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An initial molecular signature of Indian isolates of *Toxocara*

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ABSTRACT

This communication deals with molecular signature of *Toxocara canis*, *T. cati* and *T. vitulorum* collected from different geographical locations and host assemblages of India. *T. vitulorum* was collected from cattle, yak and mithun of West Bengal, Arunachal Pradesh and Nagaland, respectively. Isolated parasites were initially identified morphologically before proceeding for molecular characterization. ATP synthase subunit 6 (*atp*6) gene had a 598 bp stretch which contained both the punctuation codons but was unique in its characteristics due to presence of abbreviated stop codon (T). On the basis of phylogenetic analysis of *atp*6, 12S and transcribed spacer sequences, three species could be clustered in three different groups. Number of preferred and non preferred codons also varied in between three species of *Toxocara* of Indian origin. *Atp*6 gene had abundance in guanine (G) and thymine (T) bases which has also been described as unique characteristic for Neodermata. Restriction profile of transcribed sequences, 5.8S gene and a small fragment of 28S gene could differentiate Indian isolates of *Toxocara* in two different clades.

Key words: Molecular signature, *Toxocara canis*, *Toxocara cati*, *Toxocara vitulorum*

Carnivores and large ruminants harbour intestinal parasites belonging to the nematode family Ascarididae (Urquart *et al*. 1996). *Toxocara* was identified as important genus under this family which is of human and animal health significance. Three species of *Toxocara* viz. *T. canis*, *T. cati* and *T. vitulorum* are the common species, which have been reported worldwide due to very well known human-animal bond (Paul *et al*. 2010, Rubinsky- Elefant *et al*. 2010).

Ascaridoid nematodes of the genus *Toxocara* can be identified, classified and delineated on the basis of their morphological features, site of predilection and host species (Bowman *et al*. 2008). On the contrary, traditional methods are of no use for identification of larval and/or ova, which leaves a question mark in taxonomic analyses of *Toxocara* in paratenic and aberrant hosts and environmental samples as well (Zhu *et al*. 1998, 2001, Gasser *et al*. 2006). This has been evidenced from the literature that, micro-Ouchterlony test and primary binding assay can identify *Toxocara* larvae up to genus level in human patients suffering from generalized or compartmentalized forms of larva migrans. But species identification is not possible by immunological mining due to presence of cross reactive antigens. The shortcomings of phenotypic and immunological detection of intermediate stage of *Toxocara* can only be resolved by molecular mining of the species (Fisher 2003).

A perusal of available literature suggests that, using internal transcribed sequences, mitochondrial (mt) sequences, multi locus analysis and other DNA fingerprinting methods, taxonomic status of different species of *Toxocara* can be resolved (Jacobs *et al*. 1997, Zhu *et al*. 1998, Gibbons *et al*. 2001, Gasser *et al*. 2006, Wickramasinghe *et al*. 2009, Sheng *et al*. 2012, Fogt-Wyrwas *et al*. 2013).

Till date, there is no description on molecular signature of *T. canis*, *T. cati* and *T. vitulorum* from India, which is virtually required for proper taxonomic analysis of *Toxocara* of Indian origin. Considering the above mentioned fact, this study has been undertaken on molecular characterization of *T. canis*, *T. cati* and *T. vitulorum* collected from different geographical locations and host assemblages of India.

MATERIALS AND METHODS

Collection, isolation and processing of samples: T. canis was isolated from dogs of Arunachal Pradesh and Tamil Nadu. *T. cati* was collected from domestic cats of Arunachal Pradesh. *T. vitulorum* was collected from yak of Arunachal Pradesh, mithun of Nagaland, and cattle of West Bengal.

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Isolated parasites were cleaned in phosphate buffer saline (PBS) (pH 7.2). Samples were preserved in 70% ethanol (v/v) at -20°C for extraction of genomic DNA.

Extraction of genomic DNA: Genomic DNA was isolated from individual worms by conventional liquid-liquid extraction technique following methodology described by McManus *et al*. (1985) after minor modification. Precipitated DNA after washing by 70% ethanol (v/v) was dried and dissolved in TE buffer (pH 8.2). Purity and quantification of DNA sample was assessed by conventional spectrophotometry.

Amplification of atp6, 12S rRNA, ITS1and ITS2: To amplify specific mt genes and nuclear fragments, the following published primers were used (Wickramasinghe *et al*. 2009, Sheng *et al*. 2012).

*atp*6: Forward: 5'TWYCCWCGTTWTCGTTAGA3'
Reverse: 5'CTTAAAACAAATRCAYTTMT3'

12S rDNA: Forward: 5'GTTCCAGAATAATCGGCTA3'
Reverse: 5'ATTGACGGATGAGTTTGTACC3'

ITS1: Forward: 5'GGAAACCGCCTTAATCGCAGT3'
Reverse: 5'TTCGCTTCGCCACCCGTACA3'

ITS2: Forward: 5'CGGTGGATCACTCGGCTCGT3'
Reverse: 5'CCTGGTTAGTTTCTTTTCCTCCGC3'

Amplification was carried out with an initial denaturation step at 94°C for 5 min followed by 35 cycles. Annealing was done at 53°C followed by elongation at 72°C.

Purification of PCR product and generation of sequence information: PCR products were purified using gel extraction kit and sequence information was generated through outsourcing.

Analysis of sequence information: Sequence information was analysed by online version of MEGA, version. 6 (Tamura *et al.,* 2013). Comparison of sequence information was made with respective available sequence information of *T. canis*, *T. cati*, *T. vitulorum*, *T. malaysiensis*, *T. tanuki*, *T. leonina*, *Bayilascaris transfuga* and *Anisakis simplex*. Phylogenetic analysis was made on the basis of unweighted pair group with arithmetic mean (UPGMA) after providing 1000 bootstrap replications. Relative synonymous codon frequency (RSCF) was calculated for Indian isolates of *T. canis*, *T. cati* and *T. vitulorum*. AT and GC skew indices were calculated using standard formula described by Perna and Kocher (1995).

PCR based restriction fragment length polymorphism (PCR-RFLP): To conduct PCR-RFLP, a fragment comprising of ITS1, 5.8S gene, ITS2 plus approximately 70 nucleotides of 28S was enzymatically amplified using universal primers (forward: 5'GTAGGTGAACCTGCGGAAGGATCATT3'; reverse: 5'TTAGTTTCTTTTCCTCCGCT3') (Zhu *et al*. 1998). PCR products were further digested with 10 units of *RsaI* for 15 h at 37°C. Restriction fragments were separated on 2.5% agarose gel and size was estimated using standard markers.

RESULTS AND DISCUSSION

After PCR amplification, amplicon sizes of *atp*6, 12S rRNA, ITS1 and ITS2 were 750 bp, 500 bp, 1200 bp and 550 bp, respectively. Further analysis of the data revealed that total stretch of *atp*6 was 598 bp, which comprised of start codon and abbreviated stop codon (T). Presence of abbreviated stop codon in *atp*6 gene may be attributed as a consistent feature with annotation in the mt genomes of other ascaridoid nematodes (Okimoto *et al*. 1992, Lavrov and Brown 2001, Kim *et al*. 2006, Li *et al*. 2008).

For phylogenetic analysis, 598 bp fragment of nucleotides and 199 residues of predicted amino acids of *atp*6, 418 bp nucleotide information of 12S gene, 494 bp sequence information of ITS1 and 308 bp fragment of ITS2 were considered. Phylogenetic analysis revealed that, *T. canis*, *T. cati* and *T. vitulorum* could be clustered in three different groups and were comparable with *Toxocara* isolates reported from other parts of the globe (Figs 1-5). From the topology of the phylogenetic tree of *atp*6, it could be concluded that *T. malaysiensis* (AM412316/CAL80798) and *T. leonina*. KC902750/AGO85907 were in out group (Figs 1-2). Phylogenetic analysis of 12S gene revealed that, Korean isolate of *Anisakis simplex* (AY994157) and Chinese

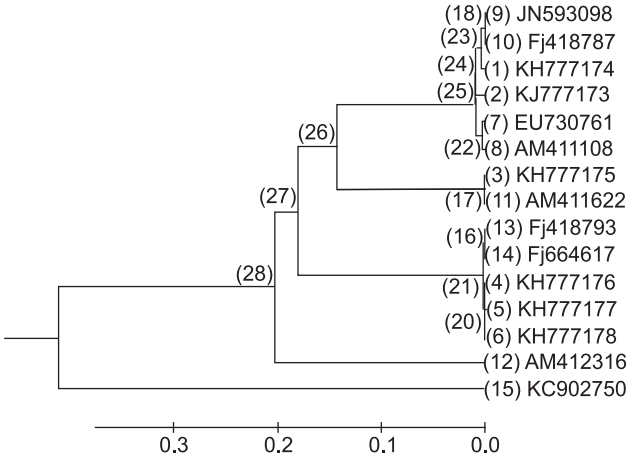

Fig.1. Phylogenetic analysis by UPGMA method on predicted amino acid sequence of *atp*6. The optimal tree with the sum of branch length is 1.29922651. The evolutionary distances have been computed using Maximum Composite Likelihood method. This analysis has involved 15 protein sequences and there were a total of 199 positions in the final set. Indian isolates of *Toxocara* (KJ777175 = *T. cati* (Arunachal Pradesh isolate), KJ777176 = *T. vitulorum* (Arunachal Pradesh isolate) , KJ777177= *T. vitulorum* (West Bengal isolate), KJ777178= *T. vitulorum* (Nagaland isolate), KJ777174= *T. canis* (Tamil Nadu isolate), KJ777173 = *T. canis* (Arunachal Pradesh isolate) , Australian isolate of *T. canis* (ACF06386), Chinese isolate of *T. canis* (CAL69956), Sri Lankan isolates of *T. canis* (AEO72443, ACR38966),Chinese isolate of *T. cati* (CAL80786), Chinese isolate of *T. malayensis* (CAL80798), Sri Lankan isolates of *T. vitulorum* (ACR38967, ACM88518) and Australian isolate of *Ts. leonina* (AGO85907).

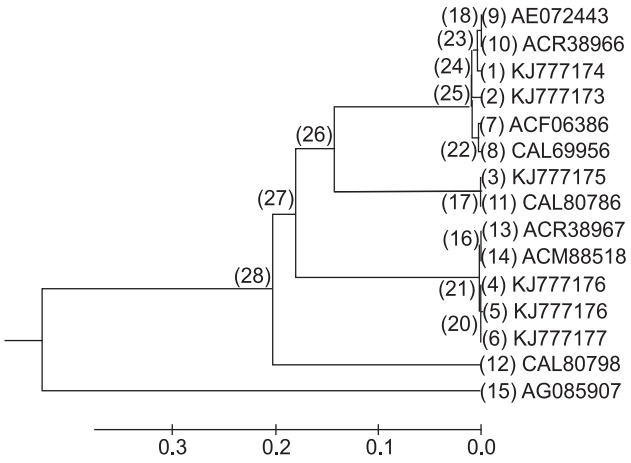

Fig.2. Phylogenetic analysis by UPGMA method on nucleotide sequence of *atp*6. The optimal tree with the sum of branch length is 1.29922651. The evolutionary distances have been computed using Maximum Composite Likelihood method. This analysis has involved 15 nucleotide sequences and there were a total of 598 positions in the final set. Indian isolates of *Toxocara* ((KJ777175 = *T. cati* (Arunachal Pradesh isolate), KJ777176 = *T. vitulorum* (Arunachal Pradesh isolate) , KJ777177= *T. vitulorum* (West Bengal isolate), KJ777178= *T. vitulorum* (Nagaland isolate), KJ777174= *T. canis* (Tamil Nadu isolate), KJ777173 = *T. canis* (Arunachal Pradesh isolate) has been analysed with Australian isolate of *T. canis* (EU730761), Chinese isolate of *T. canis* (AM411108), Sri Lankan isolates of *T. canis* (JN593098, FJ418787),Chinese isolate of *T. cati* (AM411622), Chinese isolate of *T. malayensis* (AM412316), Sri Lankan isolates of *T. vitulorum* (FJ418793, FJ664617) and Australian isolate of *Ts. leonina* (KC902750).

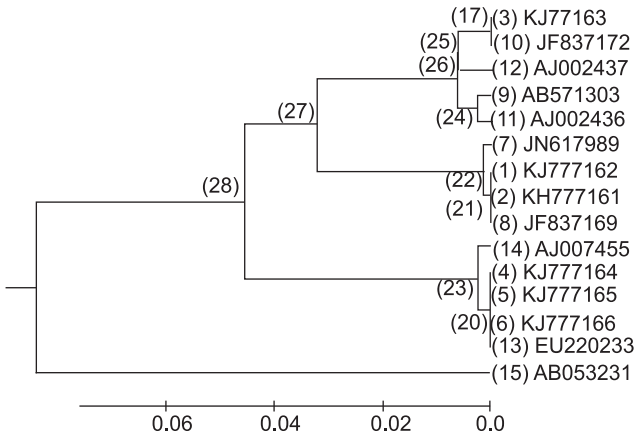

Fig.3. Phylogenetic analysis by UPGMA method on nucleotide sequence of 12S. The optimal tree with the sum of branch length is 0.73124791. The evolutionary distances have been computed using Maximum Composite Likelihood method. This analysis has involved 18 nucleotide sequences and there were a total of 401 positions in the final set. Indian isolates of *Toxocara* (KJ777169 = *T. cati* (Arunachal Pradesh isolate), KJ777170 = *T. vitulorum* (Arunachal Pradesh isolate) , KJ777171= *T. vitulorum* (West Bengal isolate), KJ777172 = *T. vitulorum* (Nagaland isolate), KJ777168 = *T. canis* (Tamil Nadu isolate), KJ777167 = *T. canis* (Arunachal Pradesh isolate) has been analysed with Chinese isolates of *T. canis* (AM411108, JN256966), Japanese isolate of *T. canis* (FJ418782), Australian isolate of *T. canis* (EU730761), Chinese isolates of *T. cati* (AM411622, JN256957, JN256961, JN256956), Sri Lankan isolate of *T. vitulorum* (FJ418789), Chinese isolate of *T. malayensis* (AM412316), Korean isolate of *Anisakis simplex* (AY994157) and Chinese isolate of *Bayilisascaris transfuga* (JN256972).

isolate of *Bayilisascaris transfuga* (JN256972) formed the out group (Fig. 3). Similarly using ITS1 sequences, Japanese isolate of *T. tanuki* (AB053231) was in out group compared to Indian isolates of *Toxocara* and homologous species sequences (Fig. 4). After analysis of ITS2 sequences, this has been observed that, *T. vitulorum* could be clustered in same group with *T. malaysiensis* (AM231609) (Fig. 5).

Topology of the phylogenetic tree was comparable with the results reported earlier on phylogenetic analysis of *atp*6 (Wickramasinghe *et al*. 2009), ITS1 (Zhu *et al*. 1998; Fogt-Wyrwas *et al*. 2013) and ITS2 (Wickramasinghe *et al*. 2009). Previous studies on phylogenetic analysis have suggested that, *T. vitulorum* was more closely related to *T. malaysiensis* (Wickramasinghe *et al*. 2009). During the present study Indian isolates of *Toxocara* could be clustered in the group of *T. malaysiensis* on the basis of ITS2 sequence, which confirmed the probable zoonotic potential of Indian isolates of *T. vitulorum*. This has been further observed that, longer homologic domains were present in ITS1 region compared to ITS2 region of Indian isolates of *Toxocara*, which is in agreement with the previous finding (Fogt-Wyrwas *et al*. 2013) (data not shown).

A total of 23 and 40-41 codons of *atp*6 could be identified as preferred and non preferred codons for Indian isolates of *T. canis*, respectively. Similarly 22 were preferred codons and 42 were non preferred codons for Indian isolate of *T. cati*. *Atp*6 of Indian isolates of *T. vitulorum* had 24 preferred codons and 40 non preferred codons. Codon usage bias depicts about non uniform usage of codons which basically helped to identify preferred and non preferred codons. In earlier studies this has been found that, numbers of preferred and non preferred codons vary in between the different species of diptera and hymenoptera (Behura and Severson 2012). The earlier finding also corroborates with our present finding on variability of preferred codons in between the 3 species of *Toxocara*.

AT skew was negative and GC skew was positive for all Indian isolates when coding region of *atp*6 was considered (Table 1). From the result of skew this could be evidenced that, T & G are the most common bases in the sequence information of *atp*6 gene. The same is the observation for other helminths like Trematoda, Monogenea and Cestoda which belong to a monophyletic taxon, the Neodermata (Le *et al*. 2004).

Digestion of ITS1, 5.8S gene, ITS2 and partial sequence of 28S yielded fragments of three variable sizes. Product sizes were 270 bp, 300 bp and 500 bp for *T. canis* and were 100 bp, 300 bp and 700 bp for *T. cati* and *T. vitulorum* (Fig. 6). Previous studies suggested that digestion with *Rsa*I could produce strong band of 510 bp, 300 bp and 270 bp

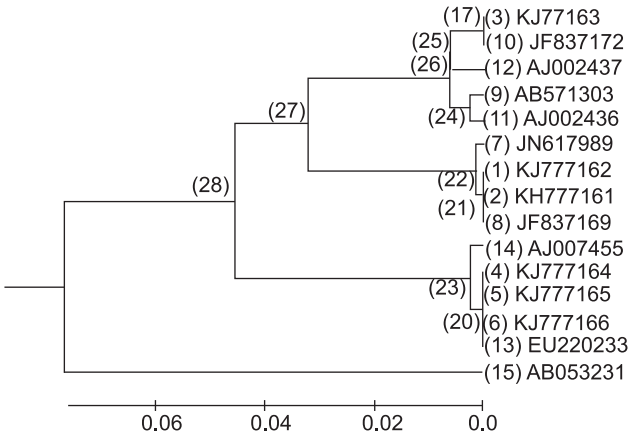

Fig.4. Phylogenetic analysis by UPGMA method on nucleotide sequence of ITS1. The optimal tree with the sum of branch length is 0.24789300. The evolutionary distances have been computed using Maximum Composite Likelihood method. This analysis has involved 15 nucleotide sequences and there were a total of 440 positions in the final set. Indian isolates of *Toxocara* (KJ777163 = *T. cati* (Arunachal Pradesh isolate), KJ777164 = *T. vitulorum* (Arunachal Pradesh isolate) , KJ777165 = *T. vitulorum* (West Bengal isolate), KJ777166 = *T. vitulorum* (Nagaland isolate), KJ777162= *T. canis* (Tamil Nadu isolate), KJ777161 = *T. canis* (Arunachal Pradesh isolate) has been analysed with Taiwan isolate of *T. canis* (JN617989), Chinese isolate of *T. canis* (JF837169) , Japanese isolate of *T. cati* (AB571303),Chinese isolate of *T. cati* (JF837172),Australian isolate of *T. cati* (AJ002436), Malaysian isolate of *T. cati* (AJ002437), United Kingdom isolate of *T. vitulorum* (EU220233), Australian isolate of *T. vitulorum* (AJ007455) and Japanese isolate of *T. tanuki* (AB053231).

Table. 1. AT/GC skew indices of Indian isolates of *Toxocara* for coding region of *atp*6

Name of the species and isolate	AT skew	GC skew
T. canis (Tamil Nadu isolate)	–0.495	0.497
T. canis (Arunachal Pradesh isolate)	–0.490	0.497
T. cati (Arunachal Pradesh isolate)	–0.466	0.460
T. vitulorum (Arunachal Pradesh isolate)	–0.531	0.525
T. vitulorum (West Bengal isolate)	–0.531	0.525
T. vitulorum (Nagaland isolate)	–0.531	0.525

for *T. canis*. For *T. cati* three prominent scorable bands could be detected approximately of 690-700 bp, 300 bp, and 100 bp (Zhu *et al*. 1998). Our finding is also comparable with the previous finding on RFLP profile of *T. canis* and *T. cati* (Zhu *et al*., 1998). However, RFLP profile of *T. vitulorum* could not be compared due to non availability of information.

Based upon the present study this could be concluded that, mt and transcribed sequences are useful for taxonomic classification of *Toxocara* and above all restriction profile could cluster *T. canis* in one group and *T. cati-T. vitulorum* in another cluster.

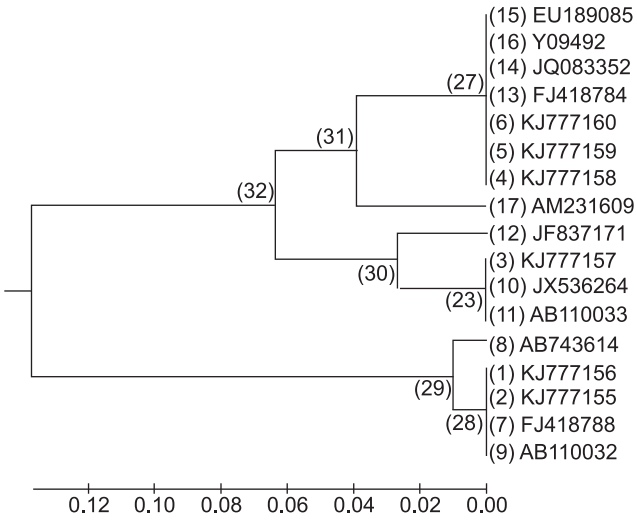

Fig.5. Phylogenetic analysis by UPGMA method on nucleotide sequence of ITS2. The optimal tree with the sum of branch length is 0.39281081. The evolutionary distance has been computed using Maximum Composite Likelihood method. This analysis has involved 16 nucleotide sequences and there were a total of 276 positions in the final set. Indian isolates of *Toxocara* (KJ777157 = *T. cati* (Arunachal Pradesh isolate), KJ777158 = *T. vitulorum* (Arunachal Pradesh isolate) , KJ777159 = *T. vitulorum* (West Bengal isolate), KJ777160 = *T. vitulorum* (Nagaland isolate), KJ777156 = *T. canis* (Tamil Nadu isolate), KJ777155 = *T. canis* (Arunachal Pradesh isolate) has been analysed with Sri Lankan isolates of *T. canis* (FJ418788), Iranian isolate of *T. canis* (AB743614), Japanese isolate of *T. canis* (AB110032) , Iranian isolate of *T. cati* (JX536264), Japanese isolate of *T. cati* (AB110033), Chinese isolate of *T. cati* (JF837171), Chinese isolate of *T. malaysiensis* (AM231609), Sri Lankan isolate of *T. vitulorum* (FJ418784), Canadian isolate of *T. vitulorum* (JQ083352), United Kingdom isolate of *T. vitulorum* (EU189085), and an isolate of *T. vitulorum* (Y09492) of unknown origin.

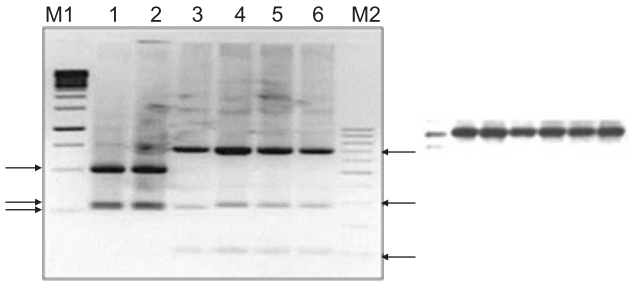

Fig.6. Molecular delineation of *Toxocara* spp. by PCR-RFLP of a fragment comprising of ITS1, 5.8 S, ITS2 and ~70 bp fragment of 28S gene. [M1= 1 kb DNA ladder; lane1= *T. canis* (Tamil Nadu isolate); lane2= *T. canis* (Arunachal Pradesh isolate); lane3= *T. cati* (Arunachal Pradesh isolate); lane4= *T. vitulorum* (Arunachal Pradesh isolate); lane5= *T. vitulorum* (West Bengal isolate); lane6= *T. vitulorum* (Nagaland isolate); M2= 100 bp DNA ladder]. [1 kb DNA ladder: last four bands from bottom (250 bp, 500 bp, 750 bp, 1000bp)] [100 bp DNA ladder: eight bands from bottom (100 bp, 200 bp, 300 bp, 400bp, 500 bp, 600 bp, 800bp, 1000 bp)].

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