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Detection and molecular characterization of ascarid nematode infection (*Toxascaris leonina* and *Toxocara cati*) in captive Asiatic lions (*Panthera leo persica*)

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Abstract

The objective of this study was to investigate the ascarid infection in Asiatic lions using scat samples, based on microscopic analysis, PCR amplification of the ITS-2 region of ribosomal DNA and sequence analysis of the amplicons. Microscopic analysis indicated the presence of eggs of *Toxascaris leonina* in eleven of the sixteen scat samples analysed and in one of these eleven scats eggs of *Toxocara cati* were also detected. In five of the scats eggs were not detectable. The presence of *T. leonina* in all the infected samples was also confirmed by PCR amplification of the ITS-2 of ribosomal RNA gene and five of these also showed amplicons corresponding to *T. cati*, respectively. *Toxocara canis* infection was not observed in any of the scat samples. Nucleotide sequence analysis of the ITS-2 region indicated 97% to 99% similarity with *T. leonina* and *T. cati*, respectively. To our knowledge, this is the first molecular characterization of ascarid infection in captive Asiatic lions from a zoological garden of India. This study also indicates that Asiatic lions are more prone to infection either with *T. leonina* or *T. cati* and the parasite is not host specific.

Keywords

Ascarid nematodes, Asiatic lion, ITS-2, *Toxascaris leonina*, *Toxocara cati*

Dogs and cats harbour four nematode intestinal parasites *Toxocara canis*, *Toxocara cati*, *Toxocara malaysiensis* and *Toxascaris leonina* belonging to the family Ascarididae (Urquhart *et al*. 1996, Gibbons *et al*. 2001). While the infection of dogs with *T. canis* is common worldwide (Minnaar *et al*. 2002, Oliveira-Sequeira *et al*. 2002), the larvae of *T. canis* are also capable of infecting humans, causing ocular larva migrans, visceral larva migrans (Schantz 1991, Taylor 2001, Despommier 2003). Larvae of *T. cati* and *T. leonina* are known to infect laboratory animals and also have the potential to cause human disease (Despommier 2003, Fisher 2003). Recently *T. malaysiensis* was reported from cat (Gibbons *et al*. 2001) and its presence was confirmed based on molecular studies (Zhu *et al*. 1998, Li *et al.* 2006).

Investigations into the endoparasitic fauna with respect to their prevalence, geographical distribution, systematics and biology would be of immense importance for the management of the health of animals (Žąsitytė and Grikienienė 2002, Opara *et al*. 2010). Normally, nematode parasites like *T. canis*, *T. cati* and *T. leonina* are detected by microscopic analysis and serological tests but both these methods are incapable of differentiating closely related taxa such as *T. canis* and *T. cati* (Van Knapen and Buijs 1993). In comparison, the second internal transcribed spacer (ITS-2) region, an insertion between the 5.8S and large subunit rRNA gene, has proven to be particularly valuable in resolving the taxonomic status of various parasitic groups including cestodes (Bowles *et al.* 1995), trematodes (Blair *et al.* 1996) and nematodes (Hoste *et al.* 1995, Stevenson *et al.* 1995). ITS-2 sequence has been utilised to identify single eggs of strongyloid nematodes to the species level (Campbell *et al.* 1995) and for differentiating ascaridoid infections in dog, fox and cat (Jacobs *et al*. 1997).

In the present study attempts have been made to detect ascaridoid nematodes infection in lions, using scat samples, based both on microscopic analysis and ITS-2 sequence analysis. The acquired data were used to determine if one or more

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Toxocara and *Toxascaris* species cause infection in Asiatic lions and to establish the phylogenetic relationships with other *Toxocara* and *Toxascaris* species previously reported from different host animals. To our knowledge, this is the first molecular characterization of ascarid infection in captive Asiatic lions from a zoological garden of India.

Scat samples of 16 different captive Asiatic lions were collected from the Nehru Zoological Park, Hyderabad, India. Fresh samples were collected in 50 ml falcon tubes in the morning between 7 to 9 AM on a regular basis. All the scat samples were analysed on the same day. Nematode eggs were collected using the sugar flotation technique (Ito 1980) with modification so as to recover the maximum number of eggs. A fresh scat sample (2–3 g) was suspended in 15 ml of autoclaved MilliQ water and the resulting slurry was filtered through a tea strainer. The filtrate was collected in a 15 ml falcon tube and centrifuged at 1500 rpm for 2 minutes. Supernatant was carefully removed and to the pellet 7 ml of flotation solution (64 g sucrose in 50 ml autoclaved MilliQ water) was added to the tube. The scat sample was mixed gently with a blunt glass rod and the slurry was made up to 14 ml with the flotation solution. The tubes were once again centrifuged at 4000 rpm for 5 minutes and the tubes were then filled up to the brim by gently adding flotation solution without disturbing the floating layer. A convex meniscus was observed in the tube. A 22 mm coverslip was placed on the surface of the meniscus and left in contact with the meniscus for 30 minutes to allow the floating faecal content to stick to the glass surface. The excess time allowed increased the chance of getting more number of eggs required for the isolation of DNA. The coverslip was carefully removed, transferred to a clean glass slide and observed under the microscope for the presence of ascarid eggs. Ascarid (*T. leonina* and *T. cati*) positive samples (with microscopic method) were carefully washed with autoclaved MilliQ water and the wash was collected in a 15 ml falcon tube. The tubes were centrifuged at 2000 rpm for 2 minutes, the sediment collected and used for DNA isolation by the standard proteinase K/phenol:chloroform protocol of Sambrook *et al*. (1989). DNA was purified using a spin column (GeneClean spin kit, MP Biomedicals, LLC, France), dissolved in TE (10 mM Tris-HCL, 1.0 mM EDTA, pH 8.0) buffer and stored at –20°C until use.

ITS-2 of *T. leonina*, *T. cati* and *T. canis* were amplified as described by Jacobs *et al*. (1997) using three primer sets *Tleo*1(5'-CGAACGCTCATATAACGGCATACTC-3')-NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3'), *Tcat*1(5' GGAGAAG TAAGATCGTGGCACGCGT-3')-NC2, and *Tcan*1(5'-AG-TATGATGGGCGCGCCAAT-3')-NC2. The anticipated size of the ITS-2 amplified fragments of *T. leonina*, *T. cati* and *T. canis* were 300 bp, 370 bp and 380 bp, respectively (Jacobs *et al*. 1997). PCR amplification was performed essentially as described by Jacobs *et al.* (1997), but using Expand High Fidelity PCR enzyme mix (2.5 U for 25 µl; Roche, Basel, Switzerland). The PCR products were gel purified using a PureLink quick gel extraction kit (Invitrogen, Carlsbad, CA, USA) and sequenced using BigDye Terminator and ABI 3700 (Applied Biosystem, Foster, CA, USA) genetic analyser. *Tleo1*-NC2 and *Tcat1*-NC2 were used for sequencing of the ITS-2 amplified fragments of *T. leonina* and *T. cati*, respectively. For accuracy both the strands were sequenced.

DNA sequences were initially edited and subjected to BLAST sequence similarity search (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the nearest phylogenetic neighbour and aligned using Autoassembler software (Applied Biosystem, Foster, CA, USA). The edited sequences along with the sequences of *T. cati*, *T. canis*, *T. malaysiensis*, *T. vitulorum*, *T. tanuki* and *T. leonina* downloaded from the Genbank database were then aligned using CLUSTAL X (Thompson *et al*. 1997). Phylogenetic analyses for ITS-2 gene were performed with *Ascaris suum* sequence as an out-group. Jukes-Cantor algorithms were utilized, and phylogenetic trees were constructed by Neighbour-joining method, Maximum parsimony and UPGMA (Kumar *et al*. 2008). For evaluation of the robustness of tree topologies bootstrap analysis was performed based on 1000 re-samplings using MEGA 4.1 program (Kumar *et al*. 2008).

Microscopic examination of the eggs collected from the scat samples of the 16 Asiatic lions revealed the presence of eggs of *T. leonina* in the scats of eleven animals and one of these scats with *T. leonina* eggs also had eggs of *T. cati* (Fig. 1a). Overcrowding of *T. leonina* could have interfered in the identification of *T. cati* eggs when examined through microscope. In five of the scats eggs were not detected. Eggs of *T. canis* were not detected in any of the 16 scats. All the

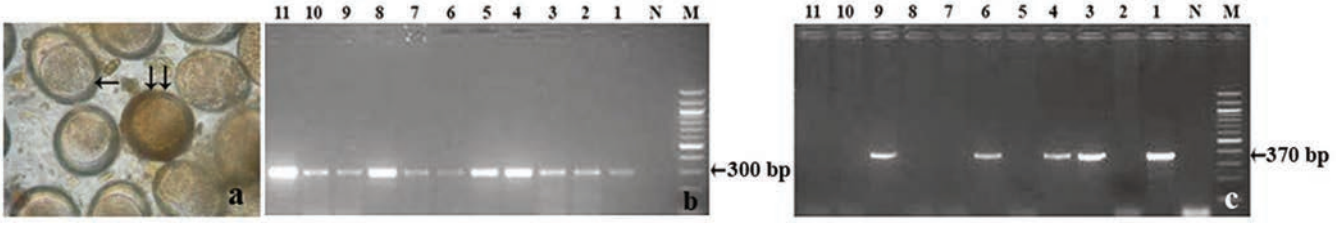

Fig. 1. Eggs of *Toxascaris leonina* (arrow) and *Toxocara cati* (two arrows) in a scat sample of an Asiatic lion (× 400) (**a**). Amplification of ITS-2 of nuclear ribosomal DNA of *Toxascaris leonina* of 300 bp (arrow) (**b**) and *Toxocara cati* of 370 bp (arrow) (**c**) using eggs isolated from the scats of Asiatic lions. Lane M represents the 100 bp DNA ladder and N the negative control, respectively

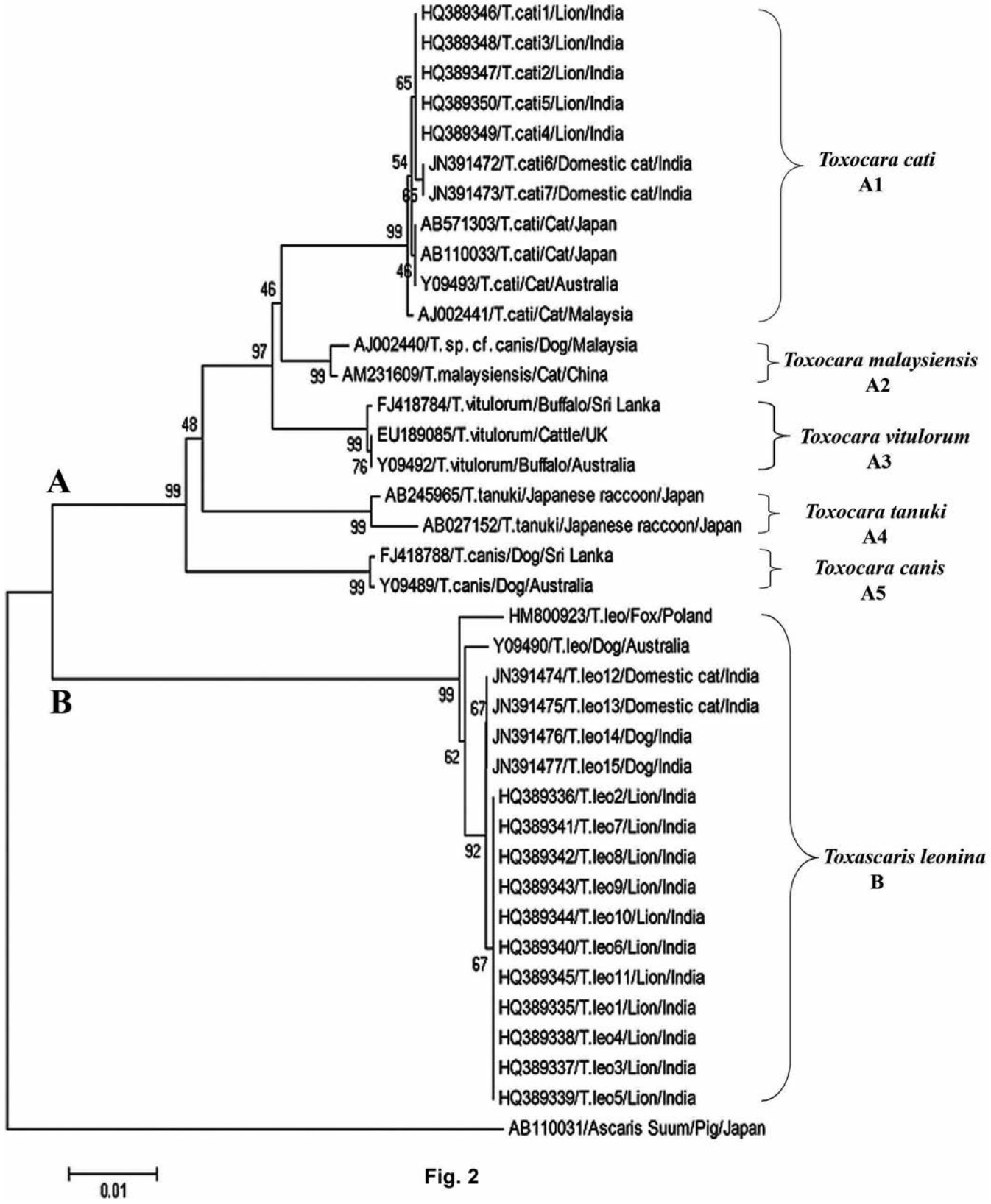

Fig. 2. Neighbour-joining phylogenetic tree based on the ITS-2 sequence of ribosomal DNA of *Toxascaris leonina* and *Toxocara cati* isolated from scats of Asiatic lions and other sequences of ascarid retrieved from the GenBank database. Sequence of *Ascaris suum* ITS-2 was used as an out-group. Ascarid clades A1, A2, A3, A4, A5 and B are labelled. Bootstrap values for 1000 replicates are shown at the respective nodes. Bar indicates 1 substitution per 100 nucleotides

Table I. Similarity of ITS-2 sequence of ribosomal DNA of *Toxascaris leonina* and *Toxocara cati* isolated from the scats of captive Asiatic lions with sequences retrieved from the GenBank

Ascaridoids/host/accession number	n	Nearest phylogenetic neighbour	Similarity of ITS-2 gene sequence of ribosomal DNA (%)
Toxascaris leonina/Asiatic lion/HQ389335 to HQ389345	11	*T. leonina* (Y09490)	97
Toxocara cati/Asiatic lion/ HQ389346 to HQ389350	5	*T. cati* (AB571303)	99

above eleven scats samples positive for the presence of ascarid eggs were used for DNA isolation. DNA from the eggs of the eleven suspected ascarid infected lions following PCR using *Tleo*1-NC2 primers, specific to *T. leonina*, yielded an anticipated single amplified product of 300 bp (Fig. 1b). Sequencing of this product and BLAST analysis indicated that

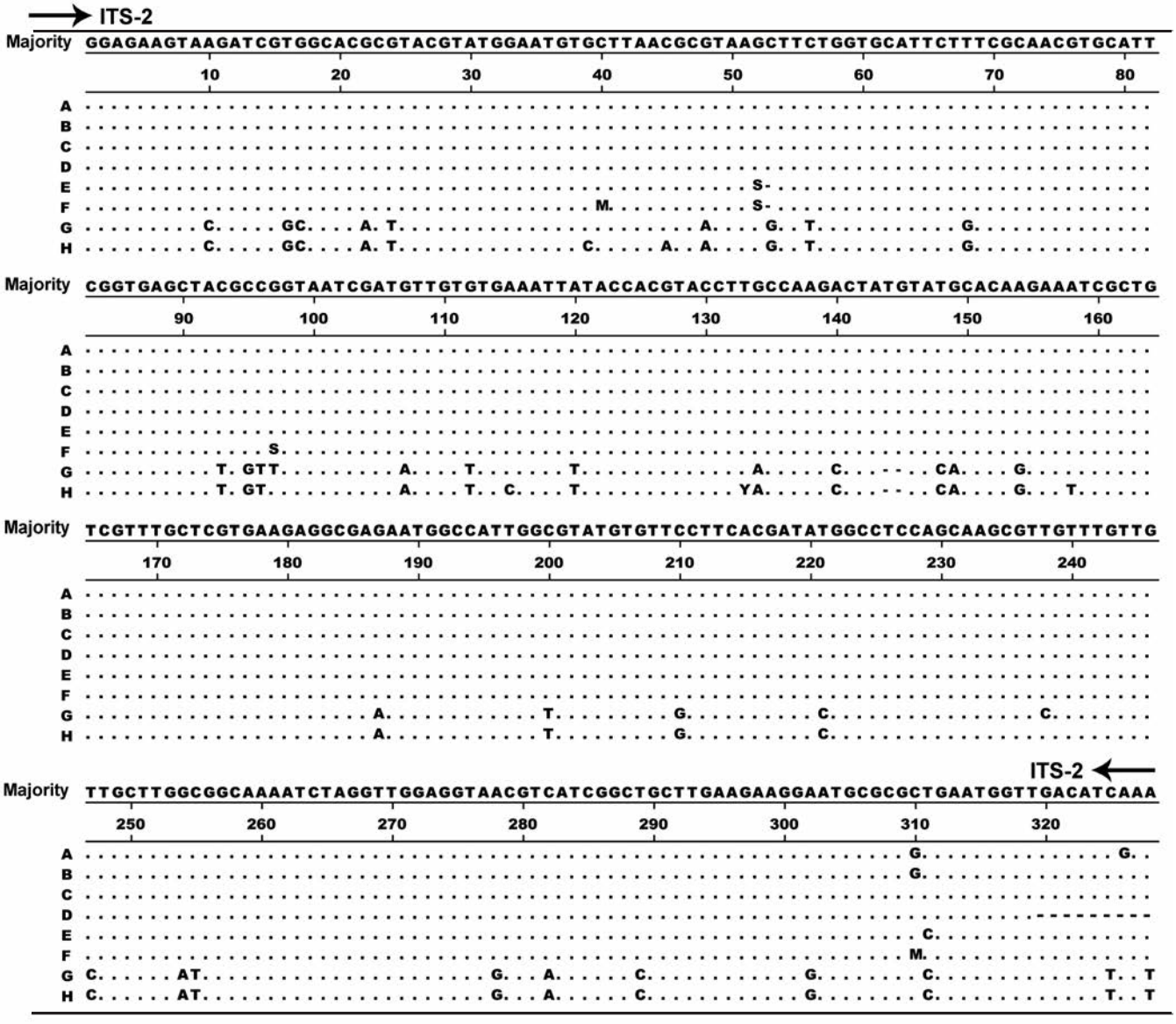

Fig. 3. Alignment of the ITS-2 rDNA sequences of *Toxocara cati* with *Toxocara malaysiensis* from different host species and countries. JN391472 *T. cati*/cat/India (**A**), HQ389346 *T. cati*/Asiatic lion/India (**B**), AB571303 *T. cati*/cat/Japan (**C**), AB110033 *T. cati*/cat/Japan (**D**), AJ002441 *T. cati*/cat/Malaysia (**E**), Y09493 *T.cati*/cat/Australia (**F**), AJ002440 *T. malaysiensis*/cat/Malaysia (**G**) and AM231609 *T. malaysiensis*/cat/China (**H**). Nucleotide similarities are indicated by a dot (**.**) and differences are mentioned in sequences. The numbers refer to alignment positions. In sequences, Bases M = A or C and S = C or G

the nearest phylogenetic neighbour was *T. leonina*, with which they exhibited 97% similarity (Table I). However, when PCR of all the above eleven samples using *Tcat*1-NC2 primers, specific for *T. cati*, was done a single anticipated amplified product of 370 bp was observed in five samples (Fig. 1c). Sequencing of this product and BLAST analysis indicated that the nearest phylogenetic neighbour was *T. cati*, with which they exhibited 99% similarity (Table I). No amplification was obtained using *Tcan*1-NC2 primers, specific for *T. canis*.

Phylogenetic analysis based on the ITS-2 gene sequences (238 nucleotides) obtained in this study and from 14 ascarid species sequences downloaded from the GenBank database indicated that the ascarid species formed two major clades (A and B) with a robust bootstrap value in all the three phylogenetic trees constructed using either Neighbour-joining, Maximum parsimony or UPGMA (Fig. 2). The phylogenetic tree based on ITS-2 sequences was able to distinguish between *Toxocara* and *Toxascaris* species and was accurate enough to distinguish different *Toxocara* species like *T. cati* (subclade A1), *T. malaysiensis* (subclade A2), *T. vitulorum* (subclade A3), *T. tanuki* (subclade A4) and *T. canis* (subclade A5) with strong bootstrap support. *T. leonina* (clade B) is the only species in the genus *Toxascaris* which causes infection in canids and felids.

Five of the *T. cati* isolates from Asiatic lions, which included HQ389346, HQ389347, HQ389348, HQ389349 and HQ389350 formed a subclade A1 along with sequences of *T. cati* (Y09493, AB571303, AB110033, AJ002441, JN391472 and JN391473) from the domestic cat from Australia, Japan, Malaysia and India indicating that *T. cati* is not host specific and present across four different countries. Percentage nucleotide identity (PNI) of ITS-2 sequence revealed that all the five isolates from Asiatic lions were identical to each other as well as with other isolates in subclade A1. This observation confirms earlier conventional studies on the worldwide presence of *T. cati* in cats (Martinez-Barbabosa *et al*. 2003, Labarthe *et al*. 2004). *T. cati* is also known to be zoonotic (Fisher 2003).

Comparison of the ITS-2 sequences (326 bp) of *T. cati* (Fig. 3) from different host species and different countries indicated 98.8% to 100% nucleotide identity implying that the sequence is highly conserved. The nucleotide identity between the two ITS-2 sequences of *T. malaysiensis* strains from Malaysia and China was also very similar (98%). But comparison of the ITS-2 sequences of *T. cati* and *T. malaysiensis* indicated a total of 45 individual nucleotide changes and the similarity ranged from 86.8% to 88%.

The sequence of ITS-2 of all the eleven isolates of *T. leonina* (HQ389335, HQ389336, HQ389337, HQ389338, HQ389339, HQ389340, HQ389341, HQ389342, HQ389343, HQ389344 and HQ389345) from the Asiatic lions formed a distinct clade B along with *T. leonina* isolate from a dog (Y09490, JN391476 and JN391477) in Australia and India, from a domestic cat (JN391474 and JN391475) in India and a fox (HM800923) from Poland (Fig. 2). All these isolates also showed 97–100% nucleotide identity with each other. Comparison of the ITS-2 sequences (238–241 bp) of *T. leonina* (Fig. 4) from different host species and different countries indicated that *T. leonina* sequence from domestic cat and Asiatic

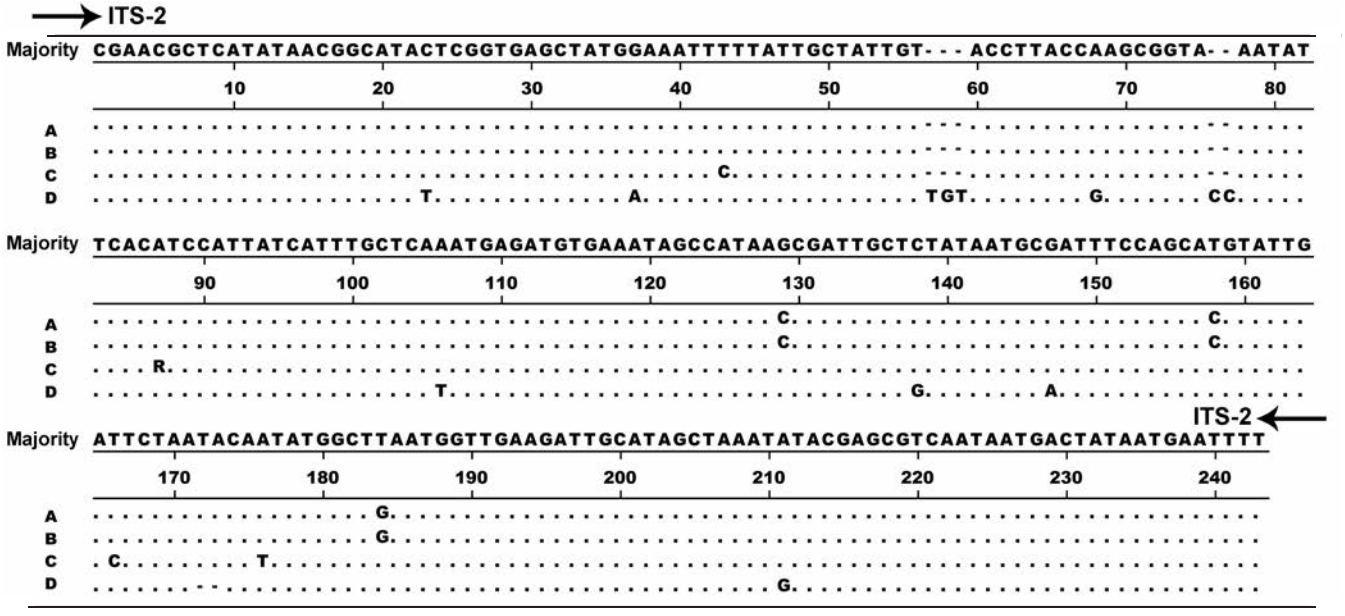

Fig. 4. Alignment of the ITS-2 rDNA sequences of *Toxascaris leonina* from different host species and countries. JN391474 *T. leonina*/cat/India (**A**), HQ389335 *T. leonina*/Asiatic lion/India (**B**), Y09490 *T. leonina*/cat/Australia (**C**) and HM800923 *T. leonina*/cat/Poland Nucleotide similarities among the taxa are indicated by a dot (**.**) and differences are mentioned in sequences. The numbers refer to alignment positions. In sequences, Bases R = A or G

lion showed 100% homology. In comparison the *T. leonina* isolates of cat from Australia and Poland showed 97.1% and 94.1% homology in the ITS-2 sequence, respectively. A total of 19 individual nucleotide changes were observed between all the *T. leonina* sequences.

Toxocara leonina was also earlier demonstrated to be parasitic in dogs and cats (Urquhart 1996, Gibbons *et al*. 2001), causing significant health problems. Larvae of *T. leonina* can also invade the tissues of laboratory animals and has the potential to cause human disease (Despommier 2003). In this study none of the animals were infected with *T. canis*. This study indicates that Asiatic lions are more prone to infection either with *T. leonina* or *T. cati* in accordance with the earlier observations made based on the microscopic method (Patnaik and Acharjyo 1970, Prescott 1981, Patton and Rabinowitz 1994, Bjork *et al*. 2000, Nashiruddullah and Chakraborty 2001, Opara *et al*. 2010). This methodology based on the ITS-2 sequence of the ribosomal RNA gene could be a valuable tool for addressing epidemiological questions related to nematode parasites in animals in wildlife sanctuaries/protected areas and captive animals and thus could help in eradication of these parasites.

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