Veterinary Parasitology 266 (2019) 80–83

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Molecular data reveal cryptic speciation and host specificity in *Toxascaris leonina* (Nematoda: Ascarididae)

Renata Fogt-Wyrwas[a,*], Mirosława Dabert[b], Wojciech Jarosz[a], Izabela Rząd[c], Bogumiła Pilarczyk[d], Hanna Mizgajska-Wiktor[a]

[a] Department of Biology and Environmental Protection, Poznań University of Physical Education, Królowej Jadwigi 27/39, 61-871, Poznań, Poland
[b] Molecular Biology Techniques Laboratory, Faculty of Biology, Adam Mickiewicz University in Poznań, Umultowska 89, Poznan, Poland
[c] Chair of Ecology and Environmental Protection, Faculty of Biology, University of Szczecin, Wąska 13, 71-415, Szczecin, Poland
[d] Chair of Biotechnology of Animal Reproduction and Environmental Hygiene, Faculty of Biotechnology and Animal Breeding, West Pomeranian University of Technology Szczecin, Doktora Judyma 6, 71-466, Szczecin, Poland

ARTICLE INFO

Keywords:
Toxascaris leonina
Cryptic-species
ITS1
cox1
nad1
Barcode gap

ABSTRACT

Toxascaris leonina (Ascarididae) is a cosmopolitan and polyxenical parasite whose host are canids and felids. To date, molecular phylogenetic studies included toxascarid representatives collected only from dogs or felids, therefore the intra-species differences between *T. leonina* collected from different host species has not been noticed. In this paper, we test the hypothesis of cryptic speciation in the *T. leonina* complex based on extended sequence data (*ITS1*, *nad1*, *cox1*) and individuals collected from dogs, felids and foxes. Phylogenetic analysis clustered *T. leonina* representatives into three well-supported clades depending on their host species, i.e. dogs and wolves, wild felids and foxes. Both genetic distances and the barcoding-gap analysis strongly support the species status of populations inhabiting different hosts. The results suggest additional genetic separation in felids. However, to determine the actual size of the *Toxascaris* complex, it would be necessary to analyse individuals collected from other canid and felid *Toxascaris leonina* host species.

1. Introduction

Toxascaris leonina (Ascarididae), morphologically very similar to *Toxocara* helminths, is a cosmopolitan and polyxenical parasite whose host are canids and felids, including domestic dogs, cats and red foxes (Okulewicz et al., 2012). So far, it has been the only species described in the genus *Toxascaris* (Sprent, 1959). Although *T. leonina* is characterized by a low level of pathogenicity and zoonotic potential (Robertson and Thompson, 2002), its final host group is diverse. Additionally, the occurrence of this nematode in all latitudes, as well as highly diverse prevalence (Vervaeke et al., 2005), encourages tracking its molecular variability in relation to phylogeny, epidemiology and biodiversity. Although *T. leonina* has been studied by molecular methods for several years, most of the research focused on the PCR-based methods of molecular identification of potentially zoonotic ascaridoid parasites (Jacobs et al., 1997; Zhu et al., 1998; Li et al., 2007, 2008). Sparse phylogenetic studies included toxascarid representatives collected from cats (Pawar et al., 2012) or dogs (Mikaeili et al., 2015); however, none of the authors seem to have noticed intra-species differences between *T. leonina* collected from different host species.

In our previous study (Fogt–Wyrwas et al., 2013) we have noticed considerable differences of the ITS sequences between the *T. leonina* populations from Polish foxes (HM800923) and the Australian dogs (Y09490). Intraspecific differences of such magnitude have neither been observed in this species in other countries, regardless of the host, nor in any species within the Ascarididae family. These data suggest that *T. leonina* originating from foxes inhabiting Poland may be a separate cryptic species. Therefore, the purpose of the present study was to test the hypothesis of cryptic speciation in the *T. leonina* complex. The research was based on extended data – additional mitochondrial DNA markers and individuals acquired from the north-western region of Poland. The secondary goal was to investigate the host specificity within this complex.

2. Materials and methods

In total, 33 *T. leonina* nematodes collected during the necropsy of red foxes (*Vulpes vulpes*) caught in the north-western (23) and the south-western Poland (10) were used for the study. The fox carcasses were obtained as a result of reduction hunting carried out in accordance with

* Corresponding author.
E-mail address: fogt@awf.poznan.pl (R. Fogt-Wyrwas).

https://doi.org/10.1016/j.vetpar.2019.01.002
Received 10 October 2018; Received in revised form 4 January 2019; Accepted 5 January 2019

Table 1
List of sequences used in this study.

Toxascaris leonina sample ID	Host origin	Country	GenBank accession numbers		
			COI	*NAD1*	*ITS*
1	*Vulpes vulpes*	Poland	KX963448	KX963447	HM800922
2	*Canis lupus familiaris*	Australia	–	AJ937267	–
3	*C. lupus familiaris*	Australia	KC902750	KC902750	–
4	*C. lupus familiaris*	Iran	KC293927	KC293948	KF577860
5	*C. lupus familiaris*	Iran	KC293943	KC293964	–
6	*C. lupus familiaris*	Iran	–	KC293968	–
7	*C. lupus familiaris*	Iran	KC293940	KC293961	–
8	*C. lupus familiaris*	Iran	KC293939	KC293960	–
9	*C. lupus familiaris*	Iran	KC293946	–	–
10	*C. lupus familiaris*	Iran	KC293931	KC293952	–
11	*C. lupus familiaris*	Iran	KC293928	KC293949	–
12	*C. lupus familiaris*	Iran	KC293929	KC293950	–
13	*C. lupus familiaris*	Iran	KC293945	KC293969	–
14	*C. lupus familiaris*	UK	AJ920064	–	–
15	*C. lupus familiaris*	Iran	KC293944	KC293967	–
16	*C. lupus familiaris*	Iran	–	KC293965	–
17	*C. lupus familiaris*	Australia	AJ920063	–	–
18	*C. lupus familiaris*	Iran	KC293937	KC293958	–
19	*C. lupus familiaris*	Iran	KC293938	KC293959	–
20	*C. lupus familiaris*	Iran	KC293930	KC293951	KF577861
21	*C. lupus familiaris*	Iran	KC293941	KC293962	–
22	*C. lupus familiaris*	Iran	KC293942	KC293963	–
23	*C. lupus familiaris*	Iran	KC293932	KC293953	KF577862
24	*C. lupus familiaris*	Iran	KC293936	KC293957	–
25	*C. lupus*	China	JF780946	JF833960	JF837174
26	*C. lupus familiaris*	Iran	KC293935	KC293956	–
27	*C. lupus familiaris*	Iran	KC293934	KC293955	–
28	*C. lupus familiaris*	Iran	KC293933	KC293954	–
29	*C. lupus familiaris*	Iran	–	KC293966	–
30	*C. lupus familiaris*	Iran	KC293926	KC293947	–
31	*Panthera leo spelaea*[Ł]	China	JF780948	JF833962	JF837176
32	*P. tigris corbetti*[*]	China	JF780949	JF833963	JF837177
33	*P. tigris altaica*[*]	China	JF780947	JF833961	JF837175
34	*P. tigris amoyensis*[*]	China	JF780950	JF833964	JF837178
35	*Lynx lynx*	China	JF780951	JF833965	JF837179
Ascaris suum	*Sus* sp.	China	HQ704901	HQ704901	–
Toxocara malaysiensis	*Felis catus*		AM412316	AM412316	AM231609

Ł - extinct species.
* *Panthera tigris tigris* (decision at the Amur tiger Global Species Management Plan GSMP, Kitchener et al. (2017)).

the applicable law. Total genomic DNA was extracted from individual specimens using a Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) according to manufacturer's protocol. A fragment of the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene for phylogenetic analysis was amplified with primers JB3 (TTTTTTGGGCATCCTG AGGTTTAT) and JB4.5 (TAAAGAAAGAACATAATGAAAATG). A fragment of the dehydrogenase subunit I (*nad1*) was amplified with primers ND1F (TTCTTATGAGATTGCTTTT) and ND1R (TATCATAACGAAAAC GAGG) primers (Li et al., 2008). A fragment of the *cox1* gene used as the DNA-barcode region (Hebert et al., 2003) was amplified using bcdF01 (CATTTTCHACTAAYCATAARGATATTGG) and bcdR04 (TATAAACYT-CDGGATGNCCAAAAAA) primers (Dabert et al., 2010). PCRs were carried out in 25 µl reaction volumes containing 12.5 µl GPB 2 x Taq PCR Mix (GenoPlast Biochemicals, Poland), 0.2 µM of each primer, and 3 µl of DNA template. Thermocycling profile of one cycle of 5 min at 94 °C followed by 35 steps of 30 s at 94 °C, 30 s at 50 °C, 30 or 60 s at 72 °C was used, with a final step of 5 min at 72 °C. The ITS 1 fragments were amplified using the primers and conditions of the PCR reactions described in Fogt-Wyrwas et al. (2013). PCR products were purified with the PCR Purification Kit (Qiagen, Germany) and sequenced with the BigDye Terminator v3.1 kit on an ABI Prism 3130XL Analyzer (Applied Biosystems, USA). Sequence chromatograms were checked for accuracy using FinchTV 1.3.1 (Geospiza Inc.) and contigs were assembled in Geneious 11.1.2 (Biomatters Ltd.).

In total, 73 sequences representing *T. leonina* from *Canis lupus familiaris* (29), *Lynx lynx* (1), *Panthera tigris amoyensis* (1), *P. t. altaica* (1), *P. t. corbetti* (1), *P. leo spelaea* (1) and *Vulpes vulpes* (1) were used in phylogenetic analysis. *Ascaris suum* and *Toxocara malaysiensis* were used as outgroups to root the trees (Table 1). Sequences were aligned using Muscle 3.8.425 algorithm as implemented in Geneious 11.1.2. The best fitting model of DNA evolution was chosen by PartitionFinder2 (Lanfear et al., 2017). TRN + G and F81 were found, respectively, for 1 st and 2nd codon position of COI and ND1 genes, while HKY was the best fit model for the3rd codon position of both protein coding genes and ITS sequences. Phylogenetic trees were reconstructed using Bayesian inference (BI) and maximum likelihood (ML) methods. BI was conducted using MrBayes 3.2 (Ronquist et al., 2012). Each run of four independent chains was performed in 10^7 generations, and the trees were sampled every 1000th generation. The final consensus tree was generated after discarding the burn-in fraction of 0.25% initial trees where the average standard deviation of split frequencies dropped below 0.005. Support for nodes in BI analysis was estimated from posterior probabilities (PP) of each bipartition calculated for the remaining trees representing the percentage of times each node was recovered by 50% majority rule consensus. ML trees were searched using Garli v.2.0 (Zwickl, 2006) with 10 search replications. Support for tree branches was calculated by the nonparametric bootstrap method with 500 replicates. Trees were edited in MEGA7 and FigTree 1.4.2 (Rambaut, 2014) and further edited with vector graphic software. Species delimitation analyses were carried out with the neighbour-joining (NJ) method as implemented in MEGA7 (Kumar et al., 2016). Pairwise distance calculations between nucleotide sequences were

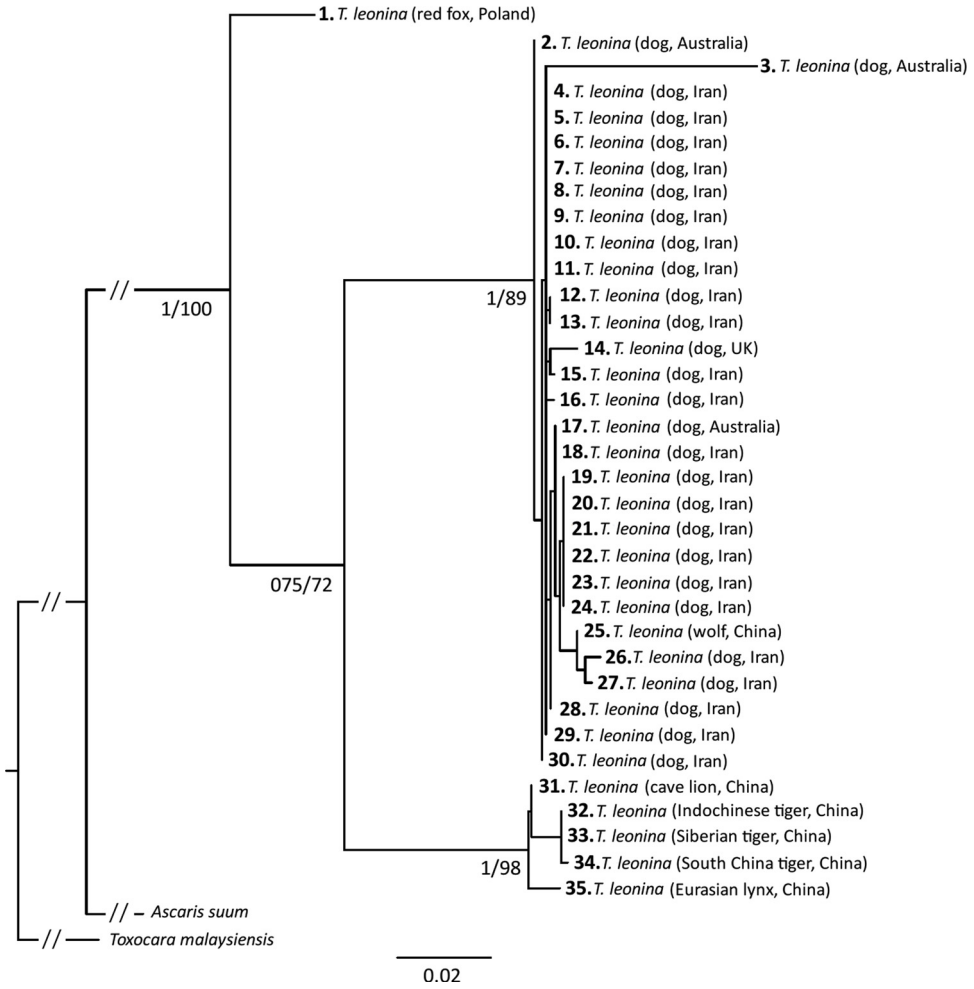

Fig. 1. Phylogenetic tree based on the *ITS*, *nd1* and *cox1* sequences of *Toxascaris leonina* collected from different hosts. Values near branches show Bayesian posterior probability (PP) and bootstrap values (BS); only supports for main clades are shown.

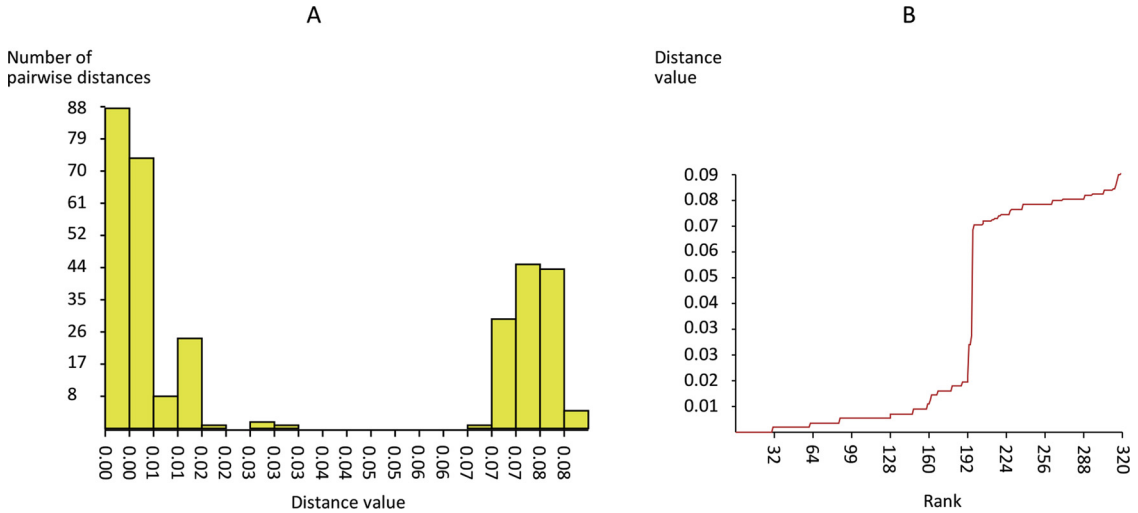

Fig. 2. Results of Automatic Barcode Gap Discovery (ABGD) analysis for DNA-barcode sequences of *Toxascaris leonina*. (A) Distribution of pairwise differences, (B) Ranked pairwise differences. Results from http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html.

computed using Kimura's 2-parameter (K2P) distance model for all codon positions using MEGA7. The cox1 and nd1 sequences from *Toxascaris* specimens were analysed using the Automatic Barcode Gap Discovery (ABGD) method to delimit genetic clusters by detecting a significant gap in the pairwise distance distribution (Puillandre et al., 2012). The online ABGD version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) was used with default settings and K2P distance model.

Nucleotide sequence data reported in this paper are available in the GenBank under the accession numbers: HM800922, KX963448,

KX963447, MH937707, MH937708, MH937709.

3. Results and discussion

All analysed sequences (*ITS*, *cox1* and *nad1*) of *T. leonina* collected from foxes in Poland presented the same haplotype (HM800922, KX963448, KX963447, respectively). Phylogenetic analysis of *ITS1*, *cox1* and *nad1* sequences from *T. leonina* collected from different hosts revealed that *T. leonina* representatives clustered into three well-supported clades depending on their host species, i.e. dogs and wolfs, wild felids and foxes (Fig. 1). Clade grouping *T. leonina* from foxes was recovered as sister to the two remaing clades. Moreover, the grouping of *T. leonina* sequences from wild felids suggests a limited gene flow among toxascarids parasitizing lions, tigers and lynxes.

To test the species-status of the *T. leonina* populations parasitizing different hosts we applied the DNA-barcoding analysis (Hebert et al., 2003). In this case, a standard DNA-barcode sequence comprising about 600 bp of the 5′ terminus of the *cox1* gene could not be directly compared with published sequences due to the lack of such data. Instead, we applied concatenated sequences of *cox1* and *nd1* gene fragments to detect the possible barcode gap among the analysed host-specific *T. leonina* populations (Fig. 2). The ABGD analysis split the sequence alignment data set into three candidate species corresponding to the hosts from which the analysed specimens were sampled. The first barcode gap occurred at a distance larger than 1.7% K2P, and was characteristic for intraspecific variation in *T. leonina* parasitizing dogs and wolfs. The second barcoding gap occurred at the distance about 3% K2P and was estimated among *T. leonina* sequences from lynx and tigers. Additionally sequence data from toxascarids parasitizing felines are needed to test whether genetic isolation is present across the populations inhabiting different feline species. Genetic distances among the main clades recovered in our phylogenetic analysis (about 7% K2P) exceed the maximum intraspecific value found in the ABGD analysis (3% K2P) which strongly supports the hypothesis of *T. leonina* cryptic speciation. Additionally, we compared the standard DNA barcode region (MH937707, MH937708, MH937709) of *T. leonina* from foxes with the only available complete sequence of the *cox1* gene originating from mitochondrial genome of *T. leonina* collected from a dog in Australia (KC902750). The nucleotide sequence of the *cox1* gene fragment used for phylogenetic analysis differs significantly from the other toxascarid *cox1* sequences, which results in long terminal branch for this specimen in our phylogenetic reconstruction (No. 3 in Fig. 1). However, the sequence fragment corresponding to the DNA-barcode region differs by 6.58% (SD 1.01) K2P and all nucleotide substitutions are synonymous.

Our study provides evidence that *T. leonina* is a species complex. Our results do not support any relationship between the genetic structure of *T. leonina* populations and the geographical region from which they originated but we have identified three *Toxascaris* species from different hosts: dogs, felids and foxes. Nevertheless, to determine the actual size of the *Toxascaris* complex, it would be necessary to analyze individuals collected from other host species belonging to canids and felids. Limited knowledge of *Toxascaris* species complex may arise from high morphological similarity of the nematodes within the Ascarididae which may lead to the misidentification of the species.

Declaration of interests

None.

Acknowledgments

This study was funded by the internal grant of Poznań University of Physical Education.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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