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Sequencing and phylogenic variation of ITS-2 region and *rrn*L gene in *Toxocara canis* of Iraqi isolation

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Abstract

Toxocara canis nematode roundworm, the main causative agent of zoonotic disease of canids and Toxocariasis in humans.This study designed to detect the ITS-2 region and *rrn*L gene Iraqi isolate and to characterize and analyse the genetic variation in*T. canis* adult nematode which collected from puppies in different geographical areas of Baghdad city. DNA extracted from worms then detected by PCR followed by sequencing and phylogenetic tree analysis using BLASTed sequences (Neighbor-Joining method) and Phylogeny.fr program(Advance method). Two aligned sequences namely Balkes I (Ba I) for (ITS-2) region and Balkes II (Ba II) for (*rrn*L) gene were deposited in NCBI with accession numbers LC328970, LC328971, respectively and at DDBJ and ENA database for the first time in Iraq. BLASTed sequences results indicate clustered the aligned sequences of Ba I and Ba II with *T. canis* specie other than *Toxocara* species with low genetic changes (0.08) and (0.04) respectively, while phylogenetic analysis results indicated that Iraqi Ba I and Ba II aligned sequences are clustered with other sequences of *T. canis* isolates retrieved from databases and recorded low genetic changes of (0.05) and (0.007).The current study concluded that the ITS-2 region and *rrn*L gene can be used as genetic markers for studying specific genetic identification and diagnosis and study genetic variation of ascaridoid nematodes.

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Introduction

The neglected nematode (roundworm) *Toxocara canis* (Ma *et al.,* 2018)which has a complex life cycle and the main causative agent of zoonotic disease of canids (Sarvi *et al*., 2018) and paratenic hosts like humans (Mughini-Gras *et al*., 2016), children were the most infected (Cassenote *et al*., 2014; Holland, 2017), and rodents (Oliart-Guzman *et al*., 2014; Krücken *et al*., 2017).Toxocariasis is a disease caused by accidental ingestion of *T. canis* infected eggs by humans (Carvalho and Rocha, 2011). when particularly embryonated egg hatching in stomach (Despommier, 2003), the larvae penetrate the intestinal wall (Owen, 2017) and migrate through organs causing the well-characterized syndrome of ocular larva migrans (OLM), visceral larva migrans (VLM), neurotoxocariasis (NT) and/or covert toxocariasis (CT) (Carvalho and Rocha, 2011; Santos *et al.,* 2017).

Whereas some epidemiological reports on *T. canis* prevalence in Iraq were revealed that 36%of stray dogs were infected with *T. canis* in Sulaimaniah city (Bajalan, 2010), In addition to high rate 67.5% of *T.canis* from fecal samples of stray dogs in Baghdad (Hadi, 2011), but 26.5% was the rate of infection with *T. canis* in Basrah city (Awad *et al*., 2017). Hadi (2017) indicated presence of toxocariasis in Iraq and recorded 27.27% at child age, adult people recorded 23.33% and old people over 50 years agerecorded 21.87% moreover, 4 from 22 healthy persons were positive and a prevalence of 18.18% used ELISA technique but there are no available genomic markers in nuclear ribosomal DNA or mitochondrial sequence data for genetic analysis of this nematode.

Using different methods, such as polymerase chain reaction (PCR) based mutation scanning and sequencing of partial or complete genes, then phylogenic have been used for genetic analysis (Chang *et al*., 2015). Different studies have reported among parasite populations existence of genetic variation and phylogenetic relationships based on the nuclear and mitochondrial gene sequences (Wang *et al*., 2012; Mikaeili *et al*., 2015).

A study by (Li *et al*., 2008) demonstrated the complete mitochondrial genomes of three *Toxocara* species of human and animal health significance. While (Chen *et al.,* 2012) studied molecular identification, taxonomy, genetic variation and diagnosis of *Toxocara* species.The ribosomal (Fogt-Wyrwas *et al*., 2013) and mitochondrial (Mikaeili *et al*., 2015) genes sequences have been used to analyze genetic variations of ascaridoid nematodes in many different parts of the world (Jacobs *et al*., 1997). Moreover, a study by (Mikaeili *et al*., 2015) based on the internal transcribed spacer ITS-2 sequences in *Toxocara* species how indecated thatwhen parasite environment changes, genetic diversity plays an important role in its survival, adaptability and makes an accurate analysis of this variation applicable for studies on pathogenesis, epidemiology, population biology, taxonomy and evolutionary biology of parasites.

The aim of this study was to detected and determined *T. canis* partial regions of ribosomal DNA (rDNA) which are internal transcribed spacer 2, 5.8S ribosomal RNA, (ITS-2) region and partial mitochondrial large ribosomal subunit (*rrn*L) gene by PCR technique and sequencing, the aligned sequences used for characterization and analysis of genetic changes in *T. canis* Iraqi isolate by phylogenetic relationships depend on Fixation index (**F_{ST}**) inorder to compared it with other isolates from different areas of the world.

Material and methods

Parasites samples collection

Fifty three *T. canis* adult worms were obtained from the stool of naturally 23 puppies infected after treatment with albendazole at a dose of 0.3 g/kg body weight. The samples were obtained from different geographical areas in Baghdad city from April to September 2017.The worms were washed with physiological normal saline, then collected in plastic cups with a screw, contain70% (v/v) ethanol until required for *T. canis* adult worms DNA extraction.

Genomic DNA Isolation Protocol

Distilled water used to wash samples to remove ethanol. Phenol-chloroform protocol (organic) method was the first choice used for total genomic DNA extraction (Sambrook and Russell, 2001).The organic method was modified from the original protocol by addition of Dithiothreitol (DTT) to the lysis buffer during yeast extraction (Klis *et al*., 2007). *T. canis* worms were suspended in 500ml of lysis buffer, followed by 10 µL of 10 mg/mL proteinase K, mix gently, and were incubated at 56°C for at least 8 hrs. until worms were completely digested and dissolved, the suspension was removed with a fresh sterile disposable pipette tip to 0.5 mL eppendorfe tube and add 0.5 ml phenolchloroform solution, vortex for 15 sec until an emulsion forms then spin in a micro centrifuge for 3-5 minutes at 10000-15000 rpm to separate the 2 phase, transfer the upper aqueous to 1.5 ml eppendorfe tube and add to the sample 1 ml cooled absolute ethanol, mix, and put sample in -20 C for 30 minwith centrifuge 10000-15000 rpm for 5 min.Discard supernatant then add 1 ml 70% ethanol and mix by vortex 10 secwith centrifuge 10000-15000 rpm for 5 min,Discard supernatantand sample dry on filter paper then add 100 TE buffer, mix by vortex 10 sec and store sample on 4 C for 2 h.

Specific primers design

Two pairs of specific oligonucleotide primers which newly designed for each ITS-2 region and *rrn* L gene of *T. canis* was used to perform a conventional PCR using purified *T. canis* worms DNA as mentioned bellow:

Sequences of designed primers for ITS2 region

ACATTGAGCACTAAAATTTCGAACGCACATTGCGCC
ATCGGGTTCATTCCCGTTGGCACG

TCTGGCTGAGGGTCAGTATATTAAGGAGTATGATG
GGCGCGCCAATTTATGGAATGTGAT
TAACGCGCAAGGTTGTGGTGCATTCGGTGAGCTAT
GCTGGTGTGGTAATGGATATTGTGC
>>>>>>>>>>>>>>>>>>>>>>>>

AATTGTACAGCGTACCTTGCCAAGGAAATATTCGCA
CAAGAAATGGCTGTCGTTTGCTCG

TAAAGAGGCAAAATTGGCCATGAGTGTATGTTGCG
TTGCTTCACGATACGGCCTCCAGCA

AACGTTGTTTATTGTTTGGTTGTGGCAGCATCCAG
GTTGGAGGTGGCGTTATNGGNCGCT
<<<<<<<<<<<<<<<<<<<<<<<<
TGAATGAGGAATGCATGGNNAATGGNTGAAATGA
GATTTT

KEYS (in order of precedence):>>>>>> forward primer , <<<<<< reverse primer

The ITS—2 region sequence primer and qualities are clarified in Table (1) and (2), respectively.

Sequence of designed primer for rrnL gene

TGTTTTTACCAGTTCGTATGGTAATAATAAGTTTTA
CATCGATGTTGTATTTTAGTTGTT
>>>>
CAGGAGAGGAGATGGTTTGAGTTTTGAGACTGTTC
TTCTTTTAGTAAACTAAACGTGATA
>>>>>>>>>>>>>>>>>>>>>>
TTAGTTTAATTCATCGTGAGATAGAATTGTTTATCT
TGTTAATTACTTGTGTTGAAGGCA
GGCAGTACGAAAGGAAATTTGTTAAAGTTTTTAAC
TTTGTTAGAGGGCGTGTCCTCTG
<<<<<<<<<<<<<<<<<<<<<<<<
KEYS (in order of precedence): >>>>>> forward primer , <<<<<< reverse primer.

The *rrn* L gene sequence primer and qualities are clarified in Table (3) and (4) respectively.

Conventional PCR was performed to all DNA samples. PCR reactions were carried out in a 20 µL reaction mix, contain (Master Mix, of Bioneer, Korea),1µL of each primer of 10 pmol concentration, 2µL of template DNA and 16µL Nuclease-free water. Several attempts have been made to determine optimal conditions for reaction. Optimal conditions have been reached one cycle of 94° C for 5 min (primarydenaturation), followed by 35 cycles of 94° C

for 20s (denaturation), 60°C for 60s for ITS2 region and 58°C for 60s for *rrn* L gene (annealing), and 72°C for 30s (extension), and a final extension of 72°C for 5min. A negative control sample containing water instead of template DNA was included in each run. A

1.5% agarose gel prepared as in Table 5 used for PCR products separated by electrophoresis in TBE (Tris 0.09 M, borate 0.09 M, EDTA 0.02 M) at 60 voltage for 1.5 hrs. Gels were stained with Red Save stain.

Detection of PCR products (Agarose Gel Electrophoresis)

Agarose gel electrophoresis is a procedure that consists of injecting DNA (PCR products) into an agarose gel and then applying an electric current to the gel. As a result, the smaller DNA fragments move faster than the larger fragments through the gel toward the positive electrode. PCR products were analyzed by electrophoresis on an agarose gel and photographed under an ultraviolet trans illuminator. PCR product sizes were compared with a marker ladder (100 pb) and products with the exact same size (Maniatis *et al.*, 1982).

Phylogenetic analyses

According to the manufacturer's instructions of the AccuPrep[w]PCR purification Kit (Bioneer, Korea), the PCR products were purified, sequenced in both directions, using the designed primers. Edited sequence results were and analyzed by BLASTn program (www.ncbi.nlm.nih.gov) and using Nucleotide Collection (nt/nr) database, in addition, to specifying the organism *T. canis* (Taxid 6265). Using BLASTed sequences (Neighbor-Joining method) for the aligned sequences Ba I and Ba II to differentiate *T. canis* species from other *Toxocara* species. Phylogenetic analyses were carried out employing (Phylogny.fr/advanced method) to find out the phylogenetic relationship of the sequence determined in the present study obtained along with relevant sequences deposited in Gen Bank.

Results and discussion

Genomic DNA extraction method

Fifty three adult worms were subject to molecular diagnosis by PCR. Phenol-chloroform protocol (organic) with the modification that included addition of an extra amount of Dithiothreitol (DTT) material was the first choice as a conventional method of extraction genomic DNA from adult worms used for the first time in Iraq.

Table 1.ITS-2 region sequence primer.

Primers	Sequences 5´ ⟶ 3´	Band size/bp
Forward(F1) (136-157)	TGGTGCATTCGGTGAGCTATG	197
Reverse(R1) (332-353)	GATGCTGCCACAACCAAACAA	

Table 2.ITS-2 region primer qualities.

OLIGO (ITS-2)	start	len	tm	gc%	any_th	3'-th	hairpin
LEFT PRIMER	136	21	60.74	52.38	0.00	0.00	0.00
seq TGGTGCATTCGGTGAGCTATG							
RIGHT PRIMER	332	21	60.20	47.62	0.00	0.00	0.00
seqseq GATGCTGCCACAACCAAACAA							
INCLUDED REGION SIZE: 400							
PRODUCT SIZE: 197, PAIR ANY_TH COMPL: 0.57, PAIR 3'_TH COMPL: 0.00							

The DNA samples characterized with high concentration 450 to 820 ng/µl and purity ranged between 1.6 to 1.8. Our method produced high intensity bands in Red save stained on agarose gels without smear formation as shown in Fig. 1.

Mikaeili *et al*., (2013) compared of six different DNA extraction protocols to extracted *T. canis* genomic DNA, including grinding, boiling, crushing, beating, freeze–thawing and application of a commercial kit, and evaluated to identify which DNA extraction protocol produce the highest yield of ribosomal and mitochondrial DNA from *T. canis* nematode for molecular genetics analyses and its results confirm that among the six DNA extraction methods, the beating method was the best effective for *T. canis*, followed by used the commercial kit, the results were disagree with this study findings, many methods used were give bad results except the result with using the organic method with addition of DDT material which have a reduction activity that required for reduced of a typical disulfide bond in biological extracellular membranes since nematode cuticle protein, collagen was a structural protein generation of extracellular matrices, are extensively cross-linked with disulfide bonds (Johnstone, 2000).

Table 3. *rrn*L gene sequence primer.

Primers	Sequences 5´ ⟶ 3´	Band size/bp
Forward(F1) (777-798)	TGTTCAGGAGAGGAGATGGTT	178
Reverse(R1) (954-974)	GGACACGCCCTCTAACAAAG	

Table 4.*rrn*L gene primer qualities.

OLIGO (*rrn* L)	startlentmgc%any_th3'-thhairpin					
LEFT PRIMER	777	21	58.09	47.62	0.00	0.00	0.00
seq TGTTCAGGAGAGGAGATGGTT							
RIGHT PRIMER	954	20	58.56	55.00	0.00	0.00	0.00
seq GGACACGCCCTCTAACAAAG							
INCLUDED REGION SIZE: 958							
PRODUCT SIZE: 178, PAIR ANY_TH COMPL: 6.09, PAIR 3'_TH COMPL: 0.11							

Molecular Identification of T. canis Adult Worms

Molecular Identification of *T. canis* adult worms based on the using of partial internal transcribed spacer 2, 5.8S ribosomal RNA, (ITS-2) region to prove the extracted DNA was belonged to *Toxocara* genus, the product of amplification was 197 bp.

In this study, another important essential mitochondrial gene (partial mitochondrial large ribosomal subunit (*rrn* L) gene was used to confirm the diagnosis purposes at the species level of *T. canis* from other *Toxocara* species, product of amplification 178 bp.

Table 5. Agarose preperation.

Agarose conc.:1.5 %---------- Weight (gm):0.75
T.B.E. 1X , (50 ml)
Voltage: 60 walt Time(h): 2
DNA Ladder-100 bp

Designed different new specific oligonucleotide primers pair for each gene using purified *T. canis* worms DNA as a template gives an accurate diagnosis for *T. canis* adult stage.

The results of PCR amplification to all samples of 197 bp and 178 bp were successfully produced for ITS-2 region and *rrn* L gene, respectively, as demonstrated in Fig. 2.

Nematode rDNA tandem repeat that started with the non-transcribed spacer (NTS) and external transcribed spacer (ETS) were included ITS-1 and ITS-2 genes which were a separate regions and flanked by conserved regions of the ribosomal DNA 18S RNA gene and 5.8S RNA gene for ITS-2 gene and 5.8S RNA gene and 28S RNA gene for ITS-2 gene permitting universal primers to be designed that bind to the 5.8S and 28S ribosomal DNA genes of many nematodes (Ellis *et al*., 1986).

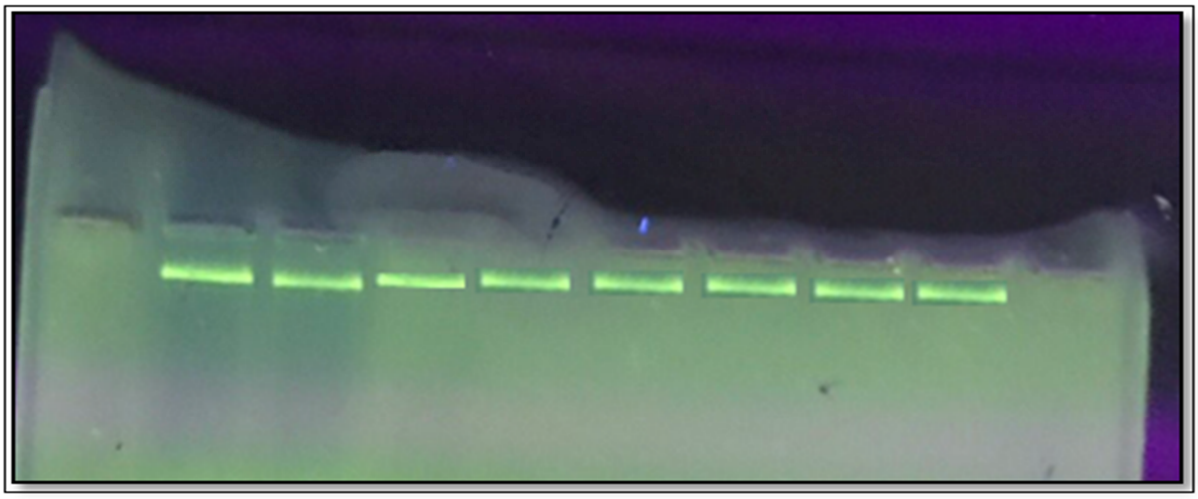

Fig. 1. The genomic DNA for all samples (Voltage 60, Gel concentration: 0.8% agarose, Time 1: hr).

Parasites ITS1 and ITS2 are located between the 18S, 5.8S and 28S coding regions of the nuclear ribosomal DNA, have been proven useful for diagnostic (Morgan and Blair, 1995; Nolan and Cribb, 2005). ITS region sequences used in molecular diagnosis have been demonstrated for other parasitic helminthes which can serve as an effective genetic marker for identification (Zhu *et al*., 2007; Wang *et al*., 2012; Fogt-Wyrwas *et al*., 2013).

Based on internal transcribed spacer (ITS-2) sequences, study by (Mikaeili *et al*., 2015) reported that there were differentiations between nematodes *T. cati*, *T. canis,*and *T. leonina*.The results of present study were agreed with previous studies and agree with (Khademvatan *et al*., 2013) study that revealed a positive *T. canis* consist of 380 bp for ITS-2 used in *T. canis* molecular diagnosis.

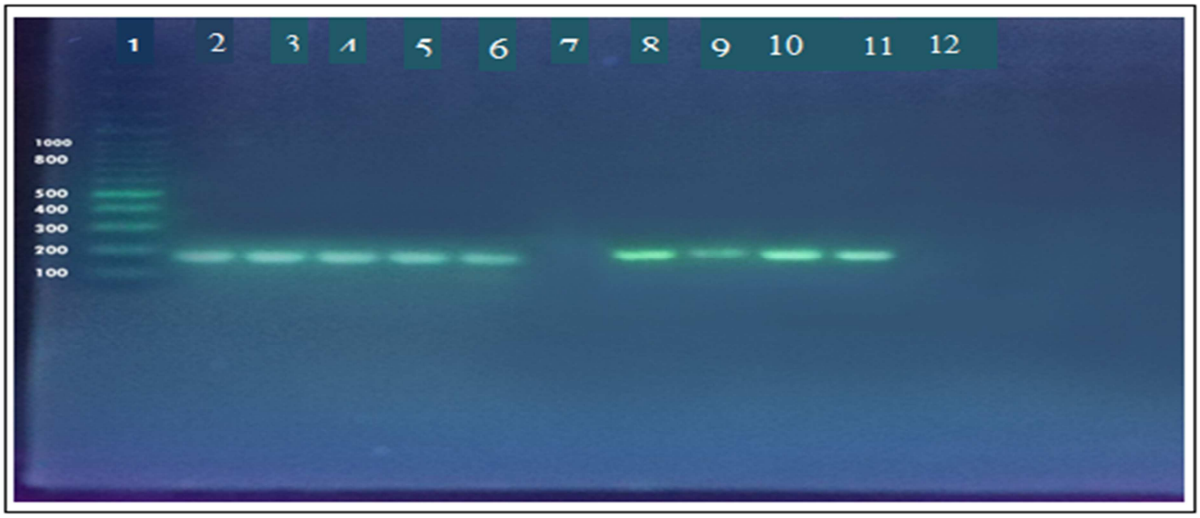

Fig. 2. DNA bands pattern of *T.canis* adult samples after PCR amplification intense of the bands 1: Molecular marker, 2-6 are positive 178bp pands for *rrn* L gene, 7 and 12: are negative controls, 8-11 are positive197bp pands for ITS-2region depend on DNA concentration (Voltage 60, Gel concentration: 1.5% agarose, Time 1.5: hrs).

The complete mitochondrial genomes for *T. canis* was demonstrated (Li *et al.*, 2008), other study find out the complete circular mitochondrial genome for *T. canis* and determined the *rrn* L gene was 923 bp in length (Jex *et al.*, 2008). This study finding out demonstrated that *rrn* L gene was good genetic marker used for detection *T. canis* adult worm samples using PCR technique in addition to ribosomal gene sequences and that agree with other studies that indecated the sequences of mitochondrial genome have been used for identification, systematic and phylogenetic relationship analyses of ascaridoid nematodes (Li *et al.*, 2008), mitochondrial DNA (mtDNA) has several features that can make it useful marker for genomic identification; usually resort to its marker when nuclear DNA marker fails to give a reliable result (Zietkiewicz *et al.*, 2012).

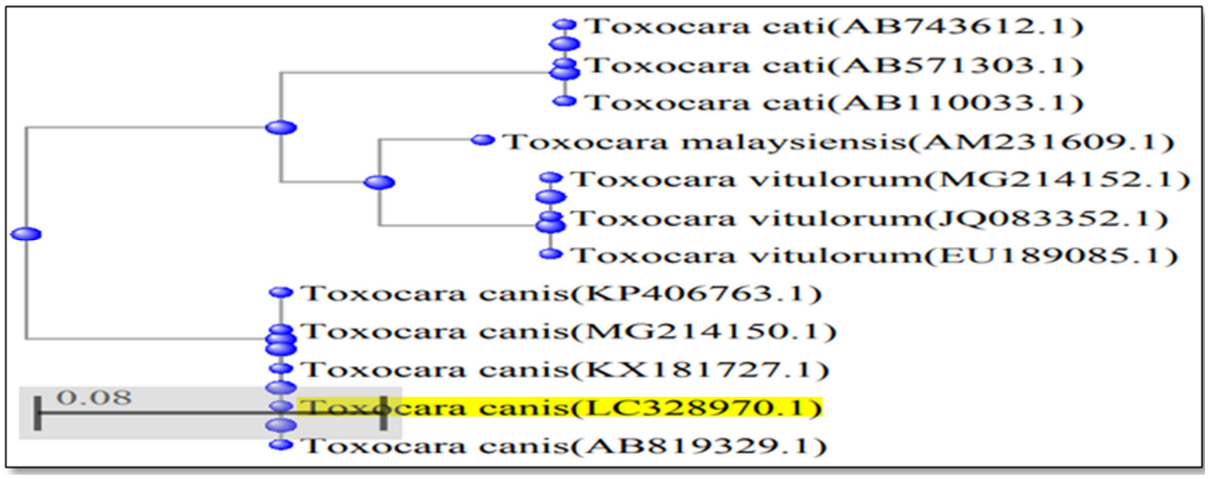

Fig. 3. Distance relationship of BLASTed sequences (BLAST distance relationship/ Neighbor-Joining method) for *Toxocara* species using *T. canis* (LC328970) Ba I nucleotide sequences.

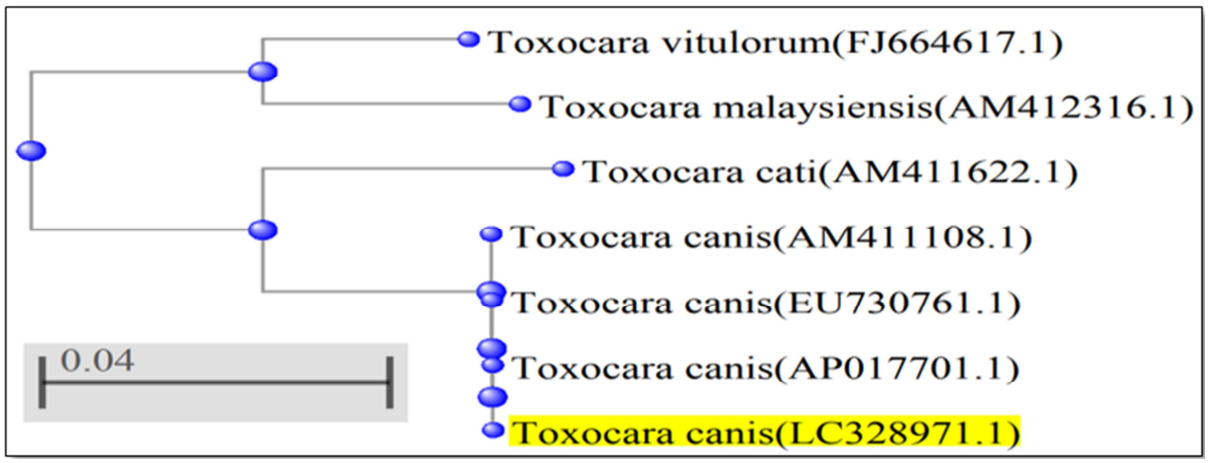

Fig. 4. Distance relationship of BLASTed sequences (BLAST distance relationship/ Neighbor-Joining method) for *Toxocara* species using *T. canis* (LC328971) Ba II nucleotide sequences.

This study finding out indicated that ITS-2 region and *rrn* L gene was good indicator which can be used as genetic markers usful for studies of genetic variability, specific identification, and diagnosis of *T. canis*.

Distance Relationship of BLASTed Sequences

Two PCR products of typical *T. canis* phenotype were sequenced, namely Balkes I (Ba I) for ITS-2 region and Balkes II (Ba II) for *rrn*L gene and deposited in NCBI with accession numbers LC328970, LC328971, respectively and at DDBJ and ENA database for the first time in Iraq.

Using the BLAST system, all Iraqi worm samples from puppies were identified as *Toxocara*. The distance relationship using BLASTed sequences (BLAST distance relationship/Neighbor-Joining method), indicate that the aligned sequences (Ba I) and (Ba II) split from the same node of the genus *Toxocara* and appear as a sister group with *T. canis* other than *Toxocara* species and revealed a low genetic changes (0.08) for Ba I and (0.04) for Ba IIas clarified in Fig. 3 and 4 respectively.

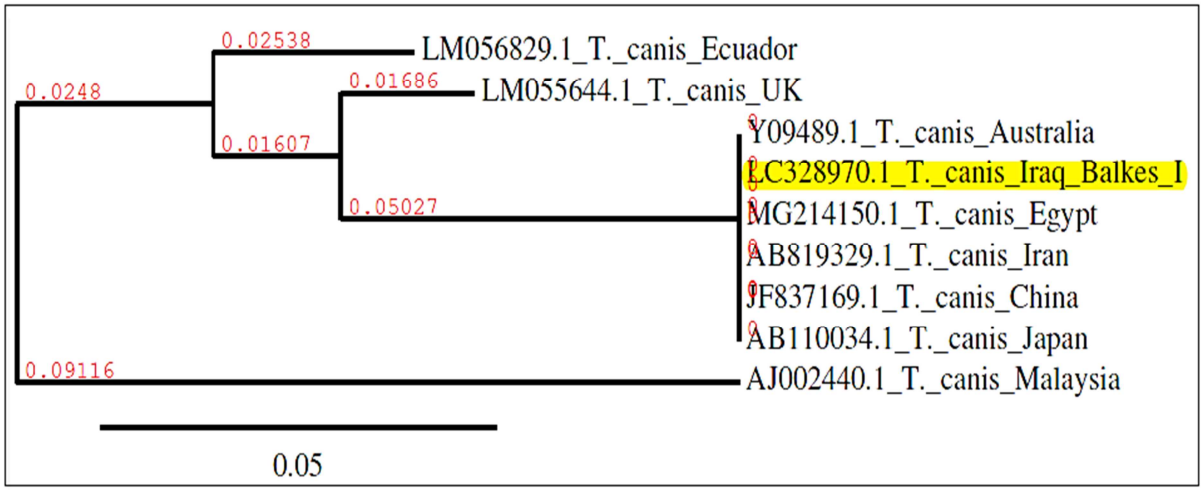

Fig. 5. Phylogenetic concatenated relationship of Ba I aligned sequences of *T. canis* adult worm from Iraq using the Phylogeny.fr/ Advance method.

This study clearly indicated that the species of the genus *Toxocara* are distinct from each other at the molecular level even though they represent similar morphological characters and that agree with other study that showed close relationship among *Toxocara* genus forms a single group consisting of several sister clades for each species analyzed (Wickramasinghe *et al*., 2009).

Phylogenic tree analysis

Phylogenic tree analyses depend on Fixation index (F_{ST}) which is a measure of population differentiation due to genetic structure (Holsinger and Weir, 2009), mounted a low genetic variation (0.05) among all comparison isolates as clarified in Fig. 5 and that means minimal or no genetic diversity among them and that populations are genetically identical. Depending on genetic distance with in phylogenic tree Iraqi isolate for aligned sequence (Ba I) recoded a high identical and exhibiting 100% homology (0.0) genetic distance with Egypt, Iran, Japan, China and Australia isolates, because it clustered in the same nodule which explains a closely related among each other and that may be due to the presence of these countries near each other and located on a single geographic line for migrating the paratenic hosts such as the birds (chickens) among these countries.

Phylogenic tree analysis indicates that Iraqi *T. canis* isolate for Ba II aligned sequence mounted a very low genetic variation (0.007) among all comparison isolates as demonstrated in Fig. 6 and genetic distance with in phylogenic tree recoded (0.00209) for India which is more closely isolate to Iraqi Ba II aligned sequence than Japan and China were mounted (0.00302) and (0.01013) respectively, these isolates are very close to Iraqi isolate because they split from the same node. Phylogenetic analysis confirmed a very low difference in Iraq isolates of *T. canis* and other countries in the world and this genetic variation recorded may be due to variance on area size of reference sequence and/ or the differences in geographical areas where isolates are collected from.

When parasite environment changes, genetic diversity plays an important role in its survival, adaptability and makes accurate analysis of this variation applicable for studies on pathogenesis, epidemiology, population biology, taxonomy and

evolutionary biology of parasites. Using different methods, such as polymerase chain reaction (PCR)-based on mutation scanning and genes sequencing of partial or complete genes, then phylogenic have been used for genetic analysis.

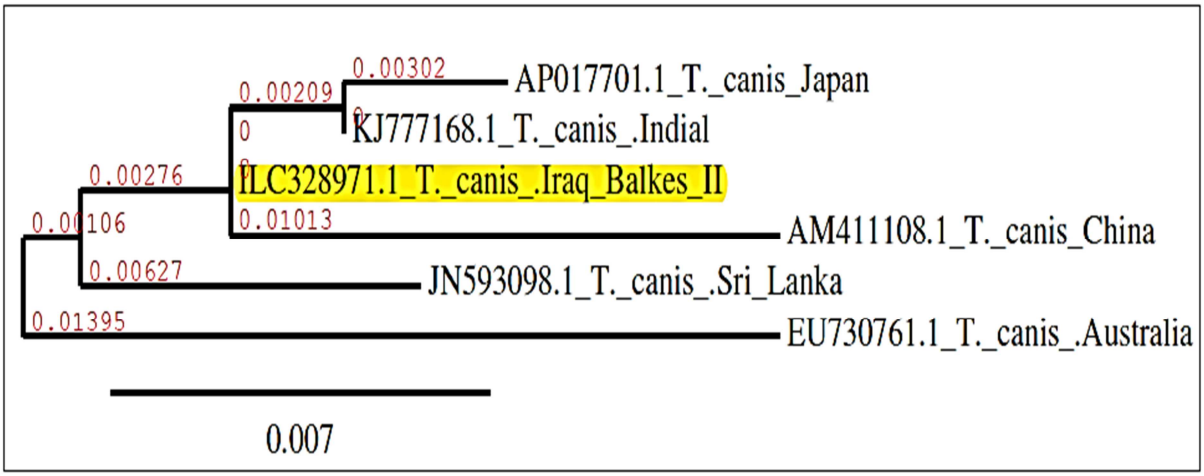

Fig. 6. Phylogenetic concatenated relationship of Ba II aligned sequences of *T.canis* adult worm from Iraq using the Phylogeny.fr/ Advance method.

Study results deal with different studies which have been reported that existence of genetic variation and phylogenetic relationships based on the nuclear and mitochondrial gene sequences among parasite populations have been used to analyze genetic variations of ascaridoid nematodes in different parts of the world (Betson *et al*., 2012; Wang *et al*., 2012).

The results demonstrated the existence of low genetic variation on the nuclear ribosomal ITS-2 region and the mitochondrial *rrn*L gene of *T. canis* isolate in Iraq among other countries, concluded that DNA sequences then phylogenetic analysis represent a useful tool to gain information about an organism evolutionary relationship.

Recommendation

Using of other targets genes to detect OLM Toxocariasis.

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