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Genetic Characterization of *Argas persicus* From Iran by Sequencing of Mitochondrial Cytochrome Oxidase I (COX1) and 16s rRNA Genes

Shahriar Yavari[1], Sedigheh Nabian[1], Elahe Ebrahimzade Abkooh[2], Parviz Shayan[1], Hamidreza Shokrani[3]

[1]*Department of Parasitology, Iranian Center for Ticks and Tick-borne Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

[2]*Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*

[3]*Department of Pathobiology, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran*

Abstract:

BACKGROUND: *Argas persicus* has a great importance for health and veterinary, it can transmit many infectious agents such as *Borrelia anserina* (avian spirochetosis) and *Aegyptianella pullorum*. Distinguishing Argasidae due to close morphological relationship is difficult.

OBJECTIVES: In the present study, we performed molecular analyses based on PCR and sequencing of Amplicon derived from 16S rRNA and COX1 genes of *A. persicus* specimens in several provinces of Iran.

METHODS: Out of seventy *Argas persicus* collected and confirmed morphologically, eight ticks were chosen from five provinces of Iran for gene analysis. Their DNA were extracted and amplificated using primers derived from 16 S ribosomal RNA and COX1 genes using PCR. Then the amplicons were sequenced and analyzed by Chromas software and sequence alignment program (Clustal W). Phylogenetic analysis was also conducted using MEGA ver. 6.06 with a maximum-likelihood method.

RESULTS: Sequencing results indicated that all eight samples belonged to *A. persicus* species. Their nucleotide sequencing revealed that the interspecific sequence differences of both genes (16S rRNA genes and COX1) between our isolates were very infrequent. All isolates from different provinces were conserved across regions except for one isolate that exhibited a difference of only 1 nucleotide. Within Phylogenetic tree, *A. persicus* formed a clade with *A. persicus* from other regions of the world (South Arica, Italy, China, and South Australia).

CONCLUSIONS: Our findings suggested a very close phylogenetic relationship between *A. persicus* specimens obtained from different regions of Iran.

Keywords:

Argas persicus, COX1, Phylogenetic analysis, 16S rRNA

Correspondence

Sedigheh Nabian, Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Tel: +98(21) 61117072, Fax: +98(21) 92664469, Email: nabian@ut.ac.ir

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Introduction

Ticks of the genus Argas (Acari, Argasidae) are spread to many parts of the world, probably via poultry hosts (Mehlhorn, 2014; Yu et al., 2015). It has been considered as a parasite of chickens, turkeys, pigeons and other birds (Davari et al., 2017; Kayedi et al., 2016; Pantaleoni et al., 2010). They have a great importance for health and veterinary, which can increase the risk of major direct damages for domestic fowl (turkeys, chickens, Guinea fowls, Helmeted, etc.), (Hoogstraal, 1979; Keirans & Durden, 2001; Koc et al. 2015); Therefore, they attracted a considerable amount of attention due to their potential impact on mentioned birds. Ticks are considered as natural reservoir hosts, which can play an important role in transmission of numerous infectious agents, such as bacteria, viruses and Rickettsia (Hosseini-Vasoukolaei et al., 2014; Orkun et al., 2014), and spirochetes (Parola & Raoult, 2001; Shah et al., 2004). *A. persicus*, also known as Tick of fowl or poultry but, carriers of the Borrelia anserine (spirochete gallinarum), which causes one of the most serious diseases influencing the poultry production (Bourne, 2013; Aslam et al., 2013; Yu et al., 2015). Furthermore, different types of virus (West Nile virus), (Kayedi et al., 2015) and bacteria, including *E.coli*, *Salmonella* sp. (Tavassoli et al., 2015), *Proteus* sp. (GINSBERG, 2013), Aerobacter, Flavobacterium. can be transmitted by *A. persicus* (Keshtkar-Jahromi et al., 2013; Shah et al., 2004). Previous studies provided some information about the distribution pattern of these ectoparasites, as well as epidemiology, morphology, transmission of diseases (Ahmed et al., 2007; Chegeni & Tavakoli, 2018; Chitimia et al., 2010; Muñoz-Leal et al., 2018; Rezaei et al., 2016), and less effort has been made in genetic characterization of *A. Persicus*.

In the current study, genetic analyses were performed for providing both a better understanding of the specific characterization of their genetic architecture. A number of studies have reported that Cytochrome oxidase I (COX1) and 16S ribosomal RNA (16S rRNA) genes are capable to provide valuable resources for the molecular phylogeny and genetics of these organisms (Cruickshank, 2002; Dermauw, 2013; Greay et al., 2016; Lu et al., 2013). It seems that there is not any information about genetic characters of Argas genus ticks in Iran. Hence, the current study was performed for the first time based upon COX1 and 16S rRNA genes of specimens obtained from several provinces of Iran.

Material and Methods

Tick sources: Argas ticks isolates were collected in January 2016 from different geographic locations of Iran including Sanandaj, Kermanshah (Gilan Gharb), Urmia, Lorestan (Poldokhtar), Lorestan (Khoramabad), Kermanshah (Dallaho), Kermanshah (Sarpole Zohab) and Hamedan in May 2016

Sample preparation: Out of seventy *Argas persicus* collected and confirmed morphologically, eight ticks were chosen from five provinces of Iran for gene analysis. The isolates of *A. persicus* were confirmed by morphological features based upon use of comprehensive keys and preserved in 70% alcohol; thereafter, samples were transported to the parasitology laboratory, Faculty of Veterinary Medicine University of

Tehran. Samples were dried on filter paper and finally homogenized. Genomic DNA was also extracted from different ticks by a DNA extraction kit (MBST, Tehran, Iran), according to the manufacturer's recommendations with a little change (Shayan et al., 2007). The ticks were carefully crushed using a germ-free pounder for around 10 min. Then, 180µl of lysis buffer was added to the crushed ticks. After shaking and homogenization, 20µl proteinase K (10 mg/ml) was added to the tube, followed by incubation of mixture at 55°C for 10 min and incubated for 24h overnight at 37 °C. Afterward, 360µl binding buffer were added to the tube and thereafter incubated for 10 min at 70°C. In the next step, 270µl ethanol 100% was added to the solution. Then, the solution was vortexed on a mini-vortex mixer and the whole volume was transferred to the MBST-column, followed by centrifugation at 8000×g. Subsequently, the columns were washed twice with 500µl washing-buffer at 8000×g. To eliminate the remaining ethanol from solution, columns were then centrifuged at 12000×g at the end of the extraction protocol. Finally, DNA samples from each isolate were eluted with 60µl elution buffer and immediately stored at −20°C. The extracted DNA was electrophoresed and analyzed on 1.5% agarose in 0.5 % TBE buffer using safe stain and a ultra-violet (UV) transilluminator. The quantity of the extracted DNA was measured by using NanoDrop spectrophotometer.

Polymerase chain reaction (PCR)

16 S rRNA and COX1 originated specific primers were applied for confirming all isolates using PCR. The following primers were used: 16S rRNA, 5'-GCTCAATGATTTTTTAAATTGCTGTGG-3' and 5'-CCGGTCTGAACTCAGATCAAGTA-3'(Black & Piesman, 1994); COX1, 5′-AGCCATTTTACCGCGATGATT-3′ and 5′- GTATTGAAGTTTCGGTTCGGT -3′. In the current study, primers were designed by oligo7 software for COX gene. PCR was performed in a final volume of 50µl, including 100 ng of template DNA, 25 µl Maxima Hot Start PCR Master Mix (2×) (Bio-Rad, United State),

1µl (20µMol) of each primer, and 20 µl of nuclease-free water. The samples were then amplified in a thermo cycler (Bio-Rad, United State). Amplification was performed with a program consisting of one cycle represents initial denaturation at 95 ˚C for 5min, followed by 36 cycles of denaturation at 95 ˚C for 45s, annealing at 55 ˚C for 45s, and extension at (72 ˚C for 45s). Finally, the process was completed by final extension at 72 ˚C for 7 min. PCR amplicons were loaded on 1.5% agarose gel, stained with Simply Blue safe staining (Invitrogen) and visualized by ultraviolet transilluminator (genius, USA). The amplicons were sequenced by Macrogen company (Korea).

Sequence and phylogenetic analysis: Nucleotide sequences of 16srRNA and COXI genes from all collected ticks were aligned with each other and other corresponding registered sequences to evaluate their similarities.

Multiple consensus sequences were modified using BioEdit sequence alignment editor (DNA Align Editor). Sequences were aligned using the program online Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo).

In the present study, partial sequence alignment of 16s rRNA mitochondrial gene was performed with 21 Argas sequences retrieved from GenBank (AY436769.1, AY436769.1, GU355920.1, L34321.1,

AF001404, GU451248.1, KR297209.1, KX258880.1, LC209198, KJ465099.1, KJ465101.1, DQ295778.1, KX855210.1, KY705381.1, KY705381.1, KX855207.1, KX855206.1, AY436768.1, GU355921.1 AB819157.1, AF001403.1, KC769587.1, AY436767.1, EU283344.1, L34322.1, AF001401.1 KJ133580.1) and eight sequences obtained in the present study.

Phylogenetic analyses (Evolutionary relationships) of *A.persicus* with other ticks based on 16srRNA nucleotide sequence were conducted based upon the use of the maximum composite likelihood method using MEGA 6.06 version. Branch support was evaluated by bootstrapping over 1000 replications. Sequences of Ornithodoros rostratus (Spain; DQ295780) and Ornithodoros brasiliensis (Brazil; GU198368) were applied as out-group.

After there, nucleotide sequences were translated to corresponding peptide sequences (https://web.expasy.org/translate). Furthermore, multiple alignment of obtained peptide sequences of *A. persicus* in this study with COX1 gene reference sequence (L34321.1) was performed.

Results

Regarding to the 16S rRNA and COX1 specific primers, sequencing indicated that eight samples belonged to *A. persicus* groups, where the morphological study confirmed this finding. In addition, generated sequences were assembled and Basic Local Alignment Search Tool (BLAST) was subsequently applied to deduce closest similarities with other Argasid species available in GenBank.

PCR amplification of each target gene of 16srRNA and COXI from individual DNA of *A.persicus* isolates resulted in amplicons

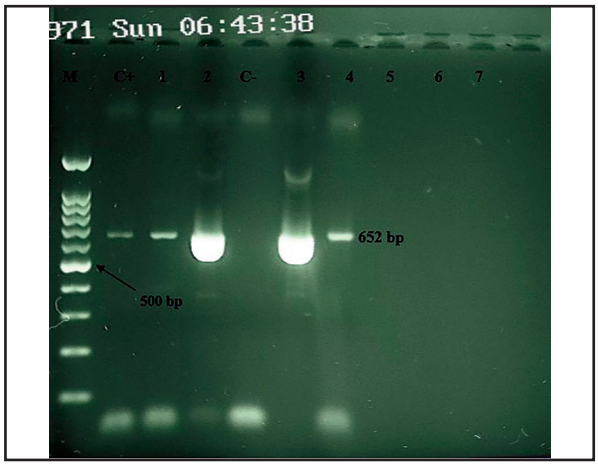

Figure 1. The result of PCR product electrophoresis using the COX primer pair. C- ; negative control, M; marker 100 bp, C +; Positive control of *A. persicus*, well No. 1, 2, 3, and No. 4 Positive samples of *A. persicus* in terms of COX gene.

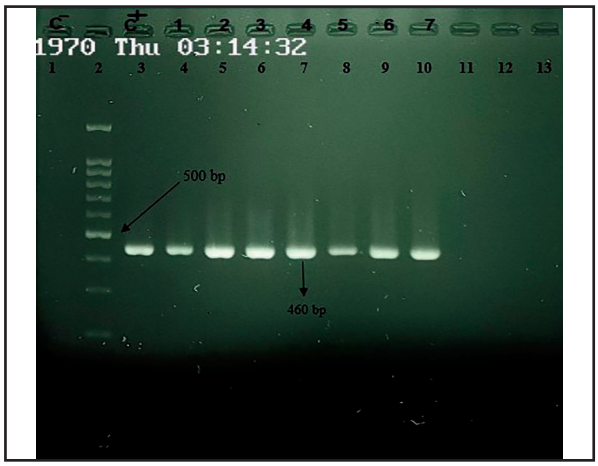

Figure 2. The result of PCR product electrophoresis using the 16s rRNA primer pair. Well No.1; Negative control, No. 2 marker 100 bp, No. 3; positive control of *A. persicus*, No. 4, 5, 6, 7, 8 and 9; positive samples of *A. persicus*.

of the expected size which were 460 bp and 650 bp in length respectively (Fig. 1, 2). All PCR products (amplicons) exhibited a distinct band. Overall, in a sample set, there was no detectable length difference among different tick species. In comparison, among all examined *Argas persicus* species sequences, the interspecific sequence differences of both genes (16S rRNA genes and COX1) were found to be very infrequent. The Multiple alignments of all tick isolates at 16s rRNA gene showed only 2 variable

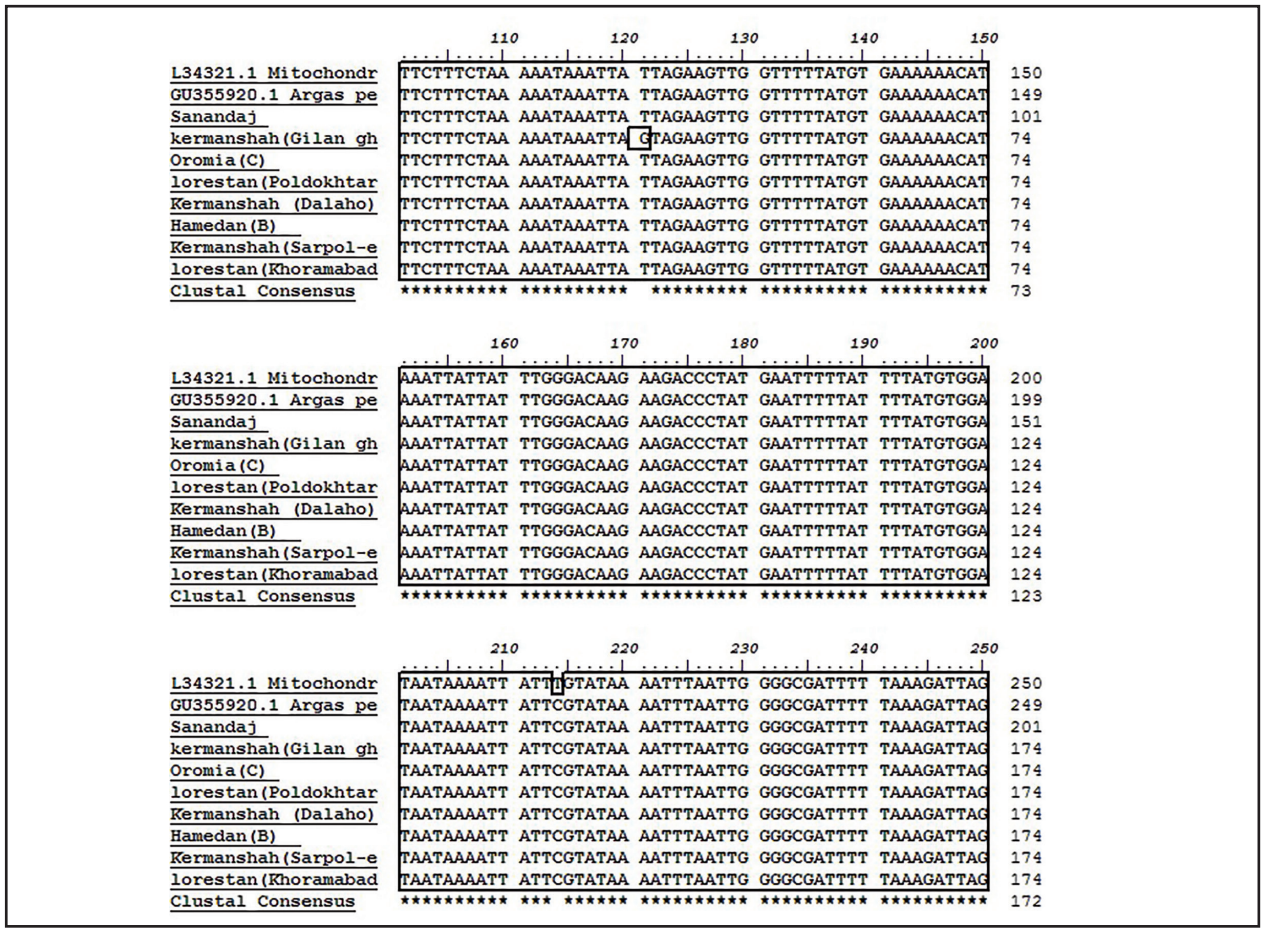

Figure 3. Multiple sequence alignment of the inferred nucleotide acid sequence for 16S rRNA (reference sequence number L34321.1; USA) with other soft ticks (*Argas Persicus*). *A. persicus* isolates (GU35592.1, South Africa), from Sanandaj, Kermanshah (Gilan Gharb), Urmia, Lorestan (Poldokhtar), Lorestan (Khoramabad), Kermanshah (Dallaho), Kermanshah (Sarpol-e Zahab) and Hamedan were aligned together. The small charts nucleotides indicate the mutation in these regions.

nucleotides, the first at position 121(G / A) only in Kermanshah isolate and the second at position 214 (T/C) in all studied isolates in comparison with reference sequence number (L34321) (Fig.3). Sequencing of 16s rRNA was showed *A. persicus* is homogeneous with the other region of the world

Describing sequencing results of COX1 analysis revealed that all isolates from different provinces (Sanandaj, Urmia, Lorestan (Poldokhtar; Khoramabad), Kermanshah (Dallaho), Kermanshah (Sarpol-e Zahab) and Hamedan were conserved across regions except for one isolate identified as Kermanshah isolate (Gilan Gharb) that had variations at one location. This variation is a transition where a purine nucleotide is changed for another purine (T/C) (Fig. 4). Our finding suggested that nucleotide variation has a frequency of 1 percent among *A. persicus* ticks obtained from Kermanshah (Gilan Gharb) and those from the South Africa (KJ133581.1), Chile (KX258880), Brazil (KX258880.1), Italia (GU451248.1), and U.S.A (L34321.1), Romania (FN394341.1), Australia (AY436770.1), United States (L34321), Egypt (AF001402) and China (KR297209.1).

Based on the finding presented herein, the major Iranian host of *A. persicus* was the domestic fowl and turkeys. On the other hand, low number of infestation was found,

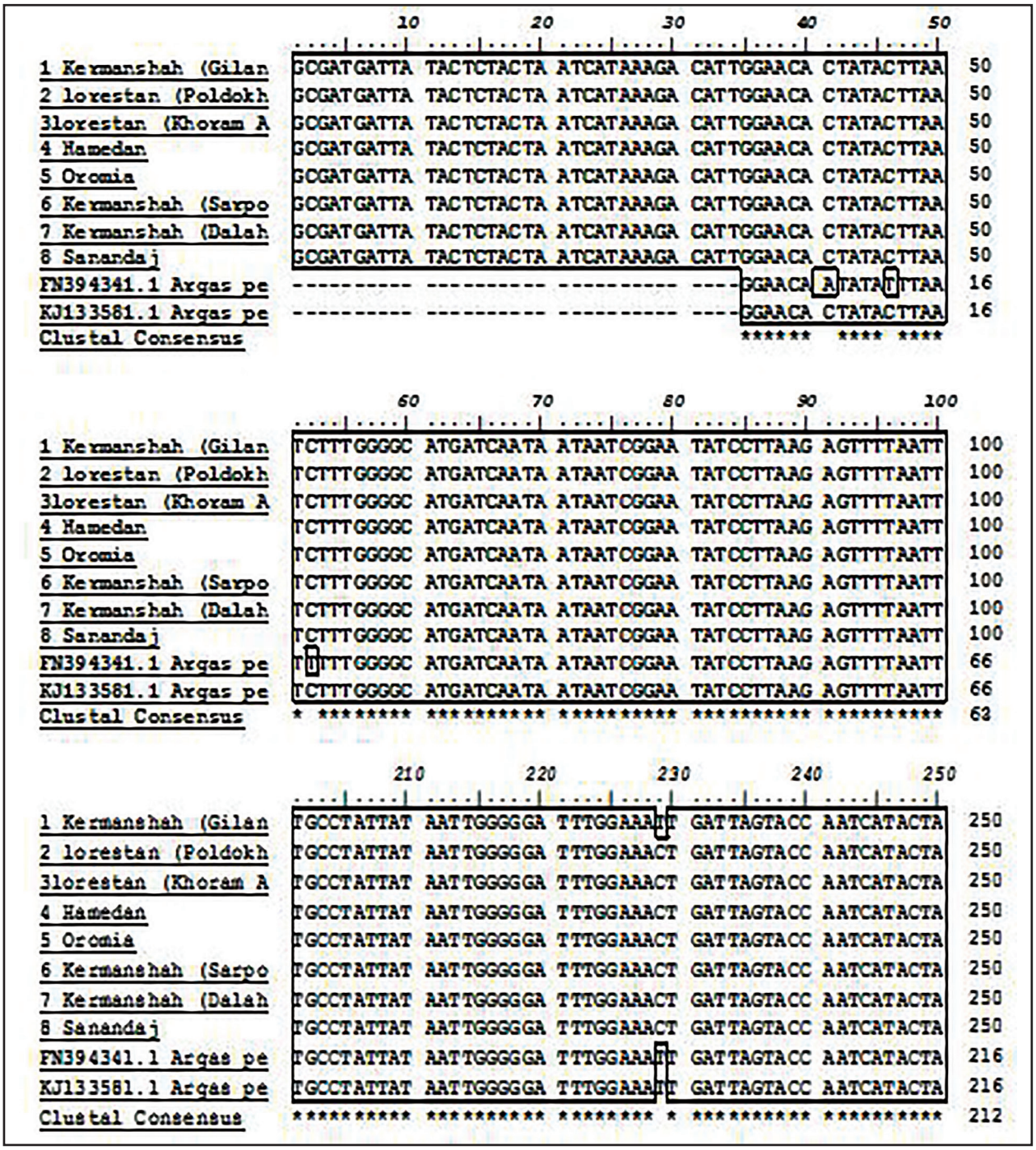

Figure 4. Multiple sequence alignment of the inferred nucleotide acid sequence for COX1 gene; Reference sequence originating from South Africa and Romania (FN394341.1), namely *Argas persicus* (KJ133581.1) compared with other soft ticks (*Argas Persicus*). COX1 gene from *A. Persicus* Sanandaj, Kermanshah (Gilan Gharb), Urmia, Lorestan (Poldokhtar), Lorestan (Khoramabad), Kermanshah (Dallaho), Kermanshah (Sarpol-e Zahab) and Hamedan were aligned together. The small charts nucleotides indicates the mutation in this regions. Sequencing findings of COX1 showed that all isolates had similar interspecific nucleotides except for Kermanshah (Gilan Gharb) isolate. Nucleotide sequence of COX region from Kermanshah (Gilan Gharb) isolate was similar to a specimen of *Argas persicus* from South Africa (KJ133581.1) and Romania (FN394341.1).

in cold areas such as Dalaho (Kermanshah) and Urmia, while tropical areas were found to have suitable habitat for tick destitution. The *A. persicus* group observed in tropical regions such as Kermanshah and Lorestan, is attracting a great deal of attention

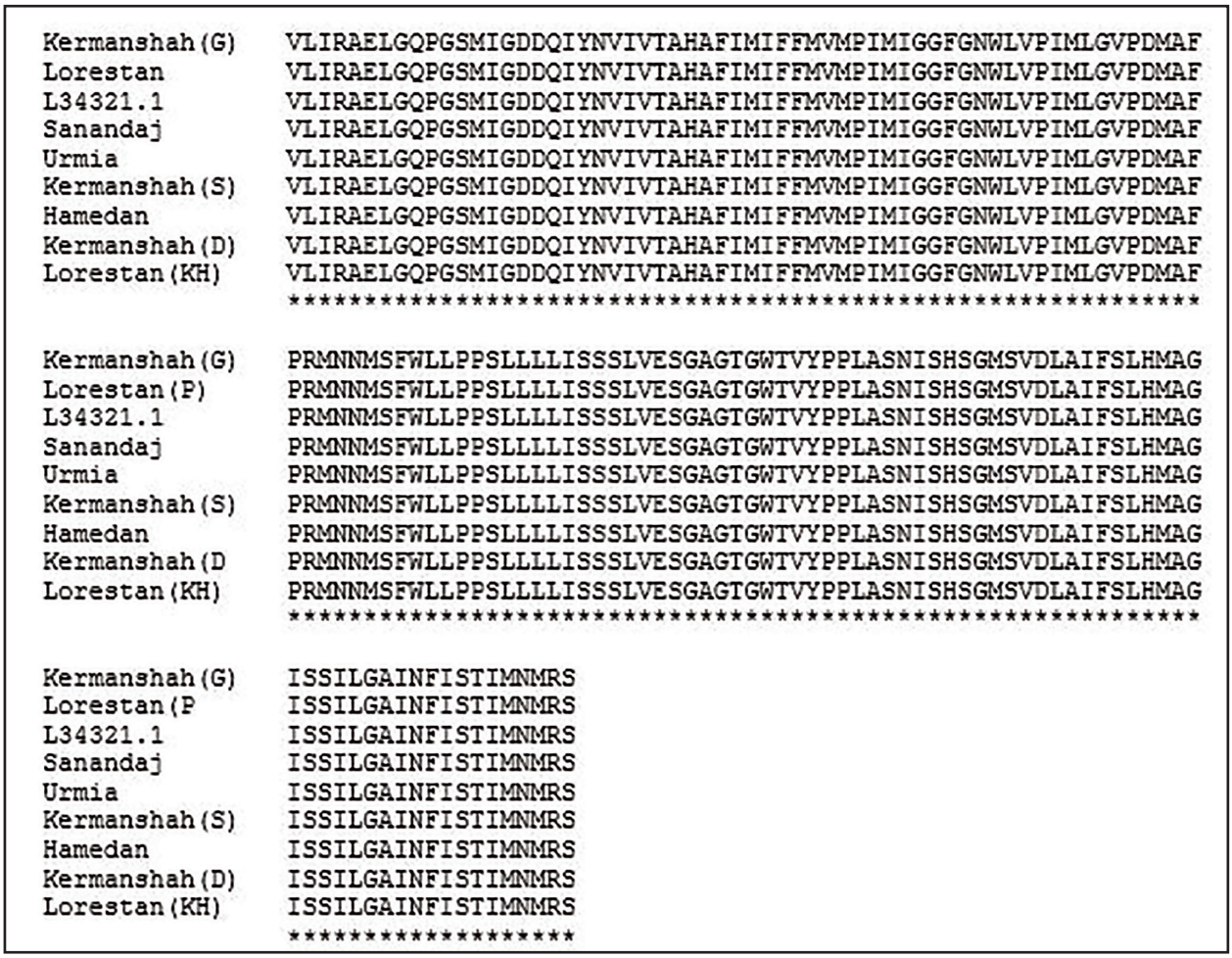

Figure 5. Multiple sequence alignment of the inferred protein sequence of reference COX1 gene (L34321.1) with other soft ticks (*A.persicus*). COX1 gene of *A. Persicus* from Sanandaj, Kermanshah (Gilan Gharb), Urmia, Lorestan (Poldokhtar), Lorestan (Khoramabad), Kermanshah (Dallaho), Kermanshah (Sarpole Zahab) and Hamedan were aligned together. Results showed that all isolates had similar amino acid sequences.

from researchers as it offers evidence for the distribution of *A. persicus* ticks in tropical regions of Iran. Results of multiple sequence alignment of COX1 proteins demonstrated that all isolates had similar interspecific nucleotides (Fig.5).

Phylogenetic analysis: Phylogenetic relationships based on 16srRNA clearly displayed *A. persicus* grouping in a similar clade supported by high bootstrap value for all branchings (Fig. 4). Within Phylogenetic tree, *A. persicus* formed a clade with *A. persicus* from other regions of the world (South Arica, Italia, China, and South Australia), (Fig.6). Our analysis revealed that, all subgenus persicargas (Argasidae group) including *A. persicus*, *A. miniatus*, and *A. walkerae* constitute the monophyletic group of Argasinae. Phylogenetic tree suggested that all mitochondrial genomes of the Argasidae family were located in a monophyletic clade and confirmed by high values of posterior probabilities.

Discussion

A. persicus is globally distributed to tropical and sub-tropical areas of the world (Hoogstraal & Kim, 1985), that is known as a fowl parasite with medical importance. It serves as the vector of avian spirocheto-

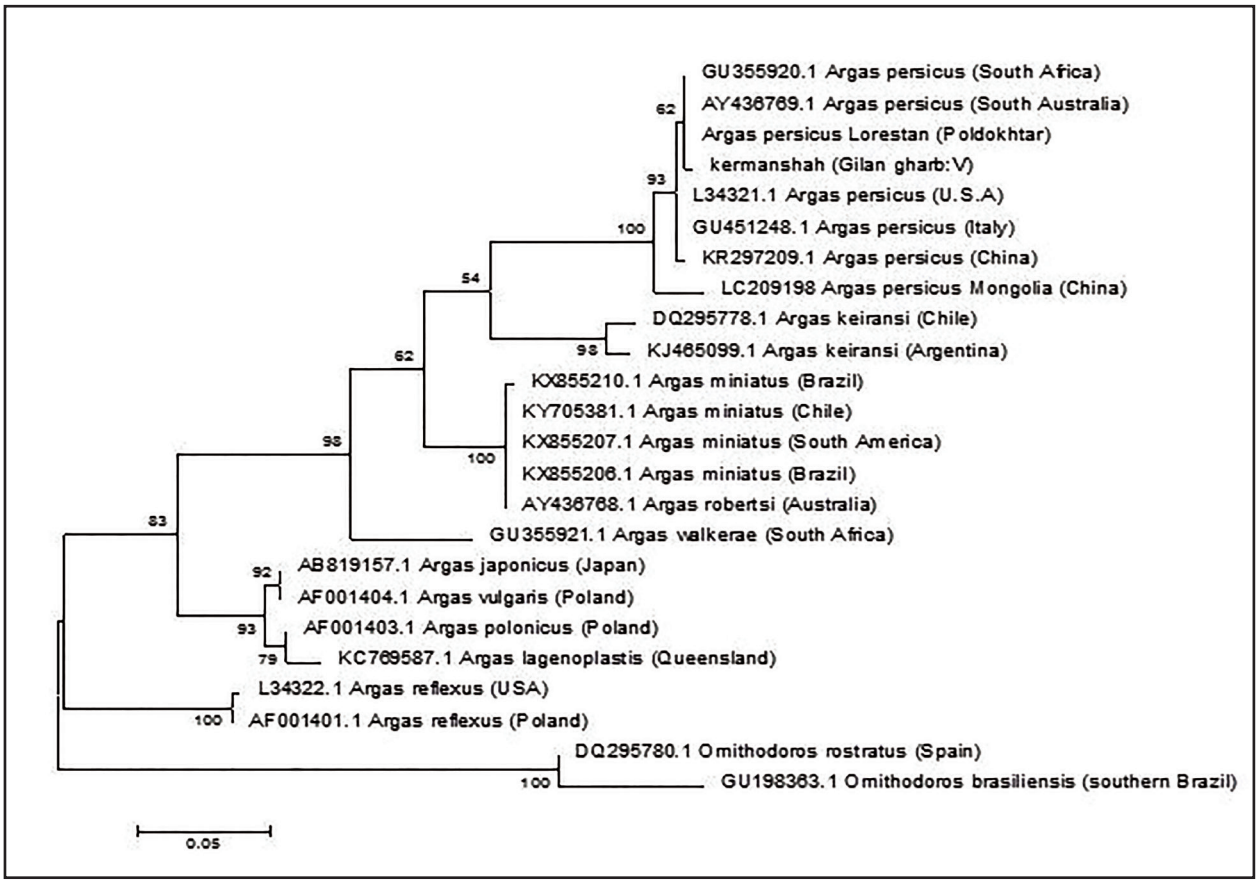

Figure 6. Phylogenetic relationship tree based upon partial nucleotide sequences of the 16s rRNA gene from different *Argas Persicus* species of Iran, that was constructed by the maximum composite likelihood method using MEGA 6.06 version. Vertical distances were arbitrary. The numbers at each branch denote bootstrap values with 1000 replicates. Kermanshah and Lorestan samples are those sequenced in the progress of this research. The source of each tick is written within parentheses and GenBank accession numbers are pointed in front of it.

sis (*Borrelia anserina*) and aegyptianellosis (*Aegyptianella pullorum*), (Khater et al., 2013; Tavassoli et al., 2015)). Additionally, it is involved in spreading West Nile virus (WNV; Flaviviridae), *Salmonella pullorum*, and *Salmonella gallinarum*, as well as *Rickettsia* spp. of the spotted fever group (Tavassoli et al., 2015; Yu et al., 2015).Taxonomic distinguishing of Argasidae ticks (soft ticks) is difficult using macroscopic and microscopic examination (Ronaghi et al., 2015); thus, molecular-genetic characterization of the Argas ticks is highly recommended, where 16S rRNA and COX1 genes are recognized as appropriate markers to investigate their phylogenetic or evolutionary characteristics (Cruickshank, 2002). For providing better phylogenic findings Black and Piesman (1994) have applied 16S rRNA to examine the phylogeny of tick's subfamilies (Ixodidae: Argasidae). In a study by Crosbie et al. (1998) a 300-bp portion of the mitochondrial 16S rRNA was used to determination phylogenetic relationships of the Dermacentor species. In accordance with our findings, they suggested that 16S rRNA appear as a suitable marker for use in phylogenetic analysis (Crosbie et al.. 1998). In addition, COXI gene used for this purpose and compared with some the other genes (Chitimia et al., 2010). The finding of our study indicated that the interspecific sequence differences were very rare among *A. persicus* isolates obtained

from dissimilar provinces. In the other same study Petney et al. (2004) revealed a variation of 0.5–1.5% between the three *A. persicus* ticks from Australia using 16s r RNA gene. Muñoz-Leal et al. (2018) reported an intriguing coincidence between two *A. persicus* species with vastly distanced geographical distributions as pointed previously by Burger et al. (2014). Another study by Burger et al. (2014) confirmed a close phylogenetic association between *A. miniatus* from Brazil and *A. robertsi* from Australia, where two specimens exhibited a difference in just one nucleotide over 400-bp of 16S rRNA region. Some evidence indicated that a very close phylogenetic relationship can be observed among Argas (Persicargas) species even in distant geographic areas. In the present study, domestic fowl was the most frequently host for *A. persicus*. Mirzaei et al. (2016) and Lafri et al. (2018) described that the fowl tick *A. persicus* has a perfect adaptation and cohabitation with domestic fowl. In agreement with our study, *A. persicus* has been reported as a common parasite of poultry (Hoogstraal & Kim, 1985; Lafri et al., 2018). Based on the data presented in our study all obtained isolates from different provinces were conserved across marker regions except one isolate that was completely similar to those from South Africa, Chile, Brazil, Italy, U.S.A, Australia, Egypt and China. Another study noted that the 16S rRNA marker region exhibited a close phylogenetic association (99–100%) between *A. persicus* from Australia and *A. persicus* miniatus from Brazil (Muñoz-Leal et al., 2018). There is satisfactory agreement between our results and the findings of Petney et al. in 2004 ,which showed a high similarity (variation of 0.5–1.5%) was found between *A. persicus* ticks from Australia and those from the United States (Ac: L34321) and Egypt (Ac: AF001402. In the current study, the 16S rRNA marker region demonstrated a close phylogenetic association between our samples and *A. persicus* of GenBank (L34321). Two sequences showed a difference by only 1 bp; it consists of 2 transitions (point mutation) between the first at position 121(G/T) and the second at position 214 (T/C), while these changes were difference from other regions of the world (Muñoz-Leal et al., 2018; Petney et al., 2004). In the current study, phylogenetic tree demonstrated that all *A.persicus* groups create a monophyletic group. Our results was consistent with previous studies from different countries, such as Australia , Brazil, Chile and Cuba (Muñoz-Leal et al., 2018), USA (Black & Piesman, 1994) and Netherland (Burger et al., 2014). It should be taken a consideration that morphological characters of Argasidae soft ticks are very similar (Keirans & Durden, 2001; Manzano-Román et al., 2012; Muñoz-Leal et al., 2018). The similarity in genetic and morphological traits of *A. persicus* isolates render them a significant challenge for resolving phylogenetic links among very closely associated species or within species that has not yet been resolved. Hence, detailed research studies are needed in terms of morphological and genetic characteristics using another gene, as well as other mechanisms underlying phylogenetic relationship. Our findings revealed that these species were highly distributed in tropical areas, when compared with cold areas such as Dalaho in Kermanshah and Urmia. However, Muñoz-Leal mentioned that *A. persicus* miniatus is distributed in tropical climatic zones, while conversely the distributions of *A. persicus* overlap in many areas with dry

climates (Muñoz-Leal et al., 2018). A high rate of *A. persicus* distribution was found during spring in the Alashtar county that is in agreement with our study (Davari et al., 2017). We conclude that there are low levels of sequence variation among *A. persicus* isolates from different provinces of Iran. Furthermore, our findings suggested a very close phylogenetic relationship between our *A. persicus* specimens and other sequences from other regions of the world. Our research was the first effort to clarify interspecific genetic variability at the mitochondrial DNA (mtDNA) level in Iranian *A. persicus* using 16srRNA and Cox1 sequences. The results provided that the 16 srRNA and Cox1 sequences could offer a more extensive documentation of their suitable capacity for characterizing genetic architecture and detection of ticks worldwide.

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Conflicts of interest

The author declared no conflict of interest.

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خصوصیات ژنتیکی کنه آگاس پرسیکوس ایران بر مبنای توالی ژن میتوکندریایی COX۱ و ۱۶SrRNA

شهریار یاوری[۱] صدیقه نبیان[۱] الهه ابراهیم زاده آبکوه[۲] پرویز شایان[۱] حمید رضا شکرانی[۳]

[۱]گروه انگل شناسی، مرکز مطالعات کنه‌ای و بیماری‌های منتقله ایران، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران
[۲]گروه پاتوبیولوژی، دانشکده دامپزشکی دانشگاه فردوسی مشهد، مشهد، ایران
[۳]گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه لرستان، لرستان، ایران

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چکیده

زمینه مطالعه: اهمیت آگاس پرسیکوس در دامپزشکی و بهداشت است و توانایی انتقال بسیاری ازعوال عفونی مانند بورلیا آسرینا (اسپیروکتوزیس ماکیان) و اجپتینلا پولروم را دارد، تشخیص ریخت شناسی در آرگاس به دلیل شباهت ظاهری دشوار است.

هدف: ایـن مطالعه تحلیل مولکولی بر اسـاس PCR دوژن COX۱ و ۱۶SrRNAوتحلیل توالی یاب محصول تکثیر آن‌ها در آگاس پرسیکوس چند استان کشور ایران را مد نظر داشت.

مواد و روش کار: از ۷۰ مورد کنه آگاس پرسیکوس جمع آوری شده که با ویژگی‌های ریخت شناسی تایید شدند، هشت کنه از پنج استان کشور برای تحلیل ژنی انتخاب شدند، DNA آن‌ها استخراج و با استفاده از پرایمرهای مشتق شده از دوژن COX۱ و ۱۶SrRNAتکثیر این دو ژن صورت گرفت ، محصول توالی یابی شد و بر اساس نرم افزار توالی یابی کروماس و مرتب سازی توالی‌ها با نرم افزار (Clustal W) تجزیه وتحلیل فیلوژنتیکی آن با استفاده از برنامه MEGA ver ۶/۰۶ با بیشترین اعتماد انجام شد.

نتایج: نتایج تعیین توالی نشان داد که تمام هشت نمونه متعلق به گونه آگاس پرسیکوس بودند. توالی‌های نوکلئوتیدی نشان داد که تفاوت‌های توالی بین دو ژن (ژن ۱۶S rRNA و COX۱) بین جدایه‌های ما بسـیار نادر بود. تمام جدایه‌ها ازمناطق مختلف استان‌های مختلف به جز یک جدایه از گیلان غرب استان کرمانشاه که تنها یک نوکلئوتید اختلاف داشت یکسان بودند، در آگاس پرسـیکوس گیلان غرب کرمانشـاه با دیگر نقاط جهان مانند آفریقای جنوبی وآمریکا ۱دراختلاف بود، آگاس پرسیکوس ایران در درخت فیلوژنی در کلاد آفریقای جنوبی، ایتالیا، چین و جنوب استرالیا قرار دارد.

نتیجه گیری نهایی: یافته‌های ما نشان می‌دهد که رابطه ی فیلوژنتیک بسیار نزدیکی بین نمونه‌های آگاس پرسیکوس در مناطق مختلف ایران وجود دارد.

واژه‌های کلیدی:
آگاس پرسیکوس، COX۱ ، تحلیل فیلوژنتیکی، ۱۶SrDNA

نویسنده مسؤول: تلفن: ۶۱۱۱۷۰۷۲ (۲۱)۹۸+ نمابر: ۹۲۶۶۴۴۶۹ (۲۱)۹۸+ Email: nabian@ut.ac.ir