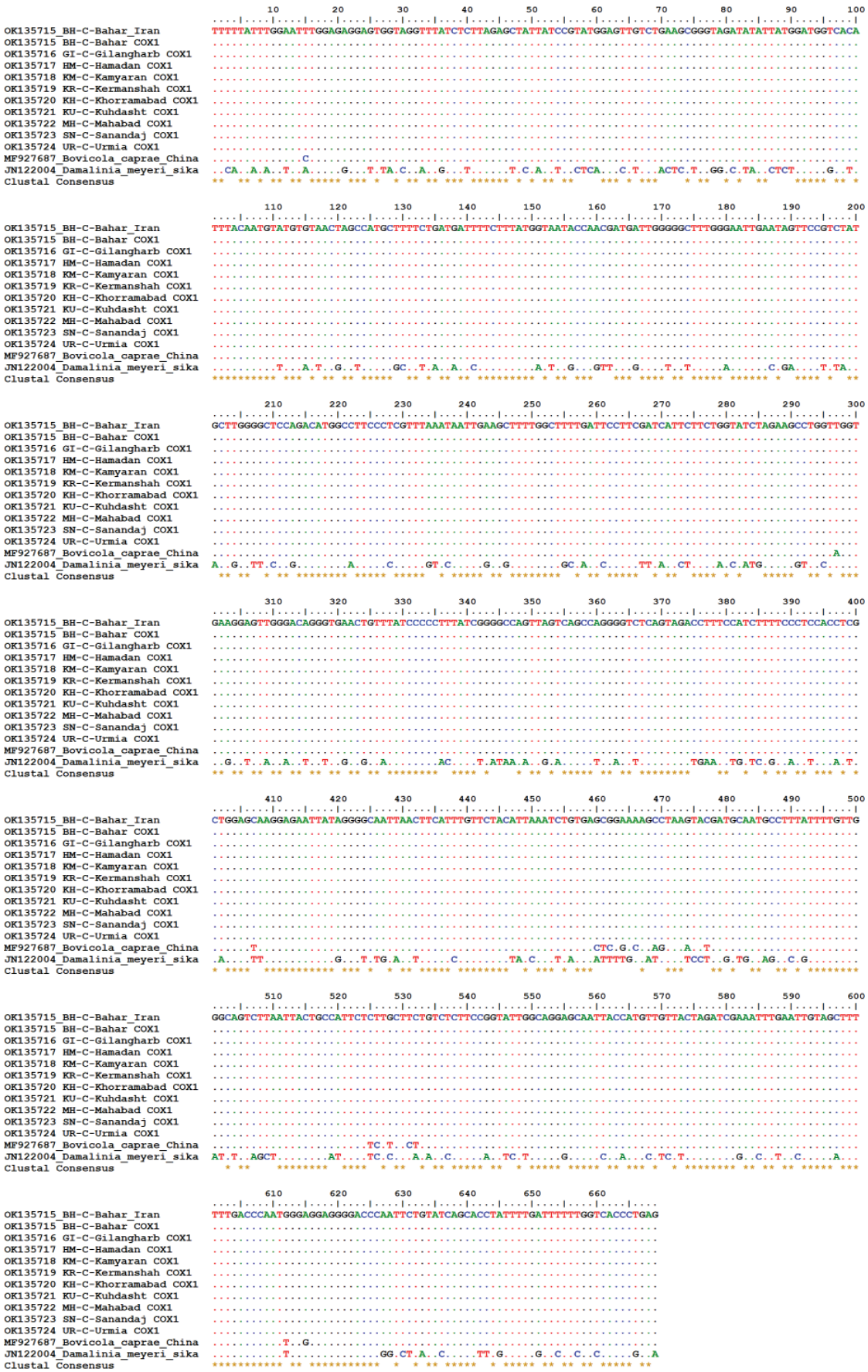

Figure 1.
Alignment results of all isolates in this study with the closest isolate in the GenBank and an outgroup sequence. The * sign in the last line of each row indicates the complete similarity in that row in terms of similar nucleotides.

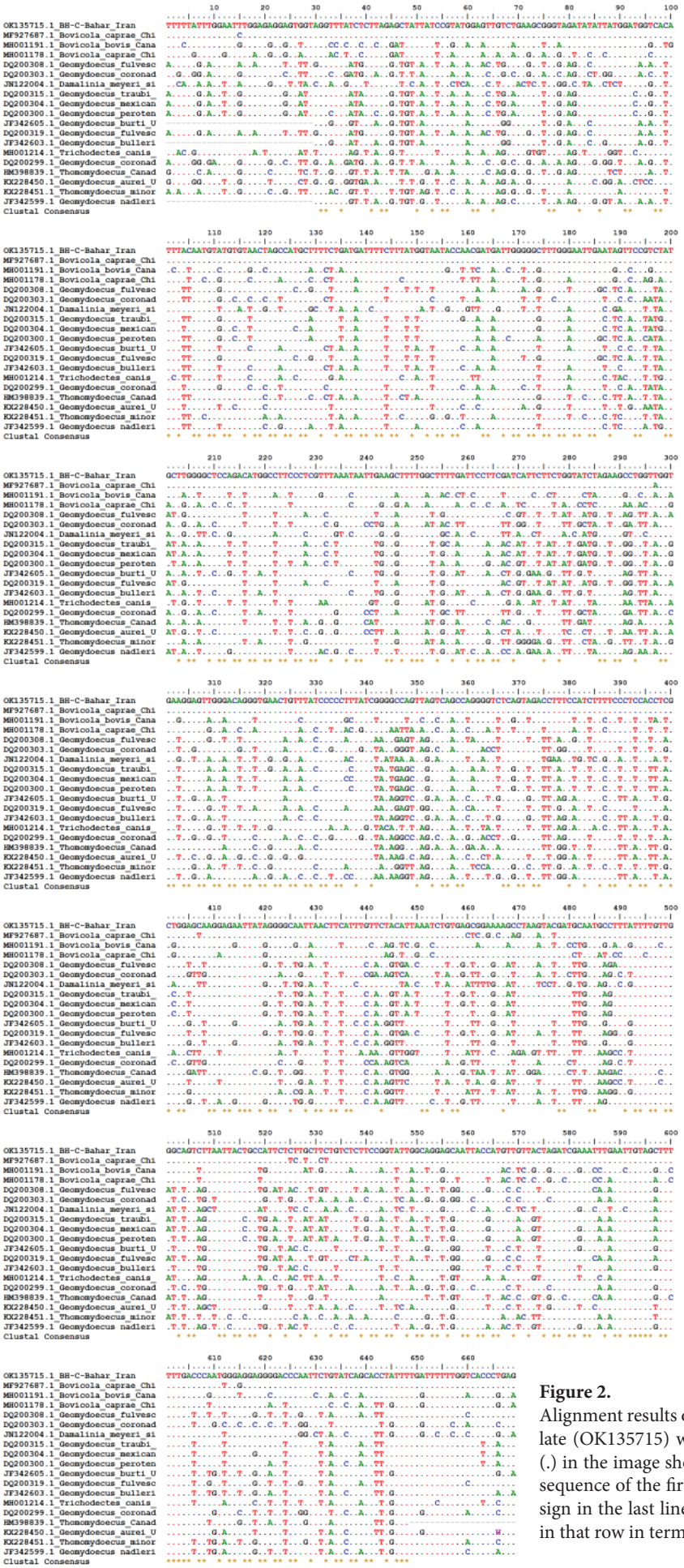

Figure 2.
Alignment results of nucleotide sequences of the city of Bahar isolate (OK135715) with sequences extracted from GenBank. Dots (.) in the image show the complete nucleotide similarity with the sequence of the first line related to the isolate of this study. The * sign in the last line of each row indicates the complete similarity in that row in terms of similar nucleotides.

Table 1.
Total number of *B. caprae* samples collected per city

Province	City	Latitude & Longitude	Species	Number of lice
Kermanshah	Kermanshah	34.1397° N, 45.9206° E	*Bovicola caprae*	122
	Gilangharb	34.3277° N, 47.0778° E	*Bovicola caprae*	130
Kurdistan	Sanandaj	35.3219° N, 46.9862° E	*Bovicola caprae*	71
	Kamyaran	34.7956° N, 46.9368° E	*Bovicola caprae*	86
West Azerbaijan	Urmia	37.5498° N, 45.0786° E	*Bovicola caprae*	102
	Mahabad	36.7684° N, 45.7337° E	*Bovicola caprae*	68
Hamedan	Hamedan	34.9083° N, 48.4393° E	*Bovicola caprae*	131
	Bahar	34.9083° N, 48.4393° E	*Bovicola caprae*	75
Lorestan	Khorramabad	33.4647° N, 48.3390° E	*Bovicola caprae*	83
	Kuhdasht	33.5275° N, 47.6111°E	*Bovicola caprae*	149

netic tree of the city of Bahar isolate (OK135715) and other sequences extracted from the GenBank showed that the isolate sequence of this study is next to the *B. caprae* sequence from China (MF927687.1) and *B. bovis* from Canada (MH001191.1). The closest resemblance was to the *B. caprae* sequence of China, shown in branch B1 (Figure 3). The phylogenetic tree of all isolates in this study along with the closest isolates in the GenBank showed a high similarity in terms of amino acid sequences. The analysis of one of the sequences related to this isolate (UAR89077.1 *B. caprae* Iran) was selected for comparison and bioinformatic studies. The alignment results of these amino acid sequences showed complete similarity to the sequences in this study (Figure 4). Drawing a phylogenetic tree based on the similarity of the amino acid sequence of the selected isolate (UAR89077.1 *B. caprae* Iran) and other amino acid sequences extracted from the GenBank showed that the isolate sequence of this study is mostly similar to the *B. caprae* sequence from China. Next to the AUV47083 sequence are *B. caprae* China and AYC65832 *B. bovis* Canada and AYC65818 *B. caprae* China, which are shown in branch B (Figure 5).

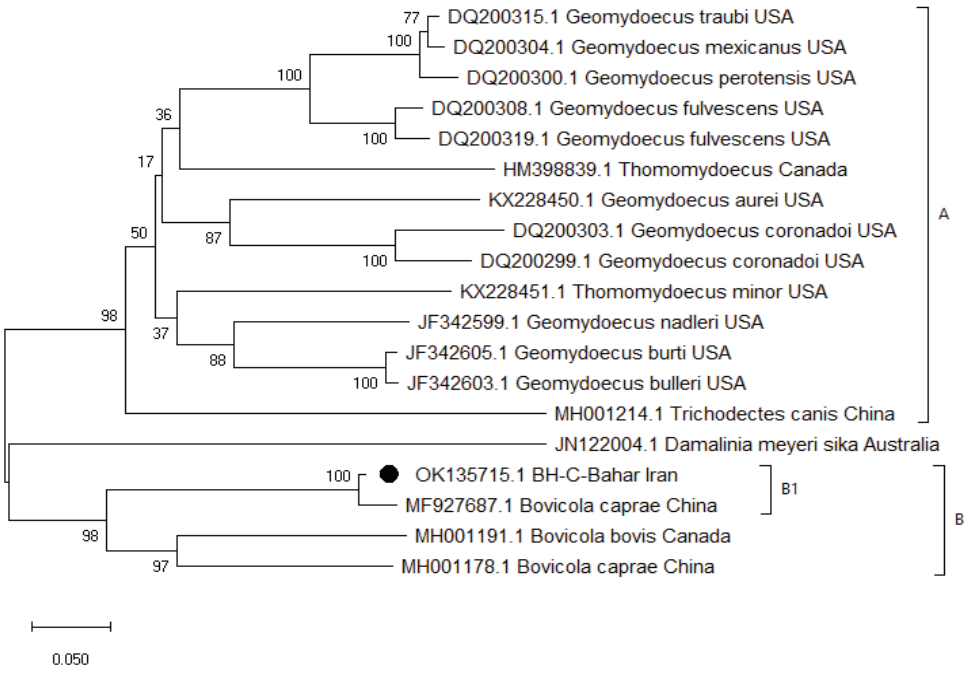

Figure 3.
Phylogenetic tree of Bahar isolate (OK135715) and other sequences extracted from the GenBank

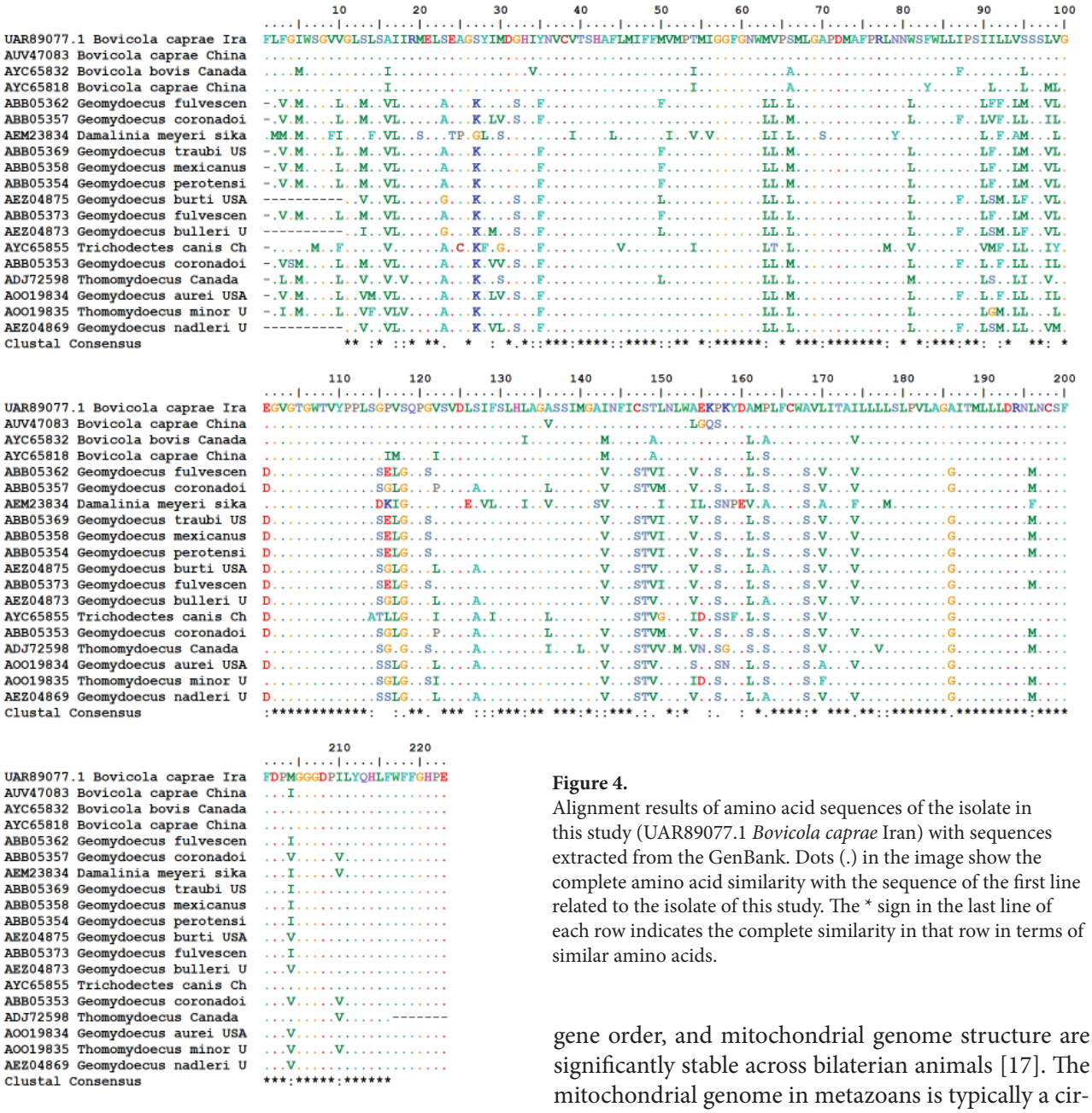

Figure 4.
Alignment results of amino acid sequences of the isolate in this study (UAR89077.1 *Bovicola caprae* Iran) with sequences extracted from the GenBank. Dots (.) in the image show the complete amino acid similarity with the sequence of the first line related to the isolate of this study. The * sign in the last line of each row indicates the complete similarity in that row in terms of similar amino acids.

Discussion

In this study, lice collected from goats in ten cities in the West and Northwest regions of Iran were studied morphologically and then phylogenetically. All collected specimens were identified as *B. caprae*. Yakhchali et al. (2006) investigated the ectoparasites of sheep and goats in Northwestern Iran and identified lice and ticks as the most common ectoparasites of small ruminants in this region and identified the species of *B. caprae* and *Linognathus stenopsis* in goats [15]. The species they identified in this region are consistent with the present research.

Many studies have revealed the value of the mitochondrial genome for the genetic study of population and intraspecific and systematic phylogeny in various organisms, including lice [16]. The gene composition, gene order, and mitochondrial genome structure are significantly stable across bilaterian animals [17]. The mitochondrial genome in metazoans is typically a circular DNA of 13-20 kb with 36-37 genes containing 12-13 protein-encoding genes, 2 rRNA genes, and 22 tRNA genes [16, 18]. However, the mitochondrial genome has an unusual structure in some species of lice and exists as small mini-chromosomes [16]. Among lice species, this fragmented structure was first found in the body lice *Pediculus humanus corporis*. The mitochondrial genome was then identified as small mini-chromosomes in some other species of lice, including *Bovicola caprae* [19-21]. The cox1 gene is a combination of highly conserved and variable regions that make this mitochondrial gene a molecular marker particularly useful for evolutionary studies [11]. The phylogeny of lice at the suborder level has not been resolved despite decades of study. Initially, all chewing lice from three suborders of Ischnocera, Amblycera, and Rhynchophthirina were collectively referred to as

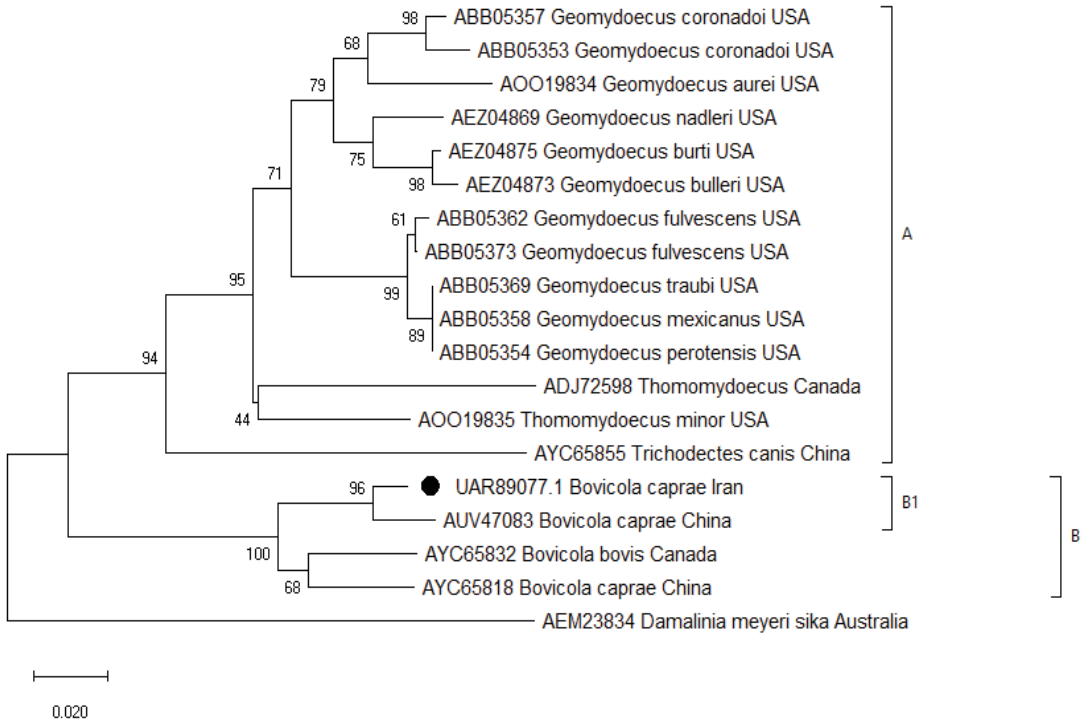

Figure 5.
Phylogenetic tree of the isolate in this study (UAR89077.1 *Bovicola caprae* Iran) and other amino acid sequences extracted from the GenBank

Mallophaga, but further studies showed that Mallophaga is paraphyletic [6]. Cruickshank et al. (2001) analyzed the EF1 sequence in 127 species from four suborders and reported that Ischnocera is paraphyletic [22]. Barker et al. (2003) analyzed 18SrRNA sequences from 33 species and reported Ischnocera as monophyletic [23]. Yoshizawa et al. (2010) analyzed five genes (*18S, histon3, wingless, rrnL, cox1*) and revealed Ischnocera as paraphyletic [24]. Recently, Johnson et al. (2018) analyzed nuclear genes of 46 species and reported Ischnocera as paraphyletic, and two species of Trichodectidae were grouped with the species of Anoplura and Rhynchophthirina [25]. Another study examined the nucleotide and amino acid sequence of protein-encoding genes *atp6, atp8, nad2, nad4, nad4l, nad6, cox1, cox2, cox3, cytb, rrnS, and rrnL* from three species of Hoplopleura. The *atp8* gene had the highest nucleotide diversity while the least diversity was observed in the cox1 gene. The latter finding is consistent with the present study [16]. Al-Shahrani et al. (2017) investigated phylogenetic differences based on two genes *cox1* and *cytb* in human head lice, and divided these lice into three classes [26]. Moreover, Mokhtar et al. (2019) examined the genetic diversity of human head lice using the genetic marker cox1 in Malaysia and reported that the collected samples belonged to clades A, B, and D [27].

This study is the first to demonstrate genetic variation in *B. caprae* lice collected from Western and Northwestern Iran using the cox1 marker. The results of morphological studies were consistent with molecular results and in general, 100% intraspecific similarity was observed in the nucleotide sequence of *B. caprae* samples isolated from ten cities based on the cox1 marker. Moreover, the alignment of the amino acid sequences also showed 100% similarity. *B. caprae* nucleic acid sequences in this study had 97.1% similarity to *B. caprae* (MF927687.1) from China and 77.1% similarity to *B. bovis* (MH001191.1) from Canada. *B. caprae* amino acid sequences in this study had 97.3% similarity to *B. caprae* (AUV47083) from China and 94.1% similarity to *B. bovis* (AYC65832) from Canada. The results of this study showed that cox1 is a useful marker to show intraspecific similarity. It is noteworthy that the cox1 gene sequence in this research contributed to the reference sequences available in the GenBank and also acts as a basis for a larger library of a goat chewing lice sequences in the West and Northwest of Iran.

The gene sequence of cox1 of the samples isolated in the present study is partial. Therefore, a comprehensive study is recommended to examine the complete sequences of this gene and compare and contrast comprehensive bioinformatic studies.

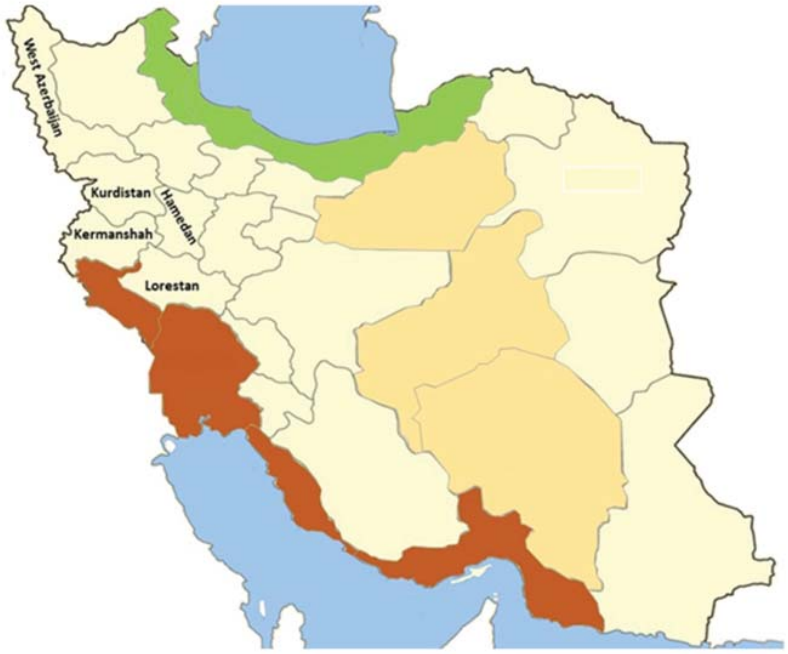

Figure 6.
The mentioned provinces in the current study

Materials and Methods

Sampling

This descriptive cross-sectional study was conducted during September 2017-March 2018 in five provinces in the West and Northwest of Iran (Figure 6). Samples were collected by direct sampling from goats' bodies in cities Urmia, Mahabad, Sanandaj, Kamyaran, Kermanshah, Gilangharb, Khorramabad, Kuhdasht, Bahar, and Hamedan. Out of 420 examined goats, 120 animals were infested by lice. Collected lice samples were fixed in 70% ethanol and transferred to the parasitology laboratory of Urmia Faculty of Veterinary Medicine and were identified using valid identification keys [7, 28, 29].

Morphological identification

All collected specimens of *B. caprae* were identified using morphological features as follows:

- Round head and antenna with three segments
- Distinct ocular points behind the antennae
- The ventral surface of the thorax with dark-colored plates
- All legs of similar size and with a single nail
- Cube abdomen with black side stripes
- Antennae in males are slightly larger than in females and have transverse bands
- Each abdominal segment has a middle spot and a row of short hair between the groove and the spots of each segment

DNA extraction and molecular diagnosis

Genomic DNA was extracted using a commercial DNA extraction kit of MBST (MBST, Tehran, Iran) according to the manufacturer's instructions. The DNA quality and concentration of each sample were evaluated using NanoDrop (Thermo Scientific 2000c, USA) by spectrophotometry and stored at -20°C until further evaluations. Primers designed by Folmer et al. (1994) as LCO1490 (5 - GGT CAA CAA ATC ATA AAG ATA TTG G-3) and HC02198 (5 -TAA ACT TCA GGG TGA CCA AAA AAT CA-3) were used to amplify a 669 bp fragment of cox1 [30]. PCR was performed in a volume of 50 µl and each microtube contained 5 µl DNA template, 5 µl 10X PCR buffer, 1 µl dNTPs (200 µM), 4 µl MgCl2 (50 mM), 1 µl of each primer (20 µM), and 1 µl Taq polymerase (Sinaclon, Iran). The PCR steps consisted of an initial DNA denaturation stage at 95°C for 5 min, and then 35 repetitions, each cycle involving denaturation at 95°C for 45 sec, primer annealing at 55°C for 45 sec, extension at 72°C for 45 sec, and a final extension step to complete polymerization at 72°C for 10 min. The PCR product was visualized using 1.5% agarose gel and UV-Transilluminator (BTS-20M, Japan). Finally, the PCR product was purified and sent along with forward and reverse primers to Takapouzist Co. (Tehran, Iran) for sequencing.

Sequencing and genomic analysis

Sequences were entered on the NCBI website to search for reference sequences with the highest similarity, and the BLAST method was used to find the positions of cox1. Afterwards, the sequences were aligned using MEGA software and any alignment error was resolved by the ClustalW method. All nucleotide sequences obtained in the GenBank were recorded with assigned access numbers, and phylogenetic relationships were investigated. To this end, a phylogenetic tree was drawn based on the maximum composite likelihood method with bootstrap test (1000 replicates) analysis

Authors' Contributions

Conceived and designed the experiments and revised the manuscript draft: KH.S., M.T. performed the experiments, analysed the data and drafted the manuscript: KH.S. All authors approved the final version of the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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