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Research paper

Phylogenetic relationships among Toxocara spp. and Toxascaris sp. from different regions of the world

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ABSTRACT

Toxocara and *Toxascaris* are parasitic nematodes that infect canids and felids although species of the genus Toxocara also infect humans. This work aimed to establish the phylogenetic and phylogeographic relationship between specimens of T. canis, T. cati, T. malaysiensis and *Toxascaris* leonina and to evaluate the degree of host specificity. In total, 437 samples (adults and pools of eggs) were collected from canids and felids from eight countries. Parasites were identified by morphology, PCR linked Restriction Fragment Length Polymorphism (PCR-RFLP) and partial sequencing of the mitochondrial gene cox1. Phylogenetic trees were constructed and genetic distance among isolates was estimated. Based on the molecular characterization all worms were identified in agreement with their respective hosts with the exception of three samples; two from cats and one from dogs identified as T. canis and T. cati, respectively. There was no clear geographical clustering of the samples despite this study including parasites from three continents. This is the first study, to our knowledge, to use molecular methods to identify T. canis in cats and T. cati in dogs with host specificity being the most common finding. Our developed PCR-RFLP method was found to be a facile and reliable method for identifying Toxocara species.

1. Introduction

Toxocara canis, T. cati, T. malaysiensis and *Toxascaris* leonina are common nematodes of canids and felids, causing toxocariosis and toxascariosis for the latter. Clinical symptoms are dependent on parasite load, location of the parasite on the body of the host, the stage of helminth development, and age of the host, with animals under six months of age being most affected (Sprent, 1983). The most common symptoms are diarrhea, vomiting and nasal discharge (Parsons, 1987). However, high worm loads in canine and feline pups can result in bulging of the abdomen, nutritional deficit, weight loss, and may in some cases lead to death (Overgaauw and Van Knapen, 2013).

Toxocara spp. are the etiological agents of human toxocariosis, caused by accidental ingestion of embryonated eggs and is one of the most common zoonotic helminth infection in the world (Ma et al., 2018) however, the study of this disease, in both animals and humans, is neglected (Fialho and Corrêa, 2016).

In veterinary clinics, species identity of parasitic worms is often

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assumed based upon the host of origin and may be complemented by specific morphological characteristics, for example, differentiation between T. canis and T. cati can be determined by the morphology of the cephalic alae. However, for closely related species a trained eye may be needed due to subtle morphological differences between species. In this way, it was only recently that T. malaysiensis was identified as a distinguished and the predominant Toxocara species infecting cats in Malaysia (Gibbons et al., 2001). T. malaysiensis was later identified in China (Li et al., 2006) and more recently in Vietnam (Le et al., 2016) which raises questions to its geographical distribution.

Mitochondrial DNA has a number of advantages when studying the evolutionary relationships among nematodes and in particular when delimiting closely related species (Hu et al., 2003). This is due to its high mutation rate and low effective population size, which favors rapid lineage sorting between species. Therefore, comparative mitochondrial analysis is useful for identifying cryptic species, which are those that cannot be identified by traditional methods, including morphological analysis, and maybe extended to those which are widespread, or have multiple host species (Nadler and DE León, 2011).

We therefore aimed to molecularly characterize nematodes of the genera Toxocara spp. and *Toxascaris* sp. from canids and felids, from different regions in the world, to establish the phylogenetic and phylogeographic relationships among isolates. We also developed a PCR linked Restriction Fragment Length Polymorphism (PCR-RFLP) to assist the identification of Toxocara spp. in dogs and cats and thereby elucidate the host specificity of the different species.

2. Methodology

2.1. Collection of nematodes and sample processing

This study was approved by the Ethics Committee at the Federal University of Uberlândia (CEUA-UFU).

Worms were obtained after anthelminthic expulsion or at necropsy and eggs isolated from canids (dogs) and felids (cats and lions) feces from eight countries, distributed on three continents (Table 1). Adult worms were washed three times in distillated water and morphologically identified as per Gibons et al. (2001). Eggs were isolated from the feces using centrifugal-flotation and zinc sulphate 33% (Faust et al., 1938). Worms and eggs were then stored in ethanol at -20 °C for subsequent molecular analysis.

2.2. DNA extraction and polymerase chain reaction (PCR)

DNA was extracted using phenol-chloroform as described by Sambrook et al. (1989), with the following modifications: After addition of 500 µL lysis buffer the samples were subjected to three manual sonication cycles (10 s each) followed by three freeze-thaw cycles (liquid nitrogen / water bath at 37 °C) and overnight proteinase K digestion. DNA was precipitated with propyl alcohol for approximately 18 h and stored (−20 °C) until molecular analysis. Negative controls (only reagents) were used in each extraction round. Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design the following primers ToxCoIF (5′-GATTTTACCTGCTTTTGGTATTATTAG-3′) and ToxCoIR (5′-CCAAAGACAGCACCCAAACT-3′) amplifying 426 base pairs of the cox1 gene based on the sequences AJ920057, AM411108 and NC010527 from Genbank.

All PCR reactions were carried out in a total volume of 20 µL using 1 µL of template DNA, 4 mM of each dNTP, 10 pmol of each primer, 2.5 mM of $MgCl_2$ and 1U of Hot Start Taq polymerase in a thermocycler under following conditions: 95 °C for 15 min (hot start Taq activation), 95 °C for 15 min (denaturation), followed by 35 cycles of 95 °C for 30 s (denaturation), 60 °C for 40 s (annealing), 72 °C for 1 min (extension) and a final extension of 72 °C for 5 min. PCR products were stained with GelRed® and visualized after 0.8% agarose gel electrophoresis.

2.3. Sequencing and bioinformatic analysis

The PCR amplicons were purified and sequenced in both directions by Macrogen Inc., in Seoul, South Korea.

The sequence quality of the forward and reverse reads was assessed using Sequence Scanner version 1.0 (Applied Biosystems, Foster City, CA, USA), and the forward and reserve sequences were joined using Vector NTI (Thermo Fisher Scientific). Only double stranded sequences were used and aligned using BioEdit Sequence Alignment Editor (Hall, 1999), and compared to sequences available in GenBank; AM411108 (T. canis; China), AJ920055 (T. canis; Australia), AJ920054 (T. canis; Australia), KC293899 (T. canis; Iran), AM411622 (T. cati; China), AJ920057 (T. cati; China), KC200179 (T. cati; Iran), AM412316 (T. malaysiensis; Malaysia), NC023504 (T. leonina; Australia), KC293927 (T. leonina; Iran), NC024884 (Parascaris_univalens; Switzerland), NC001327 (Ascaris suum; USA), NC016200 (Baylisascaris procyonis; China). Anisakis simplex (JN102304) was chosen as an outgroup.

In order to visualize the phylogenetic relationships among samples, phylograms were constructed using MEGA v.7.0 with Neighbor-Joining, Maximum Likelihood and Minimum Evolution methods, with bootstraps values established in 1000 replicates. MEGA was used to identify the best-fit substitution model. MEGA was also used to estimate the p-distances among samples.

2.4. PCR linked restriction fragment length polymorphism (PCR-RFLP)

The sequences AJ920057, AM411108 and NC010527 from Genbank and NEBcutter (New England Biolabs) were used to identify MseI as a useful endonuclease that has restriction patterns in the amplified fragment giving rise to unique fragment sizes for each of the three species; T. canis (95, 121, 210 bp), T. cati (22, 44, 172, 188 bp) and T. malaysiensis (44, 51, 121, 210).

PCR products (3 µl) were digested with two units of MseI (New England Biolabs) for two hours at 65 °C in a total volume of 10 µl. The fragments were stained with GelRed™ (Biotium) and visualized after gel electrophoresis (2% agarose) using UV light. To estimate the size of the

Table 1
Countries, number of host species and worms as well as recovery methods.

Country	Host species	Number of hosts	Number of worms	Recovery method
Brazil	Dogs and Cat	37(36D + 1C)	282	Anthelmintic treatment and Necropsy
Denmark	Dogs and Cats	64 (4D + 60C)	104	Necropsy
Germany	Dogs and Cats	3 (2D + 1C)	9	Anthelmintic treatment
Malaysia	Cats	1	5	Anthelmintic treatment
China	Dogs and Cats	6 (4D + 2D)	8	Anthelmintic treatment
Japan	Dogs and Cats	4 (2 + 2C)	4	Anthelmintic treatment
Russia	Dogs and Cats	9 (6D + 3C)	18	Anthelmintic treatment
Portugal (eggs)	Dogs and Lions	4 (1D + 3 L)	4	Isolation from faeces
TOTAL:		128(55D + 73C/L)	434	

D: Dog; C:Cat; L:Lion.

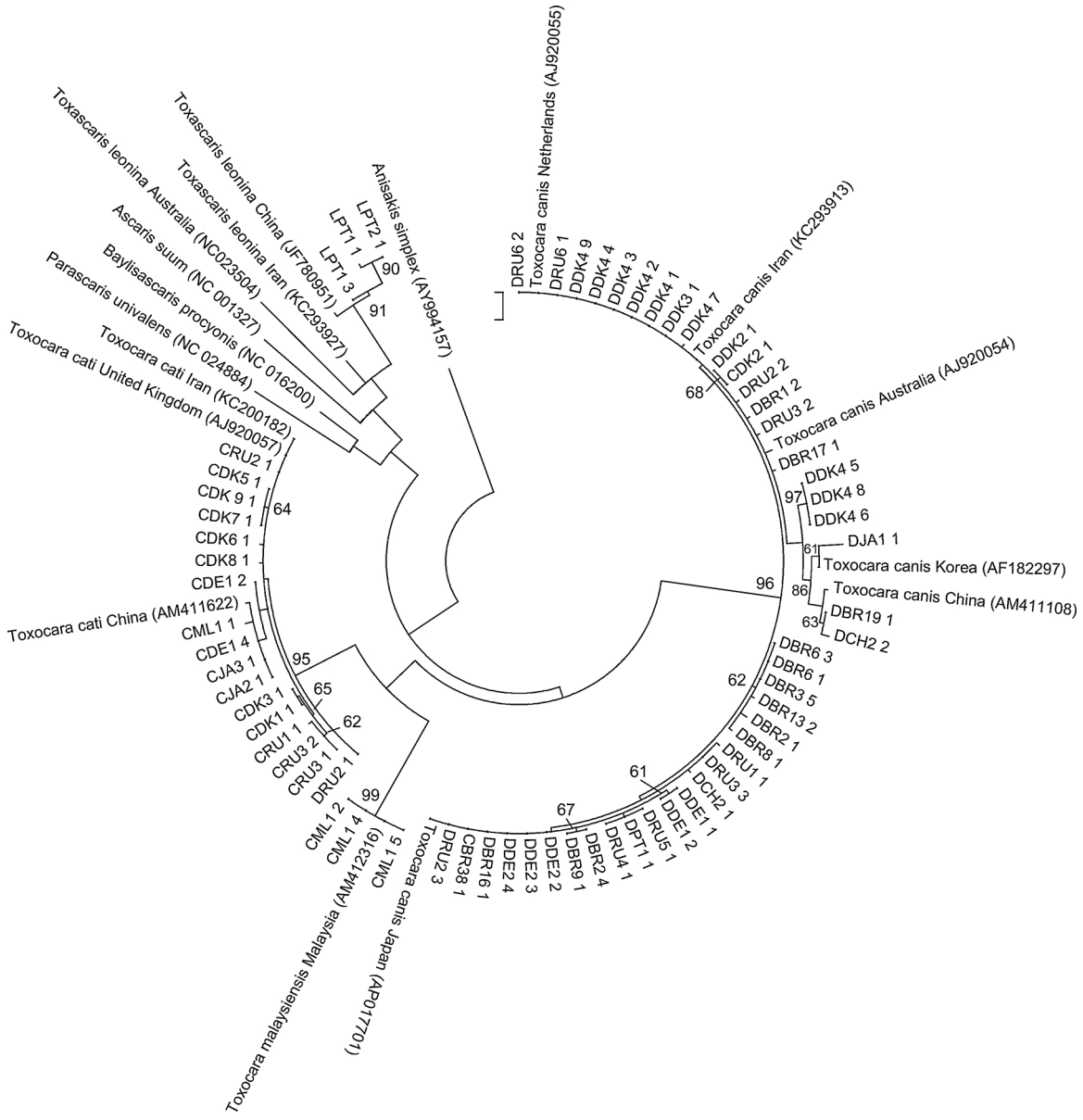

Fig. 1. Phylogenetic relationships among Toxocara canis, T. cati, T. malaysiensis and *Toxascaris* leonina from different geographical regions inferred using partial cox1 gene sequencing and the Maximum Likelihood clustering method, using the Tamura-Nei distance model with gamma distribution. The first letter indicates the host: D, dog; C, cat; L, Lion. The next two letters indicate the geographic origins (DK, Denmark; BR, Brazil; DE, Deutschland; JA, Japan; RU, Russia; PT, Portugal; ML, Malaysia; CH, China). The first numeral indicates host number and the second numeral the worm number in the host. Bootstrap values are given at the nodes if > 60. The scale bar indicates number of base substitutions per site: 0.02.

fragments a 100 bp molecular marker (Promega) was used. Negative (water) controls were included in each run.

3. Results

Based on morphology all worms from dogs were identified as T. canis and all samples from cats and lions as T. cati or T. malaysiensis or T. leonina.

All 434 samples submitted to PCR analysis were positive, presenting a band of approximately 430 bp. Among them, 68 were chosen for sequencing, comprising 21 from cats and 42 from dogs (GenBank accession numbers: MT359256-MT359318). From Brazil and Denmark, samples were chosen randomly (but maximum one from each host), whereas all samples were included from the other six countries.

All three clustering methods gave similar topology, with T. canis, T. cati and T malaysiensis and T. leonina forming main groups; thus Fig. 1 show the phylogram established using Maximum Likelihood and the Tamura-Nei distance model with gamma distribution. However, one worm from a Russian dog (DRU2_1) clustered among the T. cati samples. Two worms from cats, one from Denmark and one from Brazil (CDK2_1 and CBR38_1) clustered with the samples from dogs in the T. canis branch. In addition, three cat worms from Malaysia were identified as T. malaysiensis and three lion worms from Portugal as T. leonina (Fig. 1).

Overall, the genetic distances (p-distance) within T. canis varied from 0.0% to 4.2% and within countries values were for Brazil (0–3.3%), Denmark (0–2.1%), Russia (0–1.5%) and Germany (0 – 0.03%). Overall, the genetic distances within T. cati varied from 0% to 2.1%. Within country variation for T. cati were 0 – 0.6% for both Denmark and Russia. Variation between species ranged from 8.4% to 18.4% (Table 2, Supplementary Table 1).

All 434 samples were submitted to PCR-RFLP analysis. 319 (99.7%)

Table 2
Genetic distances (p-distance) between the different Toxocara and Toxascaris species based on partial cox1 sequencing. The distances within species are given on the diagonal.

Species	T. canis	T. cati	T. malaysiensis	T. leonina
T. canis	0–0.042[a]			
T. cati	0.101–0.125	0–0.021[b]		
T. malaysiensis	0.104–0.122	0.087–0.096	0	
T. leonina	0.087–0.107	0.104–0.122	0.104–0.113	0.003 – 0.015

[a] Lowest distance between helminths from Germany and Russia and largest distance between Japan and China.
[b] Lowest distance between helminths from Japan and Germany and largest variation between Russia and Germany.

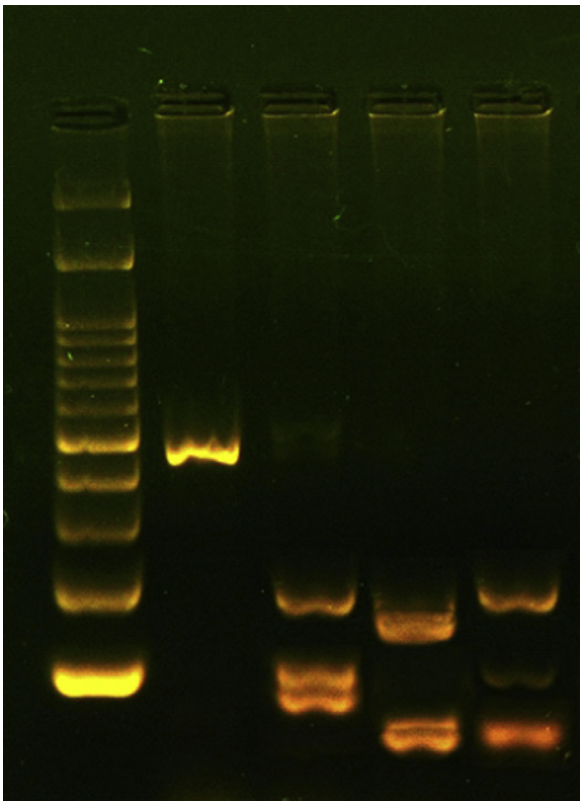

Fig. 2. PCR-RFLP band pattern of the cox1 region using the endonuclease MseI. Fragments are stained with GelRed® and visualized using UV light in a 2% agarose gel. The gel shows representatives of undigested product (lane 2) and digested products of T. canis (lane 3), T. cati (lane 4) and T. malaysiensis (lane 5). Molecular marker 100bp (lane 1).

worms, including the pool of eggs, from dogs showed a restriction pattern similar to *T. canis* whereas one (0.3%) from Russia (DRU2_1) was identified as T. cati. Of the 112 worms from cats, 107 (95.6%) were identified as *T. cati,* three (2.7%) from Malaysia as *T. malaysiensis* and two (1.7%), one from Denmark (CDK2_1) and one from Brazil (CBU38_1) as T. canis (Fig. 2). The PCR fragments from the three pools of eggs from lions remained undigested, which is expected for T. leonina. The PCR-RFLP results were therefore in accordance with the cox1 sequence analysis.

4. Discussion

Few studies discuss the morphological identification of Toxocara spp. and their specific relationship with the host as it is expected that helminths show host specificity (Costello, 2016; Chang et al., 2015; Mikaeili et al., 2015; Fogt-Wyrwas et al., 2013; Li et al., 2008, 2006; Jacobs et al., 1997). However, as nematodes tend to have very conserved morphology this may compromise identification of closely related species (Anderson et al., 1998). Herein, all worms were identified using morphology in agreement with the species of the host, i.e. T. cati and T. malaysiensis in cats and T. canis in dogs. However, molecular typing identified for the first time three worms as cross-infections and although authors such as Bhowmick (1964), Roth and Schneider (1971), Lee et al. (1993), Scholz et al. (2003) and Mundim et al. (2004) reported similar results these studies were based on morphological analysis alone. The reason for the discrepancies between our molecular and morphological identification in the current study remains elusive but may be explained by hybridization or back-crossing. As we only included a mitochondrial marker we are not able to identify hybrids between e.g. T. canis and T. cati as mitochondria is maternal inherited and if hybrids are backcrossed we may have a worm with a T. cati nuclear genome and T. canis mitochondria which may explain why two worms in cats identified as T. canis.

Across a number of parasitic nematodes, Blouin (2002) found the genetic difference between nematode species within the same genus to be in the range of 10% using the *cox*1 gene, whereas intra-species variation is usually below 2%. Likewise, intra-species variation of *Toxocara* spp. and *Toxascaris* sp. based on mtDNA genes have previously been reported to be around 2.3% for *T. cati* and 1.3% for *T. canis* and 1.0% for *Toxascaris* sp., whereas the genetic difference between these species have been found to be around 10% (Li et al., 2008; Mikaeili et al., 2015; Le et al., 2016) and is similar to our observations for within country and species variation. However, variation between countries were up to ~ 4% for T. canis with values indicative of cryptic species (Blouin, 2002). However, similar high levels of intra-species variation have been reported for Ostertagia ostertagia (Blouin et al., 1998) and Ascaris suum (Anderson et al., 1998) but not previously reported for Toxocara spp.

In the phylogeny, worms only clustered according to hosts but apparently not according to geography despite the assessment of worms from different continents. This suggests current or recent gene flow among populations, which is most likely facilitated by the recent global distribution of these domestic animals and increased transport due to re-settlement and travel (Costello, 2016). Hawash et al. (2016) also observed that whipworms (Trichuris suis) of pigs from Denmark and US clustered together suggesting recent transport of infected hosts between the continents. Conversely, they found that T. suis from Uganda and China were found in separate clusters suggesting that these populations have been separated for extensive periods.

T. malaysiensis was first identified in cats in Malaysia (Gibbons et al., 2001) and later in China and Vietnam (Li et al., 2006; Le et al., 2016) which raises questions about its distribution in other parts of Asia and globally. However, we did not identify this species among our samples except those derived from Malaysia, but further studies are required to explore the geographical boundaries and importance of T. malaysiensis.

Even though PCR-RFLP for identification of Toxocara species has previously been described (Jacobs et al., 1997), this study provides a facile method that only requires a single endonuclease (MseI) for differentiation between the three species of veterinary and medical importance, thereby reducing time and cost.

In conclusion, we found that T. cati and T. canis showed a high degree of host specificity but also provided the first molecular evidence that cross-infections may take place. We did not identify T. malaysiensis in samples outside of Asia but further studies are warranted to establish its geographical boundaries. Lastly, we described a PCR-RFLP for identification of Toxocara species.

CRediT authorship contribution statement

Natália M.N. Fava: Conceptualization, Formal analysis, Funding acquisition, Investigation, Writing - original draft, Writing - review &

editing. **Márcia Cristina Cury:** Conceptualization, Funding acquisition, Writing - review & editing. **Hudson A. Santos:** Writing - review & editing. **Nao Takeuchi-Storm:** Writing - review & editing. **Christina Strube:** Writing - review & editing. **Xing-Quan Zhu:** Writing - review & editing. **Kensuke Taira:** Writing - review & editing. **Irina Odoevskaya:** Writing - review & editing. **Olga Panovag:** Writing - review & editing. **Teresa Letra Mateus:** Writing - review & editing. **Peter Nejsum:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2020.109133.

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