Molecular characterization of *Rhipicephalus (Boophilus) annulatus* from Iran by sequences of cytochrome c oxidase subunit I (COI) and the second internal transcribed spacer (ITS2)

Ronaghi, H.[1], Nabian, S.[2,3*], Ebrahimzadeh, E.[2,3], Biranvand, F.[1], Shayan, P.[2,3]

[1]*Graduated from the Faculty of Veterinary Medicine, University of Tehran, Iran*
[2]*Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran*
[3]*Iranian Center of Ticks and Tick-born Diseases, Faculty of Veterinary Medicine, Tehran, Iran*

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Correspondence
Nabian, S.
Department of Parasitology,
Faculty of Veterinary Medicine,
University of Tehran, Iran
Tel: +98(21) 61117072
Fax: +98(21) 66933222
Email: nabian@ut.ac.ir

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Abstract:

BACKGROUND: Traditionally, morphological features of *Rhipicephalus (Boophilus) annulatus* from closely-related ticks have been considered for their identification and differentiation. However, it is difficult and requires expertise in order to accurately identify and differentiate engorged female ticks and some developmental stages such as larva and nymph from other similar ticks. Hence, molecular markers may be a suitable alternative. **OBJECTIVES:** Mitochondrial cytochrome c oxidase subunit I (COI) gene and the second internal transcribed spacer (ITS2) fragments of *Rh. (Bo.) annulatus* were sequenced to assess the use of molecular techniques for identifications and phylogenetic studies of these ticks. **METHODS:** Polymerase chain reaction (PCR) technique was performed based on the analyses of COI and ITS2 sequences of ticks collected from two different regions in Iran (Golestan and Mazandaran). **RESULTS:** The length of COI and ITS2 sequences were 1539 and 1158bp, respectively. The nucleotide similarity of COI gene was 91.3% between the ticks examined from the two different regions. The deduced amino acid sequences from COI showed 98.6% similarity between the ticks studied and showed 98.2 and 99.6% similarity with the only complete sequence of *Rh. (Bo.) annulatus* (AGH19677) registered in GenBank. The obtained complete nucleotide sequences of ITS2 from *Rh. (Bo.) annulatus* from Golestan and Mazandaran revealed 99.9% similarity, while the other ticks registered in GenBank 95 to 99% similarity (KC503267, AF271270, AF271272, JQ412126). **CONCLUSIONS:** It seems that COI and ITS2 sequences could provide suitable genetic markers for discrimination and genetic characterization of *Rhipicephalus (Boophilus) annulatus*.

Introduction

Rhipicephalus (Boophilus) annulatus is a member of Ixodidae family. This tick was formerly known as *Boophilus annulatus*; however, recently it has become a subgenus of the *Rhipicephalus* genus (Murrell and Barker, 2003). *Rh. (Bo.) annulatus* is an obligate blood-feeding ectoparasite widely distributed in the north of Iran (Rahbari et al., 2007;

Razmi et al., 2007). Heavy tick burdens can decrease production and damage hides (Jongejan and Uilenberg, 2004). They can also transmit different pathogenic agents such as *Babesia* spp. and *Anaplasma marginale* (Jongejan and Uilenberg, 2004).

Morphological features and ecological distribution are commonly used for the identification of tick specimens; however, these methods are not effective for damaged specimens, engorged female ticks, and the morphology of immature stages that has not been described so far (Nava et al., 2008). Molecular analytical tools have proven valuable and complementary for overcoming this ineffectiveness and have been used to identify and differntiate tick species (Szabó et al., 2005; Brahma et al., 2014). Molecular data can directly estimate genetic variations of specific genes among the examined taxa and identify distantly-related species (Hwang and Kim, 1999). Recently, a number of authors have utilized partial and complete ribosomal and mitochondrial DNA sequences to infer phylogenetic and systematic evolution of ticks (Barker and Murrell, 2004; Dantas-Torres et al., 2013; Brahma et al., 2014). COI and ITS2 fragments are the most frequently-used markers for inferring relationships in ticks and mites, especially between closely-related species because of high interspecific variability and intraspecific homology (Cruickshank, 2002).

To date, research has tended to focus more on morphology, distribution, epidemiology, and diagnosis as well as transmission of diseases in Iran and little has been done on molecular characterization and phylogenetic studies of ticks (Razmi et al., 2003; Spitalska et al., 2005; Rahbari et al., 2007; Razmi et al., 2007; Tahmasebi et al., 2010; Abdigoudarzi et al., 2011). The aim of the present study is first to determine full-length sequence of COI gene and ITS2 locus of *Rh. (Bo.) annulatus* of Iran and then the phylogenetic relationship of these ticks using COI and ITS2. It should be mentioned that we could register COI complete sequence of this tick for the first time in Iran.

Materials and Methods

Tick collection: Ticks were collected from naturally infected cattle in two different northern provinces of Iran, Golestan and Mazandaran. The tick specimens were washed in 70% ethanol and individual ticks were placed in single vials and preserved in 70% ethanol until use.

Morphological identification: The ticks were identified to species level on the basis of their morphological characters described by Walker et al. (2003).

DNA extraction: Total genomic DNA was isolated from the whole ticks. Each tick was rinsed in sterile distilled water and dried. Individual ticks were frozen and ground in a mortar to a fine powder under liquid nitrogen. DNA was extracted from tick specimens using DNA Extraction Kit (MBST, Iran), according to the instructions.

PCR amplification and determination of COI and ITS2 sequences: COI and ITS2 of *Rh. (Bo.) annulatus* were amplified in three and two overlapping fragments respectively by the primers designed in our department (Table 1). A set of eight primers was designed from conserved regions based on released GenBank records, including *Rhipicephalus sanguineus* (NC_002074.1), *Amblyom maelaphense* (NC_ 017758.1), *Haemaphysalis flava* (NC_ 005292.1), *Ixodes ricinus* (NJ248424), *Ixodes uriae* (NC_006078), and *Dermacentor* sp. (AF199114).

PCR reaction was performed in a total volume of 100 µl containing 10X PCR buffer, 2.5 U Taq polymerase (Sinaclon, Iran), 2 µl of each primer (20mM Sinaclon, Iran), 2 µl dNTPs (20 mM, Sinaclon, Iran), 1.5 mM MgCl2, 2 µl DNA (100 ng) and made up to 100 µl volume with sterile distilled water. The cycling conditions were 95ºC for 5 min (initial denaturation

step), followed by 32 cycles of 95ºC for 45 s (denaturation step), 46.5-55ºC for 45 s (annealing step), 72ºC for 45 s (extension step), and a final extension of 72 ºC for 5 min. Negative control was included in each amplification run. 3 µl of PCR product was electrophoresed on a 1% agarose gel in TBE buffer and stained by Red dye and visualized under UV light.

DNA sequencing: The amplified DNA sequences were purified by the PCR Purification Kit (MBST, Iran), according to the manufacturer's protocol, and were suspended in 50 µl of elusion buffer. 3 µl of each purified DNA was examined by 1% agarose gel electrophoresis to validate purification efficiency and then directly sequenced (Bioneer Co., Republic of Korea).

Data analyses: Full-length nucleotide sequences of COI and ITS2 from Golestan and Mazandaran were aligned with each other and other corresponding registered sequences to evaluate their similarities. Finally, the sequences available in the database were aligned with the resulting sequences in the study to construct a phylogenetic tree using the neighbor-joining method.

Results

PCR amplification of each target region from individual DNA of *Rh. (Bo.) annulatus* resulted in amplicons of the expected size which were 1539 bp and 1158 bp in length for COI and ITS2, respectively. Golesatn *Rh. (Bo.) annulatus* sequence of COI gene and ITS2 region were registered in GenBank under accession number KJ410769 and KJ410770, respectively.

The mean nucleotide content of the COI was 30.7% A, 40.62% T, 13.72% G, and 14.93% C. No insertion-deletion polymorphism was observed in each isolate. Between the ticks examined in our study, nucleotide similarity was 91.3%, and the consensus sequences of the aligned COI were 1539 sites. COI nucleotide sequences included 1405 conserved and 134 variable sites. Nucleotide substitutions were represented mostly by transitions (Ts) rather than transversions (Tv) by a Ts/Tv ratio of 1.27. The pairwise nucleotide sequence comparison of *Rh. (Bo.) annulatus* COI with the one registered in GenBank (KC503256.1) showed 91.1% and 99.7% similarity for Golestan and Mazandaran samples, respectively.

A long open reading frame, which encodes 512 amino acids, was obtained after the nucleotide sequences of COI. The deduced amino acid sequences showed 98.6% similarity between the ticks studied. Sequence comparison between the ticks from Golestan and Mazandaran showed 120 nucleotide differences and only 16 amino acid variations. Amino acid sequences of *Rh. (Bo.) annulatus* COI from Golestan and Mazandaran showed 98.2 and 99.6% similarity with *Rh. (Bo.) annulatus* from Romania (AGH19677), 98.4 and 99.2% with *Rh. (Bo.) microplus* (AGH19720), 98 and 98.6% with *Rh. (Bo.) geigyi* (AGH19755) as well as 98.2 and 99% with *Rh. (Bo.) kohlsi* (AGH19747), resepectively.

The ITS2 nucleotide sequence amplified from *Rh. (Bo.) annulatus* obtained from the two different regions was 1158 bp in length. No insertion-deletion polymorphism was observed in each isolate. The mean nucleotide content was 18.8% A, 17.1% T, 34.8% G, and 29.3% C. Pairwise alignment of ITS2 sequences from the tested *Rh. (Bo.) annulatus* revealed a nucleotide similarity of 99.9% with each other. *Rh. (Bo.) annulatus* from Golestan and Mazandaran displayed 99.7 and 99.6% silmilarity with *Rh. (Bo.) annulatus* from Romania (KC503267), 99.3 and 99.4% with *Rh. (Bo.) annulatus* from Texas (AF271270), 99.5 and 99.6% with *Rh. (Bo.) annulatus* from Occupied Palestine (AF271272), 95.1 and with 95.2% *Rh. (Bo.) annulatus* from Egypt (JQ412126), 98.7 and 98.8% with *Rh. (Bo.) microplus* from Brazil (KC503273), 91.3 and 91.4% with *Rh. (Bo.) kohlsi* (KC503271), as well as 89.3 and

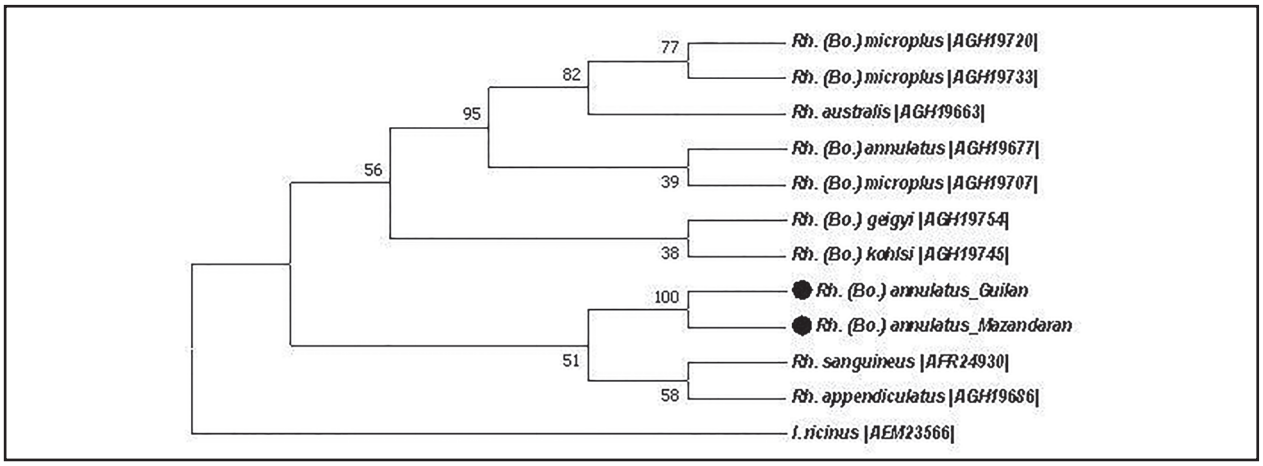

Figure 1. Phylogenetic relationship of *Rh. (Bo.) annulatus* of Iran with other ticks based on the amino acid sequence of COI was inferred by MEGA6 software. Neighbor-Joining tree (NJ) was constructed using the amino acid sequence of COI from *Ixodes ricinus* as an out group. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed.

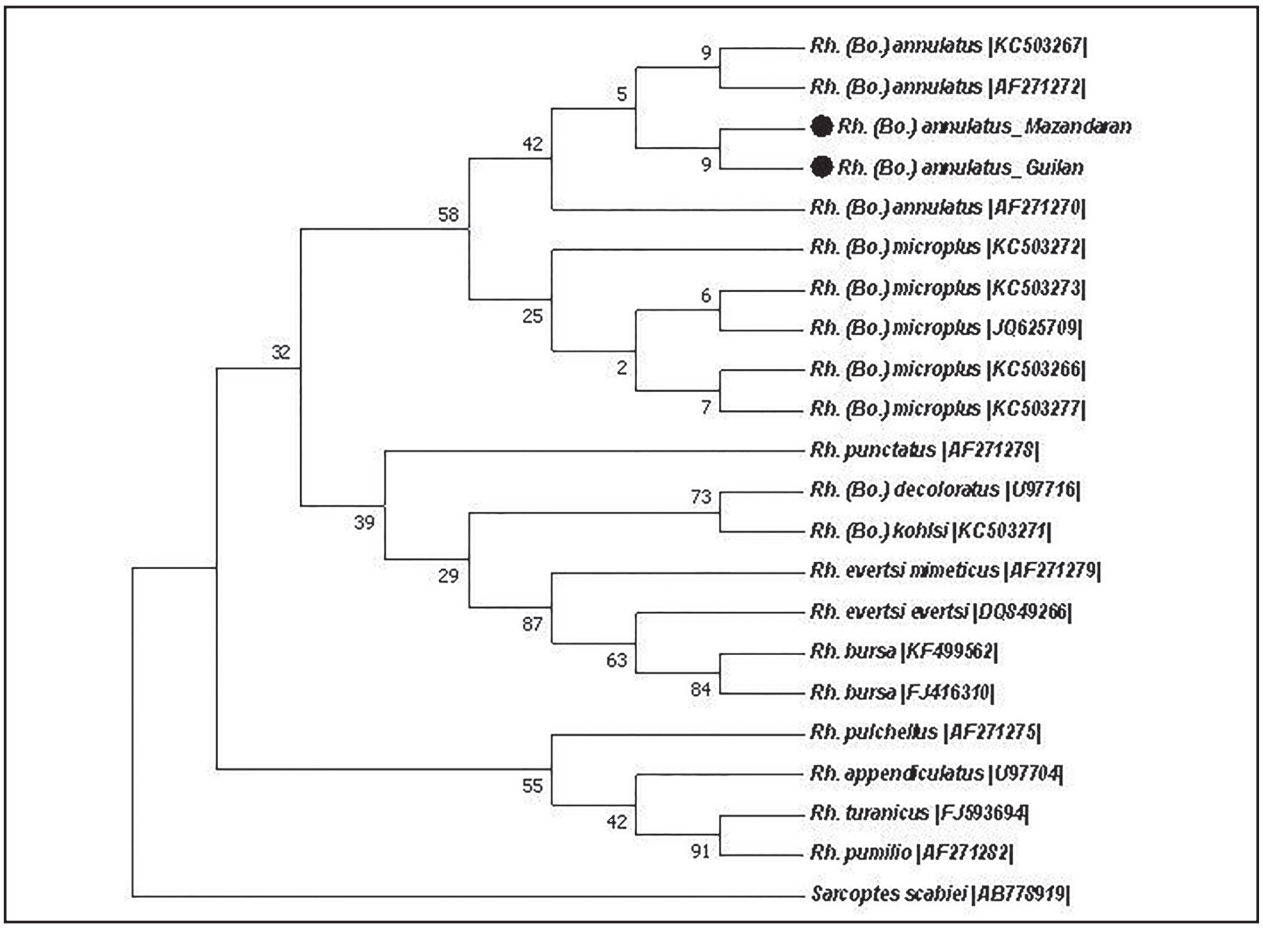

Figure 2. Phylogenetic relationship of *Rh. (Bo.) annulatus* of Iran with other ticks based on ITS2 sequence was inferred by MEGA6 software. Neighbor-Joining tree (NJ) was constructed using ITS2 sequences from *Sarcoptes scabieie* as an out group. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed.

89.4% with *Rh. (Bo.) decoloratus* (U97716.1), respectively.

The phylogenetic relationships of *Rh. (Bo.) annulatus* of Iran based on the amino acid se-

Table 1. Primer sequences used for amplification and sequencing of COI and ITS2 (F: forward, R: reverse).

Primer	Sequences 5´-3´	GenBank Accession Number	Position 5´
P1	F aaaactatttaccttcaaagt	NC_002074	1046
P2	R acaaataatggtattcgttc	NC_002074	1697
P3	F ttaccgcgatgaatatactc	NC_002074	1150
P4	R accaaagcctggtagaatta	NC_002074	1899
P5	F gaacgaataccattatttgt	NC_002074	1678
P6	R attattattgaatgatcatg	NC_002074	2778
P7	F tcgtctgtctgagggtcgga	AF199114	1
P8	R tcgtctcgcctgcatctgag	AF199114	1091

quence of COI and ITS2 nucleotide sequence with other *Rhipicephalus* species are shown in figure 1 and 2, respectively.

Discussion

Distinguishing engorged female ticks and immature stages is difficult using naked eye and in some cases by microscopic examinations (Dantas-Torres et al., 2013). Therefore, genetic characterization of these ticks is recommended. COI and ITS2 are known as suitable markers to study the evolution and phylogenies analysis (Cruickshank, 2002). ITS2 contains little intra-specific variation but considerable inter-specific difference. Therefore, it has been considered as a useful marker for inferring phylogenies and the identification of related species (Barker, 1998; White et al., 2008). In addition, the relatively high mutation rate and the apparent simplicity of mitochondrial maternal inheritance are the reasons for the widespread use of mitochondrial DNA in phylogenetic and population genetic studies (White et al., 2008).

In this study, the full length of COI was 1539 bp and had no difference in the lengths of sequences with the only complete sequence of *Rh. (Bo.) annulatus* registered in GenBank (KC503256.1). Nucleotide sequence similarity was 91.3% between the ticks from the two different regions of Iran. Mazandaran *Rh. (Bo.) annulatus* showed a high percentage of similarity (99.7%) with *Rh. (Bo.) annulatus* from Romania (KC503256.1) at COI nucleotide sequence, while nucleotide similarity was 91.1% between the ticks collected from the other part of Iran, Golestan, and the tick from Romania. Sequence analysis indicated the occurrence of a greater difference in the downstream region of the gene than upstream. It is noteworthy that Liu et al. (2007) revealed 11.23% nucleotide sequence difference in the whole mitochondrial genome between *Rh. sanguineus* from China and USA (Liu et al., 2013). It could partly be due to the different geographic origin of the specimens. In the present study, nucleotide sequence of COI from Mazandaran was more similar to that of Romania than the Golestan ticks. The reason for this interesting discrepancy with regard to geographic origin remains unknown.

The comparisons of ITS2 sequences from the tested *Rh. (Bo.) annulatus* revealed a nucleotide similarity of 99.9% with each other and presented 1-5% difference among the ITS2 sequences of *Boophilus* species registered in GenBank. According to the low variation between *Rh. (Bo.) annulatus* ITS2 from different regions, it seems that this marker is not suitable for differentiation of geographical isolates of these tick species.

Morphological features of *Rh. (Bo.) annulatus* are more similar to *Rh. (Bo.) microplus* than the others in the *Boophilus* sub-genus within the genus *Rhipicephalus*. Based on ITS2 nucleotide sequence, the above-mentioned observation could be supported in the present study. In particular, the maximum nucleotide difference between *Rh. (Bo.) annulatus* and *Rh. (Bo.) microplus* was about 3%. This percentage is lower than the others in the *Boophilus* sub-genus. This demonstrates that ITS2 may not be a suitable marker to distinguish these two species. However, sequence variation between *Rh. (Bo.) annulatus* from Iran with *Rh. (Bo.) kohlsi*, *Rh. (Bo.) decoloratus* is informative enough to distinguish them

from each other.

Prior to this report, only a partial sequence of *Rh. (Bo.) annulatus* ITS2 from Iran had been registered in GenBank (AY702974). Abdigoudarzi et al. (2011) used ITS2 to derive molecular phylogeny for hard ticks and to study the magnitude of nucleotide variation in ITS2 sequence within different geographical region of Iran. The nucleotide sequences of *Rh. (Bo.) annulatus* in this study were compared with the partial sequences of *Rh. (Bo.) annulatus* (AY702974.1) from Iran registered in GenBank and showed a similarity of 99%. Brahma et al. (2014) developed a polymerase chain reaction-restriction fragment length polymorphism diagnostic tool (PCR-RFLP) based on Hind III digestion of ITS2 to facilitate the identification of fully-fed ticks of *Ha. bispinosa* and *Rh. micropplus*.

Navajas et al. (1998) combined COI and ITS2 sequences to investigate intraspecific variation in Tetranych usurticae and discovered species-wide homogenecity in ITS2 sequences but extensive COI polymorphism. Latrofa et al. (2013) used fragments of the mitochondrial 16S, 12S and COI genes as well as ITS2 locus to investigate molecular and phylogenetic analyses of *Rh. sanguineous* and detected little interspecific divergence among ribosomal ITS2 sequence in contrast to mitochondrial genes.

Neighbor-joining tree was inferred from a multiple alignment of ITS2 sequences and the amino acid sequences of COI with the corresponding released sequence in GenBank. Based on ITS2 sequences, the phylogenetic tree showed two main branches (Figure 2). Our result revealed that *Rh. (Bo.) annulatus* is closer to *Rh (B.) microplus* than the rest of *Boophilus* species. Interestingly, *Rh. (Bo.) decoloratus* and *Rh. (Bo.) kohlsi* presented more similarity to other *Rhipicephalus species*. The phylogenic tree based on COI amino acid sequence could be as informative as ITS2 sequence applying more COI sequences of ticks. It seems that more COI sequence of ticks needs sequencing and employing for evolutionary relationship. In spite of high similarity between our sequences and *Rh. (Bo.) annulatus* from Romania, they are in different branches. Taken together, the molecular evidence presented that *Rh (B.) annulatus* from two isolates of Iran appears as a sister group.

In conclusion, we determined and established the phylogenetic status of *Rh. (Bo.) annulatus* of these isolates based on COI and ITS2 sequences. The results suggest that COI and ITS2 sequences could provide suitable genetic markers for the discrimination and genetic characterization of this species.

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مجله طب دامی ایران، ۱۳۹۴، دوره ۹، شماره ۲، ۱۲۳-۱۱۷

شناسایی ویژگی‌های مولکولی کنه ری پی سفالوس (بوافیلوس) آنولاتوس ایران با استفاده از توالی‌های ژن سیتوکروم اکسیداز یک(COI) و دومین ناحیه بین ژنی داخلی رونویسی شده خوشه ژنومی ریبوزوم هسته‌ای(ITS۲)

هومن رونقی[۱] صدیقه نبیان[۲،۳]* الهه ابراهیم زاده[۲،۳] فاطمه بیرانوند[۱] پرویز شایان[۲،۳]

۱) دانش آموخته گروه انگل شناسی، دانشکده دامپزشکی دانشگاه تهران، تهران- ایران
۲) گروه انگل شناسی، دانشکده دامپزشکی دانشگاه تهران، تهران- ایران
۳) مرکز کنه و بیماریهای منتقله توسط آن، دانشکده دامپزشکی دانشگاه تهران، تهران- ایران

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چکیده

زمینه مطالعه: به طور معمول، شناسایی کنه ری‌پی‌سفالوس (بوافیلوس) آنولاتوس از سایر کنه‌های مشابه با استفاده از ویژگی‌های ریخت شناسی صورت می‌گیرد. با توجه به این که این ویژگی‌ها جهت شناسایی دقیق گونه‌های مختلف کنه‌های ماده و برخی مراحل رشد کنه‌ها (لاروی و نوچه‌ای) به سختی امکان پذیر است لذا روش‌های بیولوژی مولکولی می‌تواند به عنوان روش‌های مکمل تشخیصی مفید واقع شود. **هدف:** توالی‌های ژن سیتوکروم اکسیداز یک(COI) و دومین ناحیه بین ژنی داخلی رونویسی شده ریبوزوم هسته‌ای (ITS۲) جهت ارزیابی روش‌های مولکولی جهت شناسایی و مطالعات ریشه اجدادی کنه ری‌پی‌سفالوس (بوافیلوس) آنولاتوس تعیین توالی شدند. **روش کار:** واکنش زنجیره ایی پلیمراز (PCR) بر روی کنه‌های جدا شده از دو منطقه ایران (گیلان و مازندران) بر مبنای توالی‌های COI وITS۲صورت گرفت. **نتایج:** اندازه توالی‌های COI و ITS۲به ترتیب ۱۵۳۹ و ۱۱۵۸ جفت باز بودند. مقایسه توالی‌های ناحیه COI کنه‌های ری‌پی‌سفالوس (بوافیلوس) آنولاتوس دو منطقه جغرافیایی با همدیگر۹۱/۳٪ مشابهت را نشان داد. توالی اسیدهای آمینه ژن COI کنه‌های مورد بررسی، با یکدیگر ۹۸/۶٪ و با تنها توالی ثبت شده (AGH۱۹۶۷۷)در بانک ژن ۹۸/۲ و ۹۹/۶٪ تشابه داشتند. مقایسه توالی‌های ناحیه ITS۲ کنه‌های ری‌پی‌سفالوس (بوافیلوس) آنولاتوس با همدیگر ۹۹/۹٪ و با توالی‌های ثبت شده در بانک ژن(KC۵۰۳۲۶۷, AF۲۷۱۲۷۰, AF۲۷۱۲۷۲, JQ۴۱۲۱۲۶) ۹۵ تا ۹۹٪ مشابهت را نشان داد. **نتیجه‌گیری نهایی:** نتایج حاصل از این تحقیق نشان می‌دهد که COI و ITS۲ می‌تواند نشانگر ژنتیکی مناسبی برای تفریق و همچنین تعیین خصوصیات ژنتیکی ری‌پی‌سفالوس (بوافیلوس) آنولاتوس باشد.

واژه‌های کلیدی: COI، ITS۲، ری‌پی‌سفالوس (بوافیلوس) آنولاتوس

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