EID DISPATCHES

Toxocara malaysiensis in domestic cats in Vietnam – an emerging zoonosis?

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We report, for the first time, the occurrence of *Toxocara malaysiensis*, but not *T. cati* in domesticated cats in Vietnam; and *T. canis* was commonly found to infect dogs. The finding of *T. malaysiensis* infection is likely of public health concern and warrants investigations of this ascaridoid in Vietnam and other countries.

Toxocariasis is a globally distributed infection of carnivores, primarily dogs and cats caused by species of the *Toxocara* genus (Nematoda: Toxocaridae), including *Toxocara canis, T. cati* and *T. malaysiensis* (McGuinness, Leder, 2014). *T. canis* and *T. cati* in pets are of the significant zonootic ascaridoid nematodes infecting humans reported worldwide (Moreira et al., 2014; Fisher, 2003), while the recently described species, *T. malaysiensis* (Gibbons et al., 2001), contributes less prevalence in cats and humans (Macpherson, 2013). In humans, *Toxocara* larvae can migrate in any organs, causing three major clinical forms of disease: visceral larva migrans/toxocariasis (VLM/VT), ocular larva migrans/toxocariasis (OLM/OT), covert or common toxocariasis (CT) and neurotoxocariasis (NT) (Macpherson, 2013). Human toxocariasis is usually not a fatal disease, but can be associated with asthma, neurodegenerative disorders, myocarditis, pleural effusion, hypereosinophilia, endophthalmitis and hepato-splenomegaly (Macpherson, 2013; Woodhall et al., 2014; Fan et al., 2015).

In Vietnam, almost nothing is known about the epidemiology of *Toxocara* species in dogs, cats and humans, and only one human case of ocular toxocariasis due to *T. canis* was molecularly confirmed (De et al., 2014). Presently in Vietnam, *T. canis* is believed to be predominant in dogs and *T. cati* in cats, but no taxonomic study has yet been conducted in pets in Vietnam. Previous studies showed that cats in Malaysia and China were infected by *T. malaysiensis*, a species that is distinct from *T. cati* (Zhu et al., 1998; Gibbons et al., 2001; Li et al., 2006), suggesting that investugations are needed in other geographical origins of the world. In the present study, we which investigated *Toxocara* from dogs and cats to establish whether *T. malaysiensis* is present in Vietnam.

The Study

A total of 29 morphologically identified *Toxocara* samples were randomly selected from a collection from a parasitoogical survey of dogs and cats from domestic households in two communes in Hanoi and in a village in Nam Dinh province of Vietnam, in 2014. These samples were used for a genetic study using PCR-coupled sequencing. Expelled adult worms and eggs combed from the fur of dogs and cats were fixed in 70% ethanol for subsequent DNA analysis (**Table 1**). We used the mitochondrial *atp*6 gene or the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA for molecular identification (Zhu et al., 1998; Li et al., 2006; Wickramasinghe et al., 2009).

Total genomic DNA was isolated from adults and eggs by using the QiaAmp DNA extraction Kit (Qiagen). PCR and direct nucleotide sequencing were performed using pairs of primers designed by us: TON2F (5'-GCTTACCCKCGTTTTCGTTATGA-3') and TOSER (5'-CCCAWAAYAGATTTAGAAGACCT-3') for *atp*6, and U3SF (5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and U28S2R (5'-ACAACCCGACTCCAAGGTC-3') for ITS-2. DNA template (10-50 ng) were added to a 50 µl-volume containing 25 µl PCR Master Mix (Promega), 2 µl of each primer (10 pmol/µl), 2 µl DMSO (dimethyl sulfoxide) and 17 µl pure water. PCR was carried out in a MJ thermal cycler PTC-100 (MJ Research, Watertown, MA, USA) with an initiation at 94˚C for 5 min, then 35 cycles of denaturation at 94˚C for 30 sec, annealing at 52˚C for 30 sec, extension at 72˚C for 2 min, followed by a final cycle of 10 min at 72˚C. Amplicons of 598 nucleotides (*atp*6) and/or 322-323 (ITS-2) were obtained from direct sequencing were used in multiple alignment.

Sequences for *T. canis*, *T. cati*, *T. malaysiensis*, *T. vitulorum* (36 ITS-2 and 14 *atp*6) and for a closely related *Toxascaris leonina* (5 ITS-2 and one *atp*6) in the GenBank database; and sequences obtained in this study from the Vietnamese samples (8 ITS2 and 29 *atp*6) were used for analysis (**Table 1; Figure 1-2**). Phylogenetic analyses (multiple alignment by GENEDOC2.7; genetic distance calculation by Kimura 2-parameter model; neighbor-joining analysis by 1,000 bootstrap tests) were conducted by using the MEGA6.06 package (Tamura et al., 2013).

As shown in **Table 2**, all 15 *Toxocara* samples from cats (designated: Tspcat group) from Vietnam showed 99.7-100% identity in the mitochondrial *atp*6 sequence with that of *T. malaysiensis* of China (Li et al., 2006) (GenBank Accession No. AM412316), whereas their identities were 89.6-90.1% to two *T. cati* sequences, one from China (AM411622) and another from India (KJ777175); and, identities were 86.7- 87.9% to 6 *T. canis* reference sequences from China, India, Sri Lanka and Australia. Four ITS-2 sequences representing the Tspcat group from Vietnam showed 99.7% identity to that of *T. malaysiensis* of Malaysian origin (AJ002440) (Zhu et al., 1998; Gibbons et al., 2001), and 97.8% to *T. malaysiensis* from Guangdong in China (AM231609) (Li et al., 2006); by contrast, the four sequences from Vietnam had only 83.5-89.9% to reference sequences for *T. cati* from Iran, India, China, Japan and Malaysia; and 78.6-81.9% to those of *T. canis* sequences (Li et al., 2006; Wickramasinghe et al., 2009; Khademvatan et al., 2014). These molecular findings show that *T. malaysiensis* was the only ascaridoid nematode to be found in all 15 *Toxocara* samples from *Toxocara*-infected cats, and no *T. cati* was detected in this study.

Toxocara sequences from dogs (designated: Tspdog group of 14 samples) in Vietnam showed identities 86.8-87.5% in the *atp*6 sequence compared with the reference sequence for *T. malaysiensis*; 88.5 - 89.1% to *T. cati*; and 98.2 -99.7% to *T. canis* (**Table 2**). ITS-2 sequences representing the Tspdog group had 95.6 - 100% sequence identity to sequences for 8 *T. canis* specimens from Iran (egg samples) (Khademvatan et al., 2014) and two *T. canis* (adult) from China (Li et al., 2006), compared with 79.3% - 81.9% to *T. malaysiensis* and 76.7% - 79.7% to *T. cati*. Thus, *T. canis* was molecularly identified in *Toxocara*-infected dogs in Vietnam. Between Tspcat and Tspdog groups of *Toxocara* spp, Vietnam, pairwise identities varied from 86.5% -

87.5% for *atp*6 and 81.9% for ITS-2 (**Table 2**), clearly confirming the presence of *T. malaysiensis* in cats and *T. canis* in dogs in Vietnam (**Table 1**).

Phylogenetic analysis based using *atp*6 and ITS-2 sequence data revealed 5 distinct groups of *Toxocara*: *T. malaysiensis*, *T. canis* related to Vietnamese samples; and *T. cati, T. vitulorum* (reference strains) and *Toxascaris leonina* as an outgroup (**Figure 1 and 2**). Phylogenetic construction showed that all the Vietnamese *Toxocara* samples from cats grouped with *T. malaysiensis*, but none with *T. cati*, supporting the proposal that *T. malaysiensis* is the only ascaroidoid nematode in house cats in Vietnam. The *Toxocara* samples from dogs from Vietnam clustered with *T. canis* reference samples, indicating dogs were infected with *T. canis*, which is commonly found throughout the world.

Conclusions

We report for the first time, the existence of *T. malaysiensis* (Gibbons et al., 2001), but not *T. cati*, in house cats in Vietnam, following previous studies in Malaysia and China (Zhu et al., 1998; Li et al., 2006). The ascaridoid samples from dogs were shown to be *T. canis*. Because domestic dogs and cats are commonly kept as pets in Vietnamese households, there is a possible risk that humans, particularly children, could become infected with *T. malaysiensis*. *Toxocara* eggs can tolerate relativey high temperatures (XX-XX •C; REF) and can remaining for long on vegetables and in soil in the environment, posing a high for transmission and circulation of *Toxocara* spp. (Fan et al., 2015). The finding of *T. malaysiensis* infection in cats sends a warning to public health authorities and draws our attention to this ascaridoid as a possible zoonotic pathogen in other geographical region in Asia and other parts of the world. It also calls for nationwide investigations of toxocariasis of animals and humans in Vietnam.

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Acknowledgments
This work was supported by National Foundation of Science and Technology of Vietnam, grant number ===== .
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Tables

Table 1: List of *Toxocara* samples, their geographic origin, markers used in this study and their final taxonomic identification

No	Sample designations	Locality (province)	Host	Sample status	Marker used		Final taxonomic identification
					ITS2	atp6	
1	Tspcat-Hctt(egg)-VN	Ha Noi	cat	eggs*	√	√	*T. malaysiensis*
2	Tspcat-Tctt1B(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
3	Tspcat-Tctt2B(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
4	Tspcat-Tctt9B(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
5	Tspcat-Tctt11(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
6	Tspcat-Tctt12(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
7	Tspcat-Tctt13(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
8	Tspcat-Tctt14(adu)-VN	Ha Noi	cat	adult	√	√	*T. malaysiensis*
9	Tspcat-Tctt202(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
10	Tspcat-Tctt289(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
11	Tspcat-Tcto25(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
12	Tspcat-Tcto27(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
13	Tspcat-Tcto100(adu)-VN	Ha Noi	cat	adult	√	√	*T. malaysiensis*
14	Tspcat-Tcto115(adu)-VN	Ha Noi	cat	adult		√	*T. malaysiensis*
15	Tspcat-ND(adu)-VN	Nam Dinh	cat	adult	√	√	*T. malaysiensis*
16	Tspdog-Hdtt(egg)-VN	Ha Noi	dog	eggs*	√	√	*T. canis*
17	Tspdog-Tdtt11(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
18	Tspdog-Tdtt202(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
19	Tspdog-Tdtt208(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
20	Tspdog-Tdtt273(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
21	Tspdog-Tdto14(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
22	Tspdog-Tdto15(adu)-VN	Ha Noi	dog	adult	√	√	*T. canis*
23	Tspdog-Tdto102(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
24	Tspdog-Tdto106(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
25	Tspdog-Tdto114(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
26	Tspdog-Tdto202(adu)-VN	Ha Noi	dog	adult	√	√	*T. canis*
27	Tspdog-TcanLL(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
28	Tspdog-TcanVN(adu)-VN	Ha Noi	dog	adult		√	*T. canis*
29	Tspdog-ND(adu)-VN	Nam Dinh	dog	adult	√	√	*T. canis*

Note: *eggs and (egg): eggs collected from the hair of the pets (cat and dog) by combing; (adu): adult worms collected from dogs and cats; Tspcat or Tspdog: groups of *Toxocara* samples collected from cat or dog, respectively; VN: Vietnam. Abbreviation of the designated samples is in the between.

Table 2: Pairwise identity (%) of the *atp*6 and ITS2 nucleotide sequences between *Toxocara* samples ot Vietnam from cats (Tspcat group) and dogs (Tspdog group) and compared with *Toxocara malaysiensis*, *T. cati* and *T. canis* in this study

Species/group	Tspcat group (Vietnam) (n = 15)		Tspdog group (Vietnam) (n = 14)	
	atp6	ITS2	*atp*6	ITS2
T. malaysiensis	99.7% - 100%	98.1% - 99.7%	86.8 - 87.5%	79.3% - 81.9%
T. cati	89.6% - 90.1%	83.5% - 89.9%	88.5 - 89.1%	76.7% - 79.7%
T. canis	86.7% - 87.9%	78.6% - 81.9%	98.2% - 99.7%	95.6% - 100%
Tspcat group			86.5% - 87.5%	81.9%

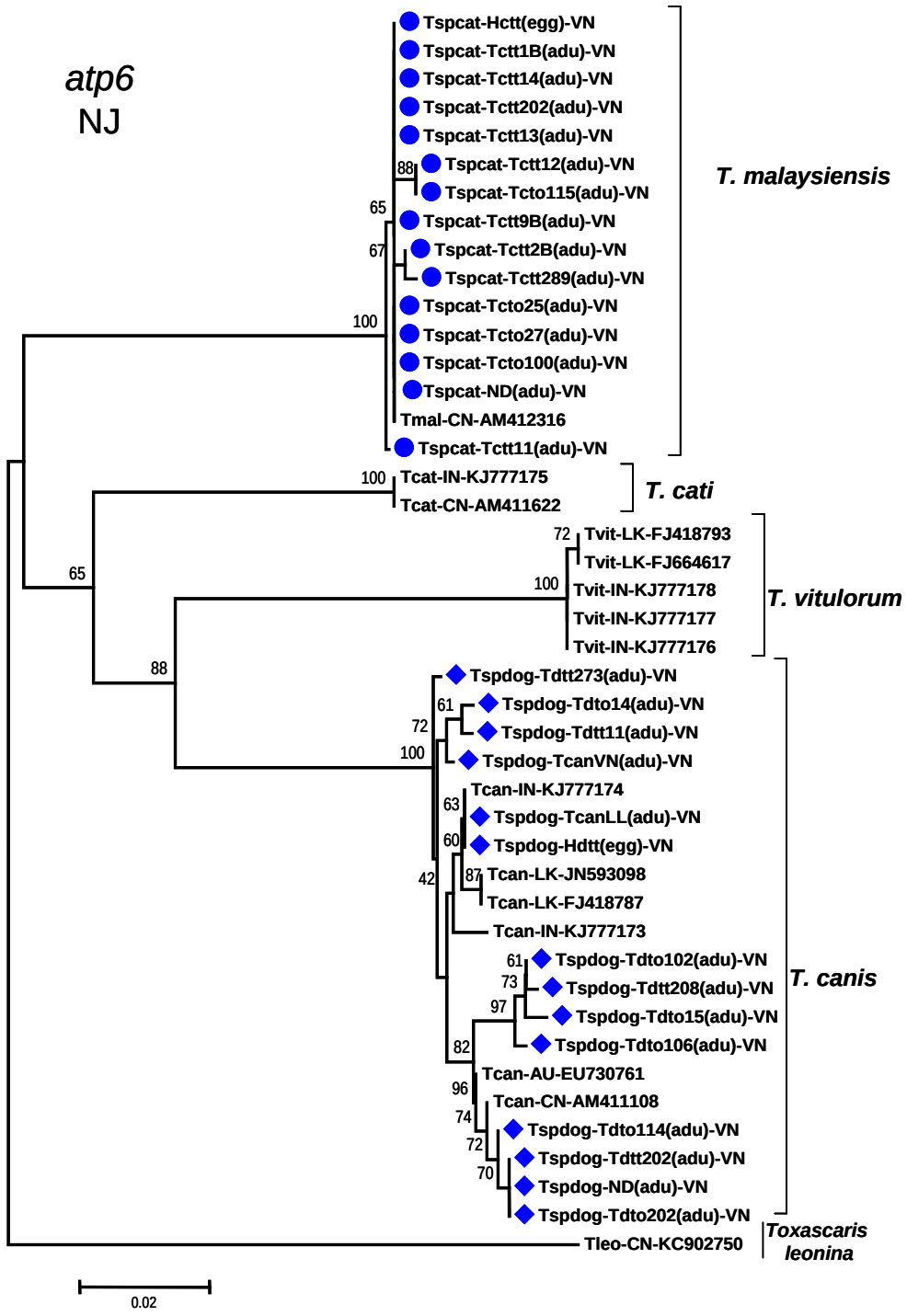

Figure 1. Phylogenetic tree of *Toxocara atp*6 nucleotide sequences (598 bp) constructed by MEGA6.06 with neighbor-joining method and 1000 bootstrap replication (Tamura et al., 2013). Forty four *atp*6 sequences were included showing the taxonomic relationship of 15 *Toxocara* samples from cats (circle) and 14 from dogs (square) of Vietnam origin. *Toxascaris leonina* is used as an outgroup. Abbreviation of the Vietnamese samples are indicated in Table 1. Reference taxonomic sequences used in tree construction were obtained from GenBank: *T. malaysiensis* (China (CN): AM412316), *T. cati* (China (CN): AM411622; India (IN): KJ777175), *T. canis* (Australia (AU): EU730761; China (CN): AM411108); India (IN): KJ777173, KJ777174; Sri Lanka (LK): FJ418787, JN593098), *T. vitulorum* (Sri Lanka (LK): FJ664617, FJ418793; India (IN): KJ777176, KJ777177, KJ777178), *Toxascaris leonina* (China (CN): KC902750). Scale bar indicates substitutions per site. Country abbreviation (2-letter) according to https://countrycode.org/ and accession number is in bracket.

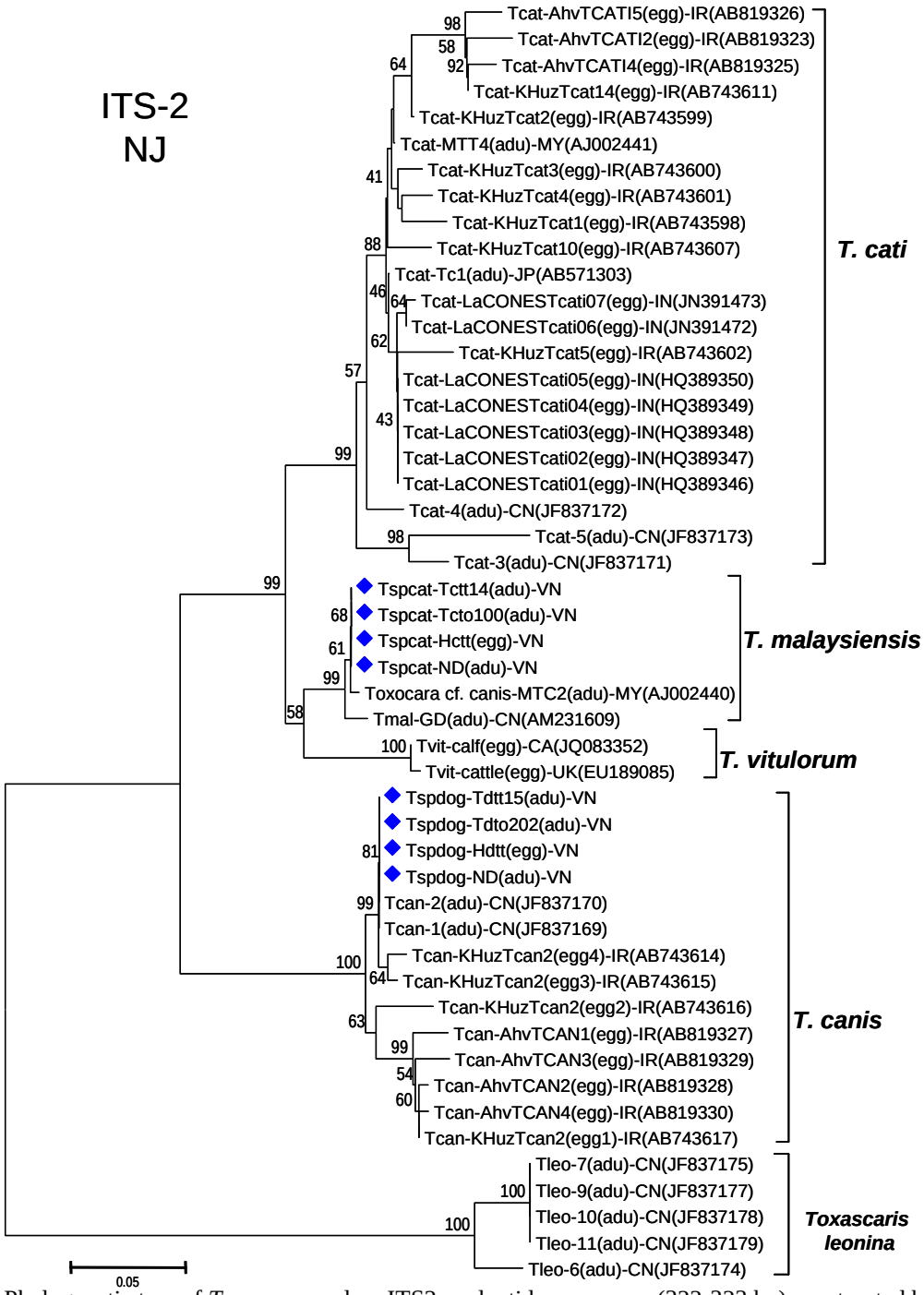

Figure 2. Phylogenetic tree of *Toxocara* nuclear ITS2 nucleotide sequences (322-323 bp) constructed by MEGA6.06 with neighbor-joining method and 1000 bootstrap replication (Tamura et al., 2013). Forty nine ITS2 sequences were included showing the taxonomic relationship of 4 represenative *Toxocara* samples of Vietnam from each infected group (cats and dogs) indicated by a square. *Toxascaris leonina* sequences are used as an outgroup. Abbreviation of the Vietnamese samples are indicated in Table 1. Reference taxonomic sequences are: *T. malaysiensis* (Malaysia (MY): AJ002440; China (CN): AM231609;), *T. cati* (Iran: AB819326, AB819323, AB819325, AB743611, AB743599, AB743600, AB743601, AB743598, AB743607, AB743602; India (IN): JN391473, JN391472, HQ389350, HQ389349, HQ389348, HQ389347, HQ389346; China (CN): JF837172, JF837173, JF837171; Japan (JP): AB571303; Malaysia (MY): AJ002441), *T. canis* (China (CN): JF837170, JF837169; Iran (IR): AB743614, AB743615, AB743616, AB819327, AB819329, AB819328, AB819330, AB743617), *T. vitulorum* (Canada (CA): JQ083352; United Kingdom (UK): EU189085), and *Toxascaris leonina* (China (CN): JF837175, JF837177, JF837178, JF837179, JF837174). Scale bar indicates substitutions per site. Country abbreviation (2-letter) is according to https://countrycode.org/ and accession number is in bracket.