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Araştırma Makalesi ***Research Article***

Genetic Characterization of *Nematodirella cameli* Based on 18S rDNA and Cytochrome c Oxidase Subunit 1 (CO1)

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Abstract

To determine the phylogenic position and genetic diversity of Nematodirella cameli two portions of nuclear ribosomal DNA, 18S rDNA and mitochondrial DNA gene, the subunit 1 of cytochrome C oxidase gene (CO1) were sequenced and compared with those previously reported for other nematodes in Trichostrongylina. The phylogenetic trees constructed based upon the 18S rDNA sequences, yielded strong support for close relationship between the *N. camel*i and *Nematodirus battus*, with a high bootstrap value of 100%. In the present research, the level of sequence polymorphism among *N. cameli* isolates was higher for CO1 with 32 polymorphic sites compared to 18S rDNA sequence. Accordingly, molecular assays based on CO1 mitochondrial marker, demonstrated the existence of at least 11 distinct haplotypes (accession nos. JX305966 to JX305976) with an intraspecific diversity of 3-7% in Iran. Whereas, all of *N. camel*i samples examined herein (n=11), had a unique 18S sequence (accession no. JX305977). In addition, *N. cameli* CO1 sequences found in this study showed maximum identities to *Haemonchus* (88%) and *Ostertagia* (87%) in BLAST analysis for existing Trichostrongylina sequences. Further information is necessary to infer interspecific and intraspecific phylogenetic relationships between genera and species in Trichostrongylina. This study describes for the first time the nuclear 18S rDNA and mitochondrial CO1 sequence data from *Nematodirella cameli* species.

Özet

***Nematodirella cameli*'nin 18S rDNA ve Sitokrom c Oksidaz Subunit 1 (C01)'e Dayalı Genetik Karakterizasyonu**

Nematodirella cameli'nin filojenik pozisyonu ve genetik çeşitliliğini belirlemek amacıyla nüklear ribozomal DNA'nın iki kısmı olan 18S rDNA ile sitokrom C oksidaz geninin (C01) subünite 1 olan mitokondriyal DNA geni sıralandı ve Trichostrongylina'daki diğer nematodlar için daha önce bildirilmiş olanlarla karşılaştırıldı. 18S rDNA sekanslarına dayandırılarak oluşturulan filogenetik ağaçlar, %100'lük yüksek bir önyükleme değeri ile *N. cameli* ve *Nematodirus battus* arasında yakın bir bağlantı olduğuna dair güçlü bir destek oluşturdu. Mevcut çalışmada, *N.cameli* izolatları arasındaki sekans polimorfizm düzeyi, C01'de 32 polimorfik konum ile 18S rDNA sekansına oranla daha yüksekti. Bununla bağlantılı olarak, İran'da C01 mitokondriyel markerine dayandırılarak yapılan moleküler testler, % 3-7 arasındaki bir intraspesifik çeşitlilik ile en az 11 belirgin haplotip varlığını gösterdi (Katılım No JX305966 ile JX305976 arası). Öte yandan, burada incelenen tüm *N.cameli* örneklerinin (n=11) özgün bir 185 dizilişi mevcuttu (Katılım No: JX305977). Buna ilaveten, bu çalışmada bulunan *N.cameli* C01 dizilişleri mevcut Trichostrongylina dizilişleri için BLAST analizinde en çok *Haemonchus* (%88) ve *Ostertagia* (%87) yapısı gösterdi. Trichostrongylina'daki cinsler ve türler arasındaki interspesifik ve intraspesifik filogenetik ilişkileri gösterebilmek için daha ileri bilgiler gerekmektedir. Bu çalışma ilk kez olarak *Nematodirella cameli* türlerindeki nüklear 18S rDNA ve mitokondriyal C01 diziliş verilerini tanımlamaktadır.

Introduction

The species genus, *Nematodirella*, a thread-necked worm in the trichostrongyloid family Molineidae, is one of the common gastrointestinal nematodes of camel (Parsani et al., 2008). Accurate identification of nematodes is an essential first step in understanding their biology, ecology, geographical distribution, habitat specificity, transmission and in designing effective control mechanisms (Susurluk et al., 2007).

In order to accelerate the characterization and predictive phylogenetic classification of undescribed nematode, new molecular tools and approaches are needed. The molecular phylogenetic framework now has sufficient depth and range of representation of nematode taxa that it is possible to place an unknown specimen in the nematode tree by analyzing its small subunit ribosomal DNA sequence (Blaxter and Floyd, 2003; Gasser and Newton, 2000; Neres et al., 2010).

Taxonomy the Nematodirinae, a subfamily containing several genera of veterinary importance, were established by Durette-Desset and Chabaud (1977, 1981) and Durette-Desset (1983). The current classification of the Nematodirinae, with 5 genera, *Murielus*, *Rauschia*, *Nematodiroides*, *Nematodirus*, and *Nematodirella* is based on a phylogeny generated from morphological characters (Hoberg et al., 2005).

According to Durette-Desset (1985), Nematodirines have been diagnosed by a suite of attributes of the anterior esophagus, synlophe, bursa and ovejectors that encompass both putative plesiomorphic character states. A comprehensive phylogeny for species of *Nematodirella* based on cuticular morphotypes was completed by Lichtenfels and Pilitt (1983). The Nematodirinae present a dorsal ray, which is divided from the base into two distinct branches, and a neodont structure on the head. The ruminant parasite genera *Nematodirella* and *Nematodirus* present a synlophe either without an orientation axis or with an indistinct axis. The most effective way to distinguish these genera is to identify differences in the didelphic genital apparati of the females. *Nematodirella* females present an anterior nonfunctional genital branch whereas both genital branches in *Nematodirus* females are functional (Durette-Desset, 1979; Rajevskaja and Badanin, 1941; Rossi, 1983). Recent molecular phylogenetic analyses based on ITS-1 and ITS-2 regions showed a close relationship between *Nematodirella cameli* and some species of *Nematodirus* (Sharifiyazdi et al., 2011). Identification and analysis of additional molecular targets may be helpful in clarifying the molecular phylogeny, classification and evolution of nematodes (Neres et al., 2010). Several genetic markers were used for phylogenetic analysis and/or characterization of polymorphisms in Nematoda around the world (Audebert et al., 2005; Blaxter et al., 1998, Blaxter et al., 2000; Chilton et al., 1997; Chilton et al., 2006; Gouy de Bellocq et al., 2001; Neres et al., 2010). Ribosomal internal transcribed spacer (ITS-1 and ITS-2) sequences are the markers used most commonly to discriminate among nematode species (Gasser and Newton, 2000; Powers et al., 1997). Relatively, little attention has been paid to mitochondrial DNA (mtDNA) as a source of species-specific markers, even though mtDNA evolves very quickly in nematodes (Blouin et al., 1998; Denver et al., 2000), and quickly reaches reciprocal monophyly between even very closely related species (Blouin et al., 1997; Hoberg et al., 1999). According to Anderson et al. (1998) (mtDNA) sequences usually exhibit more variability among closely related taxa than nuclear sequences.

To the best of our knowledge, no study has previously been conducted on the mitochondrial (CO1) and 18S rDNA sequences of *N. cameli* worldwide. Therefore, the present study was designed to molecular characterization of this nematode and to assess the level of intra specific variation among different isolates using both molecular targets.

Materials and Methods

Nematode isolation and morphological identification

Eleven nematodes (4 males and 7 females) of *N. cameli* were obtained from the Parasitology Department of Shiraz University, Iran. These parasites were previously collected during abattoir inspection from intestine of naturally infected camel from Fars provinces in Iran (Moghaddar et al., 2011; Sharifiyazdi et al., 2011). All samples were confirmed again as *N. cameli* based on morphometric criteria according to already established taxonomic keys (Durette-Desset, 1979; Rossi, 1983).

The samples subsequently fixed in 70% ethanol and stored at -80°C prior to further manipulations.

DNA isolation

Genomic DNA was extracted and purified individually from the worms using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommended protocol and used as template DNA for the PCR. Briefly, to achieve the desired results, we used at least 2-3 h incubation time for *Nematodirella* samples in 56°C with 180 µl tissue lysis buffer (ATL, Qiagen) and 20 µl (50 µg/ml) of Proteinase K with vortexing every 30 min. after adding 200 µl of lysis buffer (AL, Qiagen) containing guanidine hydrochloride and 200 µl Ethanol, the mixture was vortexed for 15 s and then added to a DNA-binding column and spun down for 1 min. The column was then washed several times using 500 µl of AW1 and AW2 buffers (Qiagen). The genomic DNA extract was diluted to a working concentration of 20 ng/µl, and 2 µl of it was used as a template in PCR.

Primers and PCR amplification

A mitochondrial partial cytochrome c oxidase subunit 1gene (CO1, ~430 bp) and 18S rDNA (~1800 bp) gene were amplified. In particular, CO1 was amplified by using the set of primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') (Bowles et al., 1992). The 18S rDNA sequences were amplified by primers NC18SF1 (5'-AAAGATTAAGCCATGCA-3') and NC5BR (5'-GCAGGTT CACCTACAGAT-3') (Brianti et al., 2012).

The following PCR conditions were applied to each assay: 50 mM KC1, 10 mM Tris–HCl (pH=9.0), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pM of each primer, and 2 U *Taq* DNA polymerase (Fermentas, USA) per 50 µl reaction using 2 µl of DNA extracted as template. For amplification, the samples were cycled in a Bio-Rad thermocycler (Bio-Rad Laboratories Inc.p, Hercules, CA, USA). Cycling conditions included an initial DNA denaturation at 94°C for 5 min, followed by 40 cycles, denaturation at 94°C for 45 s, annealing (at 48°C and 50°C for CO1 and 18S rDNA, respectively) for 1 min, and an extension at 72°C for 1 min. Sterile water was used as the negative controls. The presence of amplicons and their size were assessed by electrophoresis of 5 µl of each reaction product in 1.5% (*w/v*) Tris-acetate/EDTA agarose gel and visualized by staining with ethidium bromide (final concentration of 0.5 µg/ml) under UV light. Images were captured on a computer and printed.

Sequence analysis

The PCR products obtained for both CO1 and 18S rDNA were purified by a PCR purification kit (Fermentas, USA) and sequenced directly using a capillary DNA analyzer (ABI 3730; Applied Biosystems, Foster City, CA, USA) after sequencing reactions with a BigDye Terminator V3.1 cycle sequencing Kit (Applied Biosystems). Forward and reverse nucleic acid sequence data were used to construct a continuous sequence of inserted DNA. Further comparison of the continuous sequences was made with other available sequences of Nematodirinae NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool). Two sets of nucleotide sequence data, CO1 (393 bp), 18S rDNA (1559 bp) were separately aligned against homologous sequences existing in Genbank by MEGA 4.0 (Tamura et al., 2007). Creating multiple-sequence alignment was established using Clustal W program in the MEGA4 software for each query DNA sequence (Tamura et al., 2007). Data sequences were also used for construction of the phylogenetic trees using maximum parsimony and neighbor-jointed methods. To assess the robustness of the branches, a bootstrap test with 1000 replicates was performed, following the rule of branch consistency (Tamura et al., 2007).

Results

The PCR-amplified products were successfully obtained from 11 specimens using the primers as mentioned above. No amplification product was detectable in the negative controls. The assembling of DNA sequence in both directions (using forward and reverse primers) yielded a fragment containing 393 and 1559 consensus nucleotides for CO1 and 18S rDNA regions, respectively. GenBank (http://www.ncbi.-nlm.nih.gov) accession numbers JX305966-76 and JX305977 were provided for Iranian *N. cameli* sequences of CO1 and 18S rDNA, respectively. Molecular assays, based on CO1 mitochondrial marker, revealed that a notable genetic diversity (3-7%) exists within *N. cameli* population in Iran. Also, the CO1 sequences alignment and comparison showed that, intraspecific variations within the population of all *N. cameli* isolates were detected at 32 positions (Figure 1), in which all were silent, not affecting the translation into the amino acid sequence. Accordingly, molecular genotyping and phylogenetic analysis using the CO1 sequences have generated 11 distinct haplotypes based on neighbor-jointed method (Figure 2). Intra-specific nucleotide variations for CO 1 were related mainly to changes at the third codon positions (n=31). Only one of these silent mutations (**T**TG to **C**TG; both encoding leucine) related to the first codon. Finally, a BLAST search of *N. cameli* CO1 sequences against those previously published for other related nematodes revealed that the sequence homology 88% to *Trichostrongylus vitrinus*: GQ888711 and *Haemonchus contortus*: AF044935.

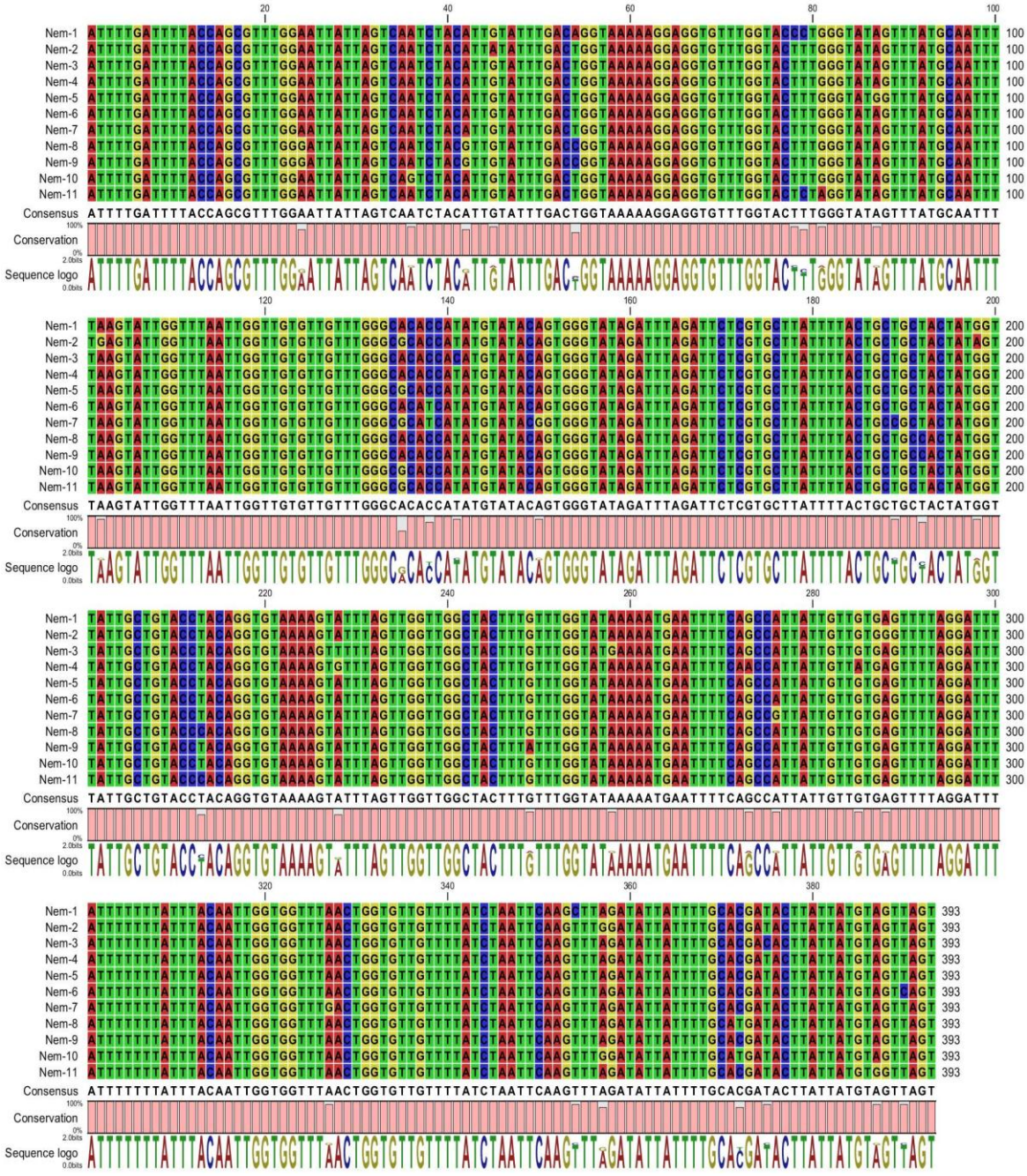

Figure 1. Partial pairwise comparison of nucleotide sequence differences in the CO1 among different haplotypes of *N. cameli* isolated from camel in Iran.

Şekil 1. İran'da deveden izole edilen *N.cameli*'nin değişik haplotipleri arasında C01'deki nükleotid diziliş farklılıklarının kısmi eşleme yönünden karşılaştırılması.

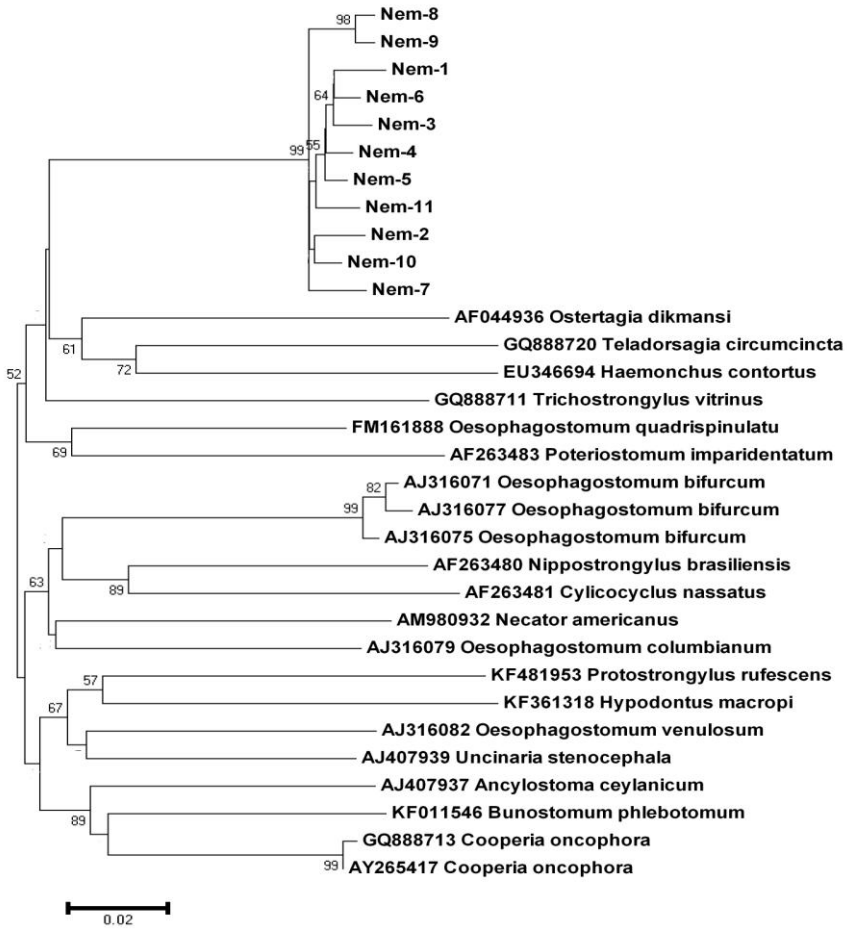

Figure 2. A phylogenetic analysis of the 11 CO1 haplotypes of *N. cameli* and other member of Trichostrongylina revealed statistical support for a close relationship between *N. cameli* and sequences from *Ostertagia dikmansi*, *Teladorsagia circumcincta* and *Haemonchus contortus* (members of the family Haemonchidae). Numbers above the branches indicate bootstrap values (%) from 1,000 replicates.

Şekil 2. *N.cameli*'nin 11 C01 haplotipleri ve Trichostrongylina'nın diğer üyesinin filogenetik analizi, *N.cameli* ile *Ostertagia dikmansi*, *Teladorsagia circumcincta* ve *Haemonchus contortus* (Haemonchidae familyası üyeleri) dizileri arasında yakın bir ilişki olduğuna dair istatistiki bir destek oluşturdu. Kolların üzerindeki numaralar 1000 tekrarlı önyükleme değerlerini (%) göstermektedir.

However, no intraspecific differences were recorded among isolates based on 18S rDNA sequence. The relative conservation of 18S region has been demonstrated in other nematodes (Blaxter et al., 1998; Nadler, 1992). In the present study, Phylogenetic tree of examined sequences of 18S rDNA using maximum parsimony method confirmed that the 18S rDNA sequence of *N. cameli* (JX305977) was most closely related to *Nematodirus battus* (U01230, AJ920360) in order of the work of Chilton et al. (2006) and Zarlenga et al. (1994). There was also, strong bootstrap support (100%) for sister-taxon relationships between *N. cameli* and *N. battus* (Figure 3). The topology of neighbor-joining trees obtained from 18S rDNA was also similar to that of the MP tree (not shown). The similarities between the sequences of the 18S rDNA gene in *N. cameli* with *N. battus* (AJ920360, U01230) were up to 99% and differences were only seen in 12-13 bp. Unfortunately there were no 18S rDNA data available for the other remaining species of *Nematodirus* and *Nematodirella* for further analysis.

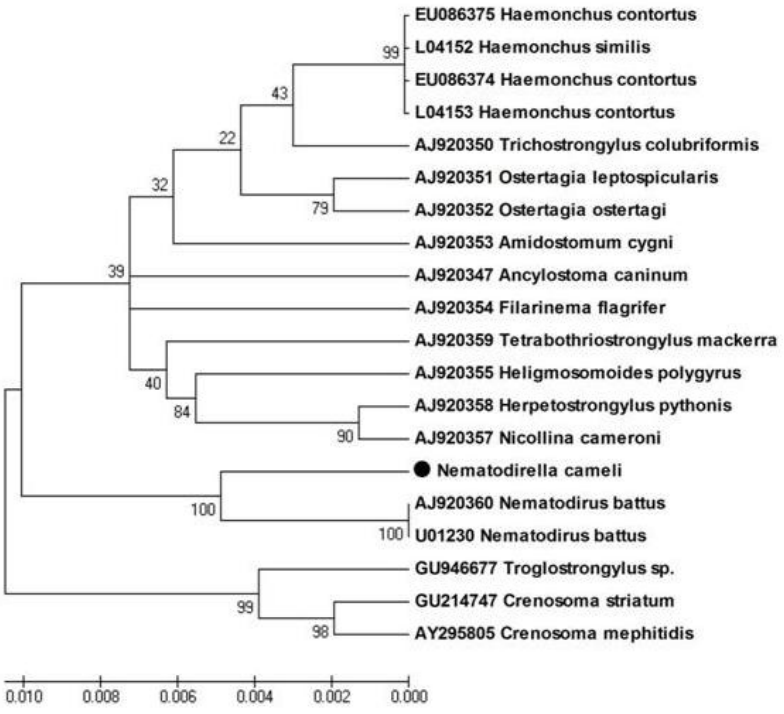

Figure 3. Phylogenetic tree based on 18S rDNA sequence data, constructed according to the Maximum Parsimony (MP) algorithms, showing the position of *N. cameli*, other related species deposited in the GenBank. Numbers above the branches indicate bootstrap values (%) from 1,000 replicates.

Şekil 3. GenBank'ta saklanan ilişkili diğer tür olan *N.cameli* 'nin pozisyonunu gösteren, Maksimum Sadelik (MP) algoritmalarına göre oluşturulmuş, 18S rDNA diziliş verisine dayalı olan filogenetik ağaç. Kolların üzerindeki numaralar 1000 tekrarlı önyükleme değerlerini (%) göstermektedir.

Discussion

Dromedary camel (*Camelus dromedarious*) is one of the highly valuable domestic animals in tropical and subtropical area that can be used for meat, milk and wool production. In addition, to the previous traditional uses, modern applications in the dairy industry lead to the development of camel dairy farms that are capable of producing camel milk on the commercial level. Gastrointestinal parasitism is the most serious constraint throughout the world, which causes significant production losses in ruminants. Also these parasites are major contributor to reduce productivity in terms of meat, milk and wool in animals (Ahmed et al., 2011). Among gastrointestinal nematodes in camel, *Nematodirella* shows highest incidence throughout the year on organized farms (Parsani et al., 2008). Sporadic reports on the occurrence of gastrointestinal helminths in camel from certain countries were available (Dakkak and Ouhelli, 1987; El Bihari, 1985). Mixed infection with two or three species in the same animal was also diagnosed by Selim and Rahman (1972). In Iran, the prevalence of infections (5.7-10%) with *N. cameli* has been found to be considerable (Borji et al., 2010; Moghaddar et al., 2011).

For years, morphological identification was the only method widely used to identify nematodes. As our knowledge of nematodes of agronomical importance increased, it became clear that morphology alone did not reveal the complete picture of observed pathological differences between populations within morphologically delimited species. As a result,

researchers have been looking for methods that can better predict observed pathological behaviors among populations within species (Abebe et al., 2011).

So far, genetic characterization and/or construct a phylogenetic classification of the *N. cameli* have been limited to ITS1 and ITS2 sequences (Sharifiyazdi et al., 2011). In the present study, we used the mitochondrial (CO1) and ribosomal (18S rDNA) sequences as genetic markers to investigate the genetic characteristics of *N. cameli* specimens obtained from camel and compared with additional closely related parasites, that exist in the GenBank database.

According to the ITS phylogenetic analyses conducted by Sharifiyazdi et al. (2011), *Nematodirella* may not be a distinct genus in Nematodirinae because of the closer phylogenetic relationship of *N. cameli* with the *Nematodirus* lineage. Due to the high degree of similarity between *N. cameli*, *N. spathiger* and *N. helvetianus* species, these authors suggested that *N. cameli* could be placed in the genus *Nematodirus*. They showed that the level of sequence homology (up to 95%) between *N. cameli* as a species of *Nematodirella* and both of the *N. spathiger* and *N. helvetianus* is actually higher than the levels of sequence homology found between *N. battus* and *N. spathiger* or *N. helvetianus* which belongs to the same genus. In the present research, level of sequence identity between two genus of *Nematodirella* and *Nematodirus* was significantly higher for 18S rDNA (99%) compared to that previously reported for ITS sequences (92-95%) by Sharifiyazdi et al. (2011). Unfortunately there were no 18S rDNA data available for the other remaining species of *Nematodirus* and *Nematodirella* to complete phylogenetic analysis. The BLAST results show that the *N. cameli* 18S rDNA sequences was the closest taxon to the *N. battus* sequences as the only existing species of *Nematodirus* in GenBank data base. Conserved sequences of the parasite based on small subunit (18S rDNA) failed to show any genetic differences in length or composition among *N. cameli* isolates.

Traditionaly, morphological and morphometric characters have been used to discriminate between *Nematodirella* and *Nematodirus*, but for diagnostic purposes and to clarify the evolutionary relationship between them, these diagnostic methods can be time-consuming, requiring a lot of skill and expertise (Maggenti, 1991; Subbotin et al., 2000). For example, according to Soulsby (1982) and Hoberg et al. (2005), the genus *Nematodirella* resemble N*ematodirus* but differ from each other by some morphological characters which required time and accurate observation. *Nematodirella* and *Nematodirus* are diagnosed by a high number of ridges in the synlophe, position of the vulva at the midbody or in the anterior and bursal rays 4 and 5 of equal size each of the genera is further diagnosed by suites of synapomorphic characters that are shared among congeners. Notably, species of *Nematodirella* are diagnosed by extremely long spicules and species of *Nematodirus* may be diagnosed by the presence of bilateral vulval fans or fin-like processes comprising the synlophe in the dorsal and ventral field (Hoberg et al., 2005).

Some discontinuity between genetic and phylogenetic data and the differences in structural anatomy for the reproductive organ (extremely long spicules) might reflect a recent evolutionary phenomenon not widely detected through the molecular evidence of a conserved region, but strikingly evident in a copulatory structure. Accordingly, Neres et al. (2010), suggest the use a fast evolving genomic region (i.e. cytichrom b or CO1) in order to clarify the taxonomic controversy observed between molecular and morphological data.

A discontinuity within Nematoda taxonomy was previously expressed by Gouy de Bellocq et al. (2001). They conducted a molecular phylogenetic study to infer relationships within the Trichostrongylina (Trichostrongyloidea, Molineoidea and Heligmosomoidea) using partial sequences from 28S rDNA. They provided resolution among these taxa, suggesting monophyly for the respective lineages based on a single-locus analysis. Also, there was a strong support for a sister taxon relationship between the Heligmosomoidea and Molineoidea. This result was not suspected, based on previous morphological analyses (Durette-Desset et al., 1994), because the Trichostrongyloidea and Molineoidea share some morphological features, such as a simple synlophe with bilateral symmetry (or synlophe absent), didelphy of the female genital apparatus, and a male caudal bursa with various types including 2-1-2 in the Molineoidea. In contrast, the Heligmosomoidea have an asymmetrical synlophe, a monodelphic (rarely didelphic) female genital apparatus, and several types of male caudal bursa except the type 2-1-2 (Gouy de Bellocq et al., 2001). Furthermore, Audebert et al. (2005) reexamined and supported this result using additional molecular analysis of ITS-1 and ITS-2 of the ribosomal DNA.

It is important to point out that the employment of molecular methods does not impoverish the field of systematic, as has been affirmed, but should be treated as part of the data for analyses of the relationships

amongst taxa (Blaxter and Floyd, 2003). Attempts are now to being made to integrate morphological and molecular analyses of nematodes, an approach which should provide a more effectives means of characterizing nematodes (Evans, 1995; Thomas et al., 1997).

Present molecular genotyping using mitochondrial DNA (mtDNA) sequences, revealed the presence of at least 11 distinct haplotypes for CO1 in *N. cameli* population. Also, The CO1 sequences alignment and comparison of them revealed 32 polymorphic sites. More detailed sequence analysis based on the CO1 sequence including comparisons with sequences available for a number of other related species in the GenBank database confirmed that the mitochondrial CO1 sequences of *N. cameli* was most closely related to those reported for *Trichostrongylus vitrinus* and *Haemonchus contortus*. These results confirmed a close relationship between three families of Molineidae, Haemonchidae and Trichostrongylidae as members of Trichostrongylina. According to the results obtained from this study, we found much higher levels of genetic diversity in mtDNA level, in contrast with 18S rDNA. These differences in pattern of genetic diversity are consistent with a high mtDNA diversity history in many strongyle species (Blouin et al., 1998). Hence, this is recommended that the analyses of genetic diversity based on partial mitochondrial DNA may prove useful to examine in greater detail the variation within *N. cameli* population. The mitochondrial marker described here (CO1) give an opportunity to explore the different origin of parasite infections in camel.

Finally, the data from our phylogenetic analyses confirms the results of an earlier study based on ITS rDNA region that suggested a close phylogenetic relationship between *Nematodirella* and *Nematodirus*. This study provided the nuclear 18S rDNA and mitochondrial CO1 sequence data from *Nematodirella cameli* for additional phylogenetic studies. However, phylogenetic analysis needs more sequence data for *Murielus*, *Rauschia*, *Nematodiroides* and other *Nematodirella* species to test morphological concepts of defined genera in the Nematodirinae.

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