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Sequence variation in mitochondrial *cox*1 and *nad*1 genes of ascaridoid nematodes in cats and dogs from Iran

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Abstract

The study was conducted to determine the sequence variation in two mitochondrial genes, namely cytochrome *c* oxidase 1 (p*cox*1) and NADH dehydrogenase 1 (p*nad*1) within and among isolates of *Toxocara cati*, *Toxocara canis* and *Toxascaris leonina*. Genomic DNA was extracted from 32 isolates of *T. cati*, 9 isolates of *T. canis* and 19 isolates of *T. leonina* collected from cats and dogs in different geographical areas of Iran. Mitochondrial genes were amplified by polymerase chain reaction (PCR) and sequenced. Sequence data were aligned using the BioEdit software and compared with published sequences in GenBank. Phylogenetic analysis was performed using Bayesian inference and maximum likelihood methods. Based on pairwise comparison, intra-species genetic diversity within Iranian isolates of *T. cati*, *T. canis* and *T. leonina* amounted to 0–2.3%, 0–1.3% and 0–1.0% for p*cox*1 and 0–2.0%, 0–1.7% and 0–2.6% for p*nad*1, respectively. Inter-species sequence variation among the three ascaridoid nematodes was significantly higher, being 9.5–16.6% for p*cox*1 and 11.9–26.7% for p*nad*1. Sequence and phylogenetic analysis of the p*cox*1 and p*nad*1 genes indicated that there is significant genetic diversity within and among isolates of *T. cati*, *T. canis* and *T. leonina* from different areas of Iran, and these genes can be used for studying genetic variation of ascaridoid nematodes.

Introduction

Toxocara cati, *Toxocara canis* and *Toxascaris leonina* are common ascaridoid nematodes of cats and dogs; adult worms live within the lumen of the small intestine. Toxocariasis is a zoonotic parasitic disease caused by the migration of second-stage larvae of *T. cati* and *T. canis* (Despommier, 2003). *Toxascaris leonina* may also be considered as a causative agent of toxocariasis (Cho *et al.*, 2009). Humans become infected through the ingestion of viable, embryonated eggs from the environment. After hatching, emerging larvae are unable to complete their life cycle and develop into mature adult worms in the human host; they may, however, migrate in the body for several months or years. The clinical

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symptoms of toxocariasis depend on the location of the larvae, and there are several forms of toxocariasis, namely, visceral larva migrans, ocular larva migrans, covert toxocariasis and neurotoxocariasis (Rubinsky-Elefant *et al.*, 2010).

Genetic diversity plays an important role in the survival and adaptability of a parasite when its environment changes. Accurate analysis of this variation is applicable for studies on pathogenesis, epidemiology, population biology, taxonomy and evolutionary biology of parasites. Different methods, such as polymerase chain reaction (PCR)-based mutation scanning and sequencing of partial or complete genes, have been used for genetic analysis. The existence of genetic variation and phylogenetic relationships based on the ribosomal and mitochondrial gene sequences among parasite populations have been reported in different studies (Betson *et al.*, 2012; Wang *et al.*, 2012). There are also some studies on genetic characterization of *T. cati, T. canis* and *T. leonina* (Jacobs *et al.*, 1997; Zhu *et al.*, 2001; Fogt, 2006; Li *et al.*, 2006, 2007, 2008a; Chen *et al.*, 2012). The ribosomal (Zhu & Gasser, 1998; Zhu *et al.*, 1998; Fogt-Wyrwas *et al.*, 2013) and mitochondrial (Li *et al.*, 2008b) gene sequences have been used to analyse genetic variations of ascaridoid nematodes in different parts of the world. However, while there are some reports on seroprevalence of toxocariasis and prevalence of ascaridoid nematodes in Iran, no data are available from genetic analysis of these nematodes.

Epidemiological studies have indicated that the prevalence of *T. cati* in cats ranges from 8 to 52.8%, and the prevalence of *T. canis* and *T. leonina* in dogs from different parts of the country ranges from 4.3 to 37% and 1.4 to 32.53%, respectively (Borji *et al.*, 2011; Mirzaei & Fooladi, 2012). In respect of the high prevalence of *T. cati, T. canis* and *T. leonina* in cats and dogs in Iran, and their medical importance, the present study was designed for characterization and analysis of genetic variation within and among Iranian isolates of *T. cati, T. canis* and *T. leonina* by sequencing partial mitochondrial cytochrome *c* oxidase subunit 1 (p*cox*1) and NADH dehydrogenase subunit 1 (p*nad*1) genes. Using these sequences, phylogenetic relationships of Iranian isolates compared with isolates from different areas of the world were studied.

Materials and methods

Collection of nematodes

Sixty adult nematodes, including 32 isolates of *T. cati* from 32 cats, 9 isolates of *T. canis* from 9 dogs and 19 isolates of *T. leonina* from 19 dogs were collected from different geographical areas in Iran (tables 1 and 2). Individual adult worms removed from infected cats and dogs were washed extensively in physiological saline. After identification to species level according to their morphological features, using existing keys and descriptions (Yamaguti, 1961), they were preserved in 70% (v/v) ethanol until extraction of genomic DNA.

Molecular and phylogenetic analyses

Samples were thoroughly washed in distilled water to remove ethanol. Total genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and the DNAs were stored at −20°C until PCR amplification. Two mitochondrial genes, partial *cox*1 (p*cox*1) and partial *nad*1 (p*nad*1), were subjected to PCR amplification. The forward JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and reverse JB4.5 (5′-TAAAGAAAGAACATAATGAA-AATG-3′) primers were used to amplify a portion of the *cox*1 gene; and the forward ND1F (5′-TTCTTATGAGAT-TGCTTTT-3′) and reverse ND1R (5′-TATCATAACGAA-AACGAGG-3′) primers were used for amplification of the partial *nad*1 gene (Li *et al.*, 2008b). All PCR reactions were carried out in a 25 µl reaction mix, containing 12.5 µl of PCR premix (2 × Master Mix RED Ampliqon, Denmark), which included 1.25 U *Taq* DNA polymerase, 200 µM dNTPs and 1.5 mM $MgCl_2$; 25 pmol of each primer and 5 µl of template DNA. The temperature profile was one cycle of 94°C for 5 min (primary denaturation), followed by 35 cycles of 94°C for 30 s (denaturation), 50°C for 30 s (annealing), and 72°C for 30 s (extension), and a final extension of 72°C for 5 min. A sample containing water instead of template DNA was included in each run as a negative control. PCR products were separated by electrophoresis on a 1.5% agarose gel in TBE (Tris 0.09 M, borate 0.09 M, EDTA 0.02 M) at 80 V for 1 h. Gels were stained with 0.5 µg/ml ethidium bromide

Table 1. Geographical origin and accession numbers of p*cox*1 and p*nad*1 sequences of *Toxocara cati* isolates from cats in Iran.

Geographical origin	Isolate	Accession no. *cox*1	Accession no. *nad*1
Fars	T.cat1	KC200179	KC200213
	T.cat3	KC200181	KC200215
	T.cat4	KC200182	KC200216
	T.cat5	KC200183	KC200217
	T.cat6	KC200184	KC200218
	T.cat7	KC200185	KC200219
	T.cat9	KC200187	KC200221
	T.cat10	KC200188	KC200222
	T.cat11	KC200189	KC200223
Tehran	T.cat8	KC200186	KC200220
	T.cat20	KC200198	KC200232
	T.cat21	KC200199	KC200233
	T.cat22	KC200200	KC200234
	T.cat23	KC200201	KC200235
	T.cat24	KC200202	KC200236
	T.cat25	KC200203	KC200237
	T.cat28	KC200206	KC200240
Mazandaran	T.cat2	KC200180	KC200214
	T.cat31	KC200209	KC200243
	T.cat33	KC200211	KC200245
Ardabil	T.cat13	KC200191	KC200225
	T.cat14	KC200192	KC200226
	T.cat15	KC200193	KC200227
	T.cat16	KC200194	KC200228
	T.cat17	KC200195	KC200229
	T.cat26	KC200204	KC200238
	T.cat27	KC200205	KC200239
	T.cat29	KC200207	KC200241
	T.cat30	KC200208	KC200242
Gilan	T.cat18	KC200196	KC200230
	T.cat19	KC200197	KC200231
Golestan	T.cat12	KC200190	KC200224

Table 2. Geographical origin and accession numbers of p*cox*1 and p*nad*1 sequences of *Toxocara canis* and *Toxascaris leonina* isolates from dogs in Iran.

Species	Geographical origin	Isolate	Accession no. *cox*1	Accession no. *nad*1
T. canis	Ardabil	T.can1	KC293899	KC293915
		T.can3	KC293901	KC293917
		T.can4	KC293902	KC293918
		T.can6	KC293904	KC293919
		T.can7	KC293905	KC293920
	Khorasan Razavi	T.can10	KC293908	KC293922
		T.can11	KC293909	KC293923
		T.can17	KC293913	KC293925
	Tehran	T.can9	KC293907	KC293921
T. leonina	Ardabil	T.leo1	KC293926	KC293947
		T.leo2	KC293927	KC293948
		T.leo3	KC293928	KC293949
		T.leo4	KC293929	KC293950
		T.leo8	KC293933	KC293954
		T.leo27	KC293945	KC293969
	Khorasan Razavi	T.leo5	KC293930	KC293951
		T.leo6	KC293931	KC293952
		T.leo7	KC293932	KC293953
		T.leo10	KC293935	KC293956
		T.leo11	KC293936	KC293957
		T.leo12	KC293937	KC293958
		T.leo13	KC293938	KC293959
		T.leo14	KC293939	KC293960
		T.leo15	KC293940	KC293961
		T.leo16	KC293941	KC293962
		T.leo17	KC293942	KC293963
		T.leo18	KC293943	KC293964
		T.leo21	KC293944	KC293967

(Roche, Germany) and the bands were visualized using a UV transilluminator and digitally photographed. PCR products were purified using the AccuPrep® PCR purification Kit (Bioneer, Korea), according to the manufacturer's instructions, and were sequenced in both directions, using the same primers as used in the PCR. Sequence results were edited and analysed by the Geneious software (www.geneious.com) and the consensus sequences were compared with each other and GenBank reference sequences using the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and BLAST program (http://www.ncbi.nlm.nih.gov/). Three separate phylogenetic analyses of sequence data were conducted: (1) using p*cox*1 sequences determined in the present study only; (2) using p*nad*1 sequences determined in the present study only; and (3) using concatenated p*cox*1 + p*nad*1 sequences obtained in this study along with relevant sequences deposited in GenBank. Phylogenetic analyses were carried out employing Bayesian inference (BI) and maximum likelihood (ML) methods. BI was conducted using the program MrBayes v.3.1.2 (http://mrbayes.csit.fsu.edu/index.php). Posterior probabilities (pp) were set for 2,000,000 generations (ngen = 2,000,000). The program TreeviewX v.0.5.0 (Page, 1996) was used for trees. A maximum likelihood tree was constructed and pairwise comparisons were made of the level of sequence differences within and among species using the MEGA 5.0 software (Tamura *et al.*, 2011). Bootstrap analyses (using 1000 replicates) were carried out to determine the robustness of the finding.

Results and discussion

For all isolates, amplicons of about 450 and 370 base pairs (bp) were successfully produced by PCR for p*cox*1 and p*nad*1, respectively. Sequences of the Iranian isolates obtained in this study were deposited in GenBank and the accession numbers of these sequences are given in tables 1 and 2. Phylogenetic studies based on the analysis of DNA sequence differences represent a useful tool to gain information on an organism's evolutionary relationships. Previous studies have reported the existence of genetic variation among nematode populations. However, few studies have been done on the molecular characterization of *Toxocara* and *Toxascaris* species. For the first time, Jacobs *et al.* (1997) reported the differentiation of nematodes of *T. canis*, *T. cati* and *T. leonina* based on internal transcribed spacer (ITS)-2 sequences. In addition to ribosomal gene sequences, sequences of the mitochondrial genome have been used for identification, systematic and phylogenetic relationship analyses of ascaridoid nematodes (Li *et al.*, 2008b). The present study was designed for analysis of genetic variation within and among Iranian isolates of *T. cati, T. canis* and *T. leonina* by sequencing partial mitochondrial genes. Intra-species variation within Iranian isolates of *T. cati*, *T. canis* and *T. leonina* amounted to 0–2.3%, 0–1.3% and 0–1.0% for p*cox*1 and 0–2.0%, 0–1.7% and 0–2.6% for p*nad*1, respectively; meanwhile, inter-species sequence differences among the three ascaridoid nematodes were significantly higher, being 9.5–16.6% for p*cox*1 and 11.9–26.7% for p*nad*1. Concatenated p*cox*1 + p*nad*1 sequences revealed 27, 7 and 8 different haplotypes

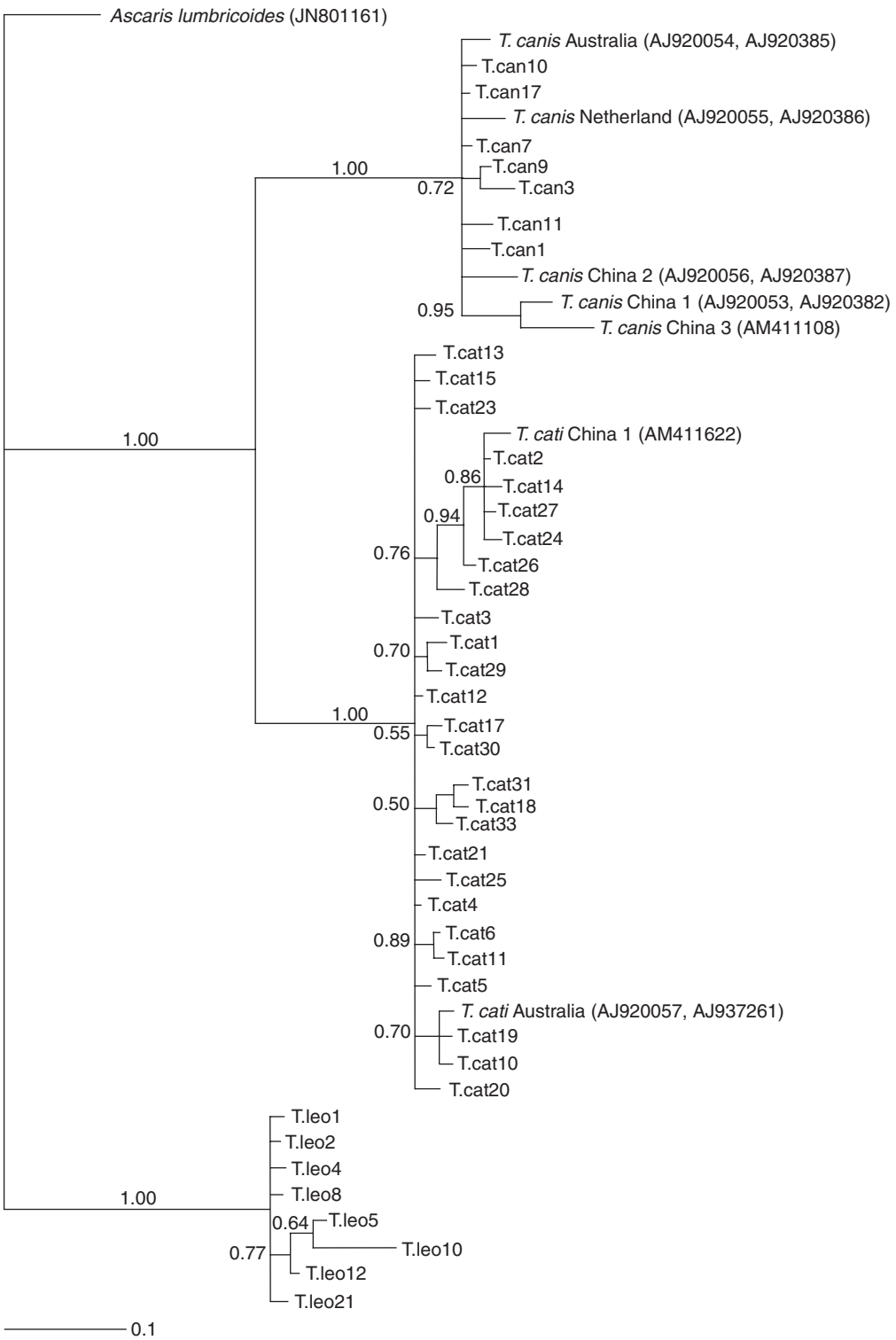

Fig. 1. Phylogenetic relationship of concatenated p*cox*1 + p*nad*1 sequences of isolates of *Toxocara cati*, *Toxocara canis* and *Toxascaris leonina* from Iran, using the Bayesian inference method with *Ascaris lumbricoides* (AN: JN801161) as the outgroup; nodal support is given as a pp value.

among Iranian isolates of *T. cati, T. canis* and *T. leonina*, respectively (fig. 1). The sequences of some samples collected from different provinces in Iran were identical; for example, p*nad*1 sequences of seven isolates (T.cat4, T.cat6, T.cat7, T.cat8, T.cat9, T.cat12 and T.cat21) collected from three provinces in Iran were identical. Based on these results, there is no correlation between homogeny of these isolates and their geographical origin.

For the first time, Zhu *et al.* (1998) used ITS-1 and ITS-2 sequences for characterization of an ascaridoid nematode of cats from Kuala Lumpur, Malaysia. This nematode was previously identified morphologically as *T. canis* (Lee *et al.*, 1993), but a molecular approach using the ribosomal DNA (rDNA) sequences characterized it as *Toxocara* sp. cf. *canis* (Zhu *et al.*, 1998). A detailed morphological study of this nematode was carried out by Gibbons *et al.* (2001) and the parasite was described and named *Toxocara malaysiensis*. The first report of *T. malaysiensis* in cats outside of Malaysia was from China; the nematodes collected from cats in China were morphologically and genetically consistent with *T. malaysiensis* (Li *et al.*, 2006). These studies showed that this species has a broader geographical distribution. In the present study, using the BLAST system, all 32 isolates from cats were identified as *T. cati.*

The p*cox*1 and p*nad*1 sequence analysis of *T. cati, T. canis* and *T. leonina* has not been done before in Iran, and the present study was designed for characterization and analysis of genetic variation among and within Iranian isolates and comparison with data available from other countries. Based on pairwise comparisons, there was sequence variation within and among the three ascaridoid species. Li *et al.* (2008b) described genetic variation among and within *T. canis*, *T. cati*, *T. malaysiensis*, *Toxocara vitulorum* and *T. leonina* from different geographical origins using an single-strand conformation polymorphism (SSCP)-sequencing approach. In the present study, BLAST analysis of p*cox*1 sequences indicated that eight isolates of *T. cati* (T.cat4, T.cat7, T.cat8, T.cat9, T.cat15, T.cat16, T.cat19 and T.cat23) from four provinces in Iran were identical and exhibiting 100% homology with an isolate from Australia (accession no. AJ920057). The p*cox*1 sequence of two isolates of *T. cati* (T.cat2 and T.cat27) showed 100% homology with two reference sequences of *T. cati* from China (accession nos. AM411622, NC_010773). Seven isolates of *T. leonina* collected from dogs in Iran (T.leo5, T.leo7, T.leo11, T.leo12, T.leo13, T.leo16 and T.leo17) did not differ from an isolate of *T. leonina* collected from a grey wolf (*Canis lupus*) in China (accession no. JF780946), across the p*cox*1 gene. None of the p*nad*1 sequences of *T. cati*, *T. canis* and *T. leonina* from Iran had 100% homology with reference sequences in GenBank, except for an isolate of *T. canis* (T.can9) which showed 100% homology to an isolate from Australia (accession no. AJ920383). Phylogenetic analysis not only confirmed the differences within Iranian isolates of *T. cati*, but showed a large difference between isolates of *T. cati* from Iran and three isolates of *T. cati* from China (accession nos. JF780941, JF780942, JF780945 for p*cox*1 and JF833957–JF833959 for p*nad*1); the three isolates of *T. cati* from China were collected from different hosts (jungle cat, leopard cat and Asian golden cat).

In conclusion, this study demonstrated the existence of genetic diversity in mitochondrial genes within and among isolates of *T. cati*, *T. canis* and *T. leonina* in Iran. The results can be used for studies of genetic variability, specific identification of ascaridoid nematodes and diagnosis of toxocariasis, and the results clearly highlight the applicability of mitochondrial gene sequences as genetic markers. For a deeper understanding of genetic diversity among populations of *T. cati*, *T. canis* and *T. leonina,* analysis of more isolates from various geographical areas, other felid hosts and variable genetic markers are recommended.

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Conflict of interest

None.

Ethical standards

This research project was reviewed and approved by the Ethics Committee of Tehran University of Medical Sciences, Iran.

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