# Molecular characterization and phylogenetic analysis of ascarid nematodes from twenty-one species of captive wild mammals based on mitochondrial and nuclear sequences

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SUMMARY

Although ascarid nematodes are important parasites of wild animals of public health concern, few species of ascarids from wild animals have been studied at the molecular level so far. Here, the classification and phylogenetic relationships of roundworms from 21 species of captive wild animals have been studied by sequencing and analysis of parts of the ribosomal 18S and 28S genes and the mitochondrial (mt) 12S gene. Phylogenetic relationships were inferred by 3 methods (NJ/MP/ML) based on the data of single gene sequences and concatenated sequences. Homology analysis indicated that the 18S sequences were conserved among roundworms from all 21 species and that 28S showed interspecies variability. Divergence levels displayed in 12S suggested that 12S appears to be either intra- or interspecifically variable. Evolutionary trees indicated that the ascarids split into 2 families, 4 genera and 7 species, with high bootstrap support for each clade. Combined trees suggested that *Baylisascaris ailuri* is more closely related to *B. transfuga* than to *B. schroederi*. This study provides useful molecular markers for the classification, phylogenetic analysis and epidemiological investigation of roundworms from wild animals.

Key words: giant panda, wild mammals, Ascarid nematodes, mitochondrial gene, nuclear gene, classification, molecular phylogeny.

## INTRODUCTION

Ascarids are ubiquitous helminthic parasites and have been found in many rare wild mammals, such as the giant panda (*Ailuropoda melanoleuca*), red panda (*Ailurus fulgens*), ursids, primates, canids and felids. These parasites colonize in the small intestine and can cause significant health problems in host animals, including humans (Yang and Wang, 2000; Sorvillo *et al*. 2002; Nejsum *et al*. 2005; Lim *et al*. 2008; Zhang *et al*. 2008). Of these, the species *Ascaris*, *Toxocara* and *Baylisascaris* (family Ascarididae) and *Toxascaris* (family Toxocaridae) are responsible for the majority of health problems in captive wild animals. Also human infection with these ascarids can cause extensive damage to hepatic and pulmonary parenchyma, and ocular larva migrans (OLM), visceral larva migrans (VLM) and spinal or neurological forms that may be especially harmful to young individuals (Despommier, 2003). Ascarids are parasites of strong host specificity and the accurate identification and differentiation of these ascarid species is of great importance in veterinary and medical science (Roberts and Janovy, 2005).

To date, classification of species and the phylogenetic relationship of ascarid nematodes have been proposed based on morphological characters (Gibson, 1983; Sprent, 1983). However, structural features of ascarids, particularly the external parts, show considerable variation among genera and species. Additionally, their life cycles also show a diversity of patterns. Although many general features of ascarid life cycles have been established, detailed studies have been limited to relatively few species and, in some cases, recent investigations have yielded new findings. Analyses based on molecular characters rather than on morphology are employed in the study of ascarid phylogeny to verify previous findings. The application of molecular approaches may provide

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important evidence to clarify the taxonomic status and the relationships among different species of ascarids. Mitochondrial (mt) and nuclear genes have been employed as useful gene markers to explain relationships among the ascarids without considering morphology characters. Explicit evolutionary hypotheses were based on rDNA data for some ascarids, providing useful markers for the taxonomy of ascarids (Nadler, 1992; Nadler and Hudspeth, 1998). However, rDNA cannot provide entirely satisfactory solutions for the species and genus classification of *Toxocara* and *Baylisascaris*. This is mainly because rDNA contains fewer informative sites and is less useful at a lower level (Blaxter *et al*. 1998). 28S contains a variable region, and is regarded as evolutionarily conserved. Compared with rDNA, mt genes are more variable both between and within species, and are therefore more sensitive for the relationship analysis among genera. It has been confirmed that mt genes are useful molecular markers in the classification, population genetics and phylogenetic evolution (Hu *et al*. 2003*a*). More recently, 2 mitochondrial genes (ATPase 6 and a partial sequence of 12S rRNA) and 2 nuclear ribosomal genes (ITS-2 and part of the 28S region) have been compared between *Toxocara canis* and *Toxocara vitulorum*. The results revealed that 12S showed more variation than the 28S region and was more suitable for species identification (Wickramasinghe *et al*. 2009). Subsequent molecular evolutionary investigations have proven 12S to be an effective genetic marker for the interspecific molecular analysis of other parasites (Yatawara *et al*. 2007; Li *et al*. 2010). Studies based on single rDNA or mt gene loci allow only limited inference of molecular analyses (Nadler, 1995). To address the potential limitations of single locus molecular analyses, studies have been developed to use a combined analysis of nuclear and mt genes (Razo-Mendivil *et al*. 2010; Wyngaard *et al*. 2010). Compared with single-locus trees, combined data trees were more accurate for certain branches both in Steinernema and Ascaridoidea (Nadler and Hudspeth, 2000; Nadler *et al*. 2006).

However, for ascarids parasitizing wild mammals, current surveys mainly stagnate at morphological and biological study levels. There is little molecular information available for these roundworms. In this study, distinction between species and phylogenetic relationships among roundworms from the giant panda and 20 other species of captive wild animals was estimated using sequences from 2 nuclear genes (18S rDNA and 28S rDNA) and 1 mitochondrial gene (12S rRNA). The findings of this research will provide data for the molecular characterization, discrimination, and phylogenetic analysis of roundworms from wild animals. These findings could be used as a tool to further investigate the epidemiology and control of these parasites.

## MATERIALS AND METHODS

### *Specimen collection and identification*

After treatment with pyrantel pamoate, adult roundworms were obtained from 21 captive wild animals housed in the Chengdu Zoological Garden, Sichuan Province, China (Table 1). After washing in physiological saline solution, the morphological identification of these worms was performed based on the taxonomic keys of Sprent (1968), Tang and Tang (1987) and Wu *et al*. (1987). Subsequently, all adult specimens of 21 ascarid species were collected and stored at −20 °C until prepared for nucleic acid extraction.

### *DNA extraction, amplification, and sequencing analysis*

Total genomic DNA was extracted using a modified method from the standard phenol/chloroform extraction process (Gasser *et al*. 1993; Jacobs *et al*. 1997). PCRs were performed to amplify the partial gene sequences of 28S, 18S and 12S, using the following primers: 28S rDNA, forward primer 28SF (5′-CCCGATTGATTCTGTCGGC-3′) and reverse primer 28SR (5′-TGATCCTTCTGCAGG TTCACCTAC-3′); 18S rDNA gene, forward primer 18SF (5′-AGCGGAGGAAAAGAAACTAA-3′) and reverse primer 18SR (5′-TGATCCTTCTGCAGG TTCACCTAC-3′); 12S rRNA gene, forward primer 12SF (5′-AGCGGAGGAAAAGAAACTAA3′) and reverse primer 12SR (5′-TGATCCTTCTGCAGG TTCACCTAC-3). PCRs were conducted in a 25 $\mu$l reaction mixture containing 0·5 $\mu$M of each primer, 200 $\mu$M deoxynucleoside triphosphates, 1·5 mM $MgCl_2$, 10 buffer and 0·5 U proof-reading polymerase (Finnzymes DNAzyme EXT, MJ Research, Alameda, CA, USA). PCR cycling parameters for the rDNA amplification included denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50–58 °C (optimized for each rDNA region) for 1 min, and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 7 min. PCR cycling conditions for 12S included denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 1 min, followed by a post-amplification incubation at 72 °C for 5 min. All PCRs were performed in a Mastercycler Gradient 5331 thermocycler (Eppendorf, Germany). PCR products were then purified using a PCR purification kit (Watson Biomedical, W5212) and were subjected to automated sequencing. To ensure maximum accuracy, each PCR amplicon was sequenced twice independently and, in case of discrepancies, a third PCR product was sequenced. The consensus DNA sequences were edited with and aligned in DNAStar software (DNAStar, USA). All

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Table 1. Species information and GenBank Accession numbers for all studied species of tested animals and of their ascarid parasites

(Asterisks denote new sequences obtained in this study.)

| Parasite samples | Host | 12S | 18S | 28S |
|---|---|---|---|---|
| Toxocaridae | | | | |
| *Toxocara* | | | | |
| *Toxocara malaysiensis* | F Cat | AM412316 | | |
| *T. cati* | F Cat | AM411622 | EF180059 | |
| *T. vitulorum* | Buffalo | FJ418789 | EF180078 | FJ418790 |
| *T. canis* | F Dog | AM411108 | AF036608 | FJ418783 |
| *T. cati* | *Felis temmincki* | JN256961* | JN256975* | JN256995* |
| *T. cati* | *Prionailurus bengalensis* | JN256956* | JN256973* | JN256993* |
| *T. cati* | *Felis chaus* | JN256957* | JN256974* | JN256994* |
| *T. canis* | *Alopex lagopus* (Blue fox) | JN256964* | JN256976* | JN256996* |
| *T. canis* | *Alopex lagopus* (Silver fox) | JN256966* | JN256977* | JN256997* |
| Ascarididae | | | | |
| *Ascaris* | | | | |
| *Ascaris suum* | Pig | FJ418791 | AF036587 | U94752 |
| *A. lumbricoides* | Human | | U94366 | U94751 |
| *Ascaris* sp. | *Pan troglodytes* | JN256953* | JN256986* | JN257006* |
| *Ascaris* sp. | *Hylobates hoolock* | JN256952* | JN256985* | JN257005* |
| *Ascaris* sp. | *Macaca mulatta* | JN256954* | JN256987* | JN257007* |
| *Parascaris* | | | | |
| *Parascaris equorum* | Horse | | U94378 | U94764 |
| *Toxascaris* | | | | |
| *Toxascaris leonina* | Lion | | U94383 | U94769 |
| *T. leonina* | *Panthera tigris tigris* | JN256955* | JN256978* | JN256998* |
| *T. leonina* | *Panthera tigris amoyensis* | JN256960* | JN256981* | JN257001* |
| *T. leonina* | *Panthera tigris altaica* | JN256958* | JN256979* | JN256999* |
| *T. leonina* | *Panthera tigris* | JN256962* | JN256983* | JN257003* |
| *T. leonina* | *Puma concolor* | JN256959* | JN256980* | JN257000* |
| *T. leonina* | *Felis lynx* | JN256963* | JN256984* | JN257004* |
| *T. leonina* | *Canis lupus* | JN256965* | JN256982* | JN257002* |
| *Baylisascaris* | | | | |
| *Baylisascaris procyonis* | Raccoon | | U94368 | U94753 |
| *B. transfuga* | Bear | | U94369 | U94754 |
| *B. scheordrial* | *Ailuropoda melanoleuca* | JN256968* | JN256992* | JN257013* |
| *B. ailuri* | *Ailurus fulgens* | JN256967* | JN256991* | JN257012* |
| *B. transfuga* | *Ursus thibetanus* | JN256970* | | JN257011* |
| *B. transfuga* | *Ursus arctos pruinosus* | JN256971* | JN256989* | JN257009* |
| *B. transfuga* | *Ursus arctos* | JN256972* | JN256990* | JN257010* |
| *B. transfuga* | *Ursus maritimus* | JN256969* | JN256988* | JN257008* |
| Outgroup | | | | |
| *Porrocaecum depressum* | | | U94379 | U94765 |
| *Contracaecum multipapplillatum* | | | U94370 | U94756 |
| *Chabertia ovina* | | NC013831 | | |

sequences were deposited in GenBank and the Accession numbers of the sequences are shown in Table 1.

### *Data analysis*

The divergence among different genes and relationships among different genera was performed using DNAstar software. Multiple sequence alignments were finished with ProAlign Version 0.5. For each alignment, a ProAlign guide tree was constructed using corrected (for multiple hits) pairwise distances. Program (Java) memory and bandwidth were increased as required to complete the alignment. The average minimum posterior probability of sites was used as the criterion for detecting and removing unreliably aligned sequence, since this value is strongly correlated with correctness as determined by simulation studies (Loytynoja and Milinkovitch, 2003).

### *Phylogenetic analysis*

In this study 18S and 28S were considered as representing a single locus, and were therefore concatenated to one rDNA dataset for the construction of phylogenetic trees. Based on this dataset, 12S was also concatenated with 18S and 28S. Before the

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Table 2. Nucleotide composition of the 12S rRNA, 18S rDNA and 28S rDNA genes

| Gene | Length | A | T | C | G | Variation sites | Info-sites |
|---|---|---|---|---|---|---|---|
| 12S rDNA | 499 bp | 20·3% | 38·4% | 9·9% | 31·4% | 271 | 161 |
| 18S rDNA | 1708 bp | 27·4% | 25·4% | 22·1% | 27·4% | 72 | 24 |
| 28S rDNA | 751 bp | 30·3% | 25·5% | 21·4% | 30·3% | 252 | 179 |

3 genes were concatenated, the partition homogeneity, also referred to as the incongruence length difference, was checked by using PAUP* 4.0 (Swofford, 2002) software to test whether the degree of incongruence among the actual loci in the data set was significantly different from random partitions of the same data. We implemented 1000 replicates of the ILD test with 100 random addition sequences. All datasets were analysed by the following 3 different methods. (i) Maximum parsimony (MP) trees were constructed by PAUP* 4.0b10, using heuristic searches with 100 random additions of taxa, tree-bisection-reconnection (TBR) algorithm and 1000 random-addition sequence replicates with 10 trees held at each step. The optimal topology was then obtained using Kishino-Hasegawa. (ii) Neighbour-joining (NJ) was constructed using MEGA 4.0 under default parameters bootstrapped with 1000 replicates to evaluate the confidence for each node of the tree topology. Branches with less than 50% support are not shown in the tree (Tamura *et al*. 2007). (iii) Maximum likelihood (ML) was performed by PHYML v. 3.0.1 (Guindon and Gascuel, 2003), using the subtree pruning and regrafting (SPR) method with a BioNJ starting tree. Branch supports were evaluated by bootstrapping analysis of 1000 resembling for NJ and MP trees, and 100 resembling for ML trees.

## RESULTS

### *Sequence characteristics*

The aligned portions of the 12S, 18S and 28S genes of the roundworms from 21 wild animals were 490 bp, 750 bp, and 1700 bp long, respectively. 12S displayed a significant AT bias, as found in other mt genes sequenced previously. In addition, there were more variable sites observed in 12S rRNA than those in 18S rDNA and 28S rDNA (Table 2).

Homology and divergence analyses indicated that the identity of 18S gene sequences among all roundworms was 98·5–100%. The divergence of 28S gene sequences from *T. cati* and *T. canis* were 14·9–15·8% and 14·7–15·1%, respectively. For 12S, the divergence of the sequences was 11·7–12·8% between *T. cati* (isolated from *Felis temmincki, F. chaus, Prionailurus bengalensis*) and *T. canis* (isolated from *Alopex lagopus, A. spitz*), and from 20·3–23·4% between *Toxocara* and *Toxascaris* (isolated from *Panthera tigris*, *P. tigris altaica*, *P. tigris amoyensis*, *P. tigris tigris*, *Puma concolor*, *F. lynx*, *Canis lupus*). The divergence of 12S in the genera *Baylisascaris* and *Ascaris* were 5·2% and 2·7%, respectively. Additionally, a high degree of variation of the 12S region was observed among members of the same species and genus.

### *Phylogenetic analyses*

Phylogenetic relationships were inferred using 3 methods. The NJ/MP/ML trees were constructed based on 18S rDNA, 28S rDNA, 12S rRNA, and the combined 3 genes. As shown in Figs 1, 2, and 3, the topologies of the trees were similar except for the position of *Toxascaris*.

Equal-weighted parsimony analysis of the 12S data (499 nt characters) yielded 3 trees with a consistency index (C. I.) = 0·76 and a length of 623. High bootstrap value supports the formation of the 2 clades containing Ascarididae and Toxocaridae with maximum statistical support (NJ/MP/ML bootstrap values = 100). Within the Ascarididae, the MP majority-rule (50%) consensus tree received 3 groups. *Ascaris*, *Toxascaris* and *Baylisascaris*, each as a monophyletic group, received moderate support (68%), whilst among the Toxocaridae, each group appeared to receive higher support (Fig. 1).

For the combined rDNA analysis, no characters were excluded from a total of 2457 characters. All characters had equal weight (2181 constant, 106 variable and 170 parsimony-info sites). Analysis of these data yielded 12 trees with a C. I. = 0·73 and a length of 471. The MP bootstrap consensus tree yielded 2 monophyletic clades with a high bootstrap value of 100%. Interestingly, the same topologies were also inferred for 12S (Fig. 2).

The concatenated dataset of 3 genes consisted of 2950 characters with 358 parsimony informative sites. MP analysis based on these rDNA + 12S data yielded 3 trees with a C. I. = 0·76 and a length of 623. A higher support for each clade was received than those in the 12S rRNA and rDNA trees. Within the Ascarididae, the *Ascaris* and *Baylisascaris* grouped with a moderate support (Fig. 3). In addition, the position of *B. ailuri* in the concatenated trees was consistent with that in 12S rRNA trees, in contrast with that in the rDNA trees (Fig. 3).

All evolutionary trees suggested that the ascarids from 21 species of wild animals were classified into 2 families, 4 genera and 7 species. The topologies

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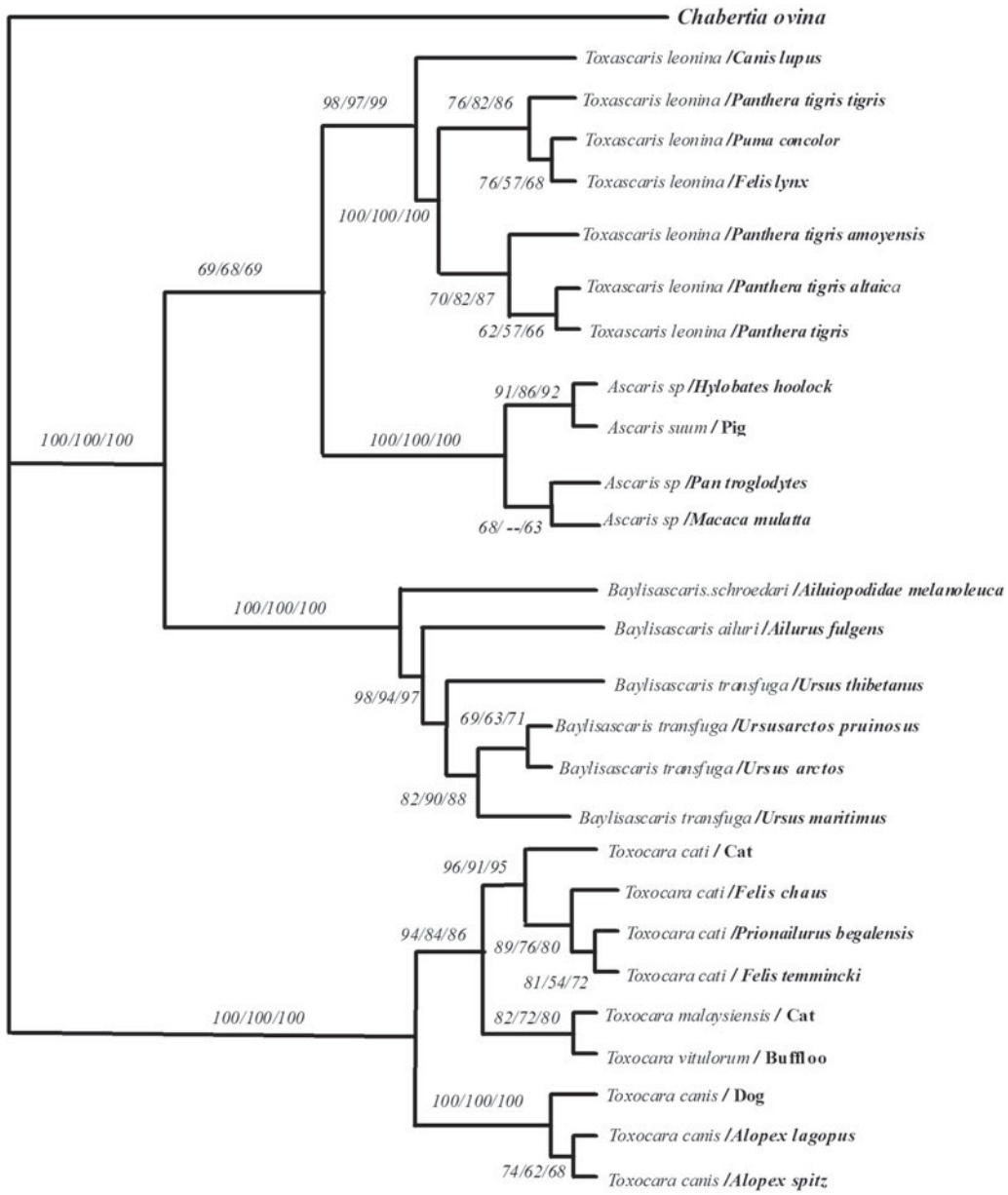


Fig. 1. Phylogenetic relationships among 21 *Ascaris*-infected species of wild animals. Trees are inferred from NJ/MP/ML methods based on 12S gene data. Bootstrap values are shown above nodes on the trees (only values $\geqslant$50% are shown).

showed that roundworms from *A. melanoleuca*, *A. fulgens*, *U. arctos pruinosus*, *U. thibetanus mupinensis* and *U. arctos lasiotus* were gathered together into 1 group with conspicuous confidence level in all trees. The Ascaridae in our constructed trees include roundworms from *Pan troglodytes*, *Hylobates hoolock*, *Macaca mulatta* and from *Panthera tigris*, *P. tigris altaica*, *P. tigris amoyensis*, *P. tigris tigris*, *Puma concolor*, *Felis lynx*, *Canis lupus* and pig. However, roundworms from *F. temminck*, *F. chaus*, *Prionailurus begalensis*, *Alopex lagopus* and *A. spitz* were assigned to the Toxocaridae. All taxa were included in a big branch tree. Additionally, the combined trees suggested that *B. transfuga* had a closer genetic relationship to *B. ailuri* than to *B. schroederi*, and it received higher support (94%) (Fig. 3).

## DISCUSSION

### *Gene variability*

In the present study, the high identities of 18S and 28S genes in roundworms obtained from 21 species of wild animals indicate the conservation of these 2 genes among different hosts. There was no variability in the nuclear gene 18S among the 4 populations (*Toxocara*, *Toxascaris*, *Baylisascaris* and *Ascaris*), while 28S showed some differences between *Toxascaris* and *Toxocara* from Canidae and Felidae, thus indicating that the 2 rDNA genes may be suitable for the higher level analysis. The base composition of 12S rRNA showed a strong AT-bias, as found in other nematodes reported previously (Hu *et al*. 2003*b*). In general, mt DNA evolves faster

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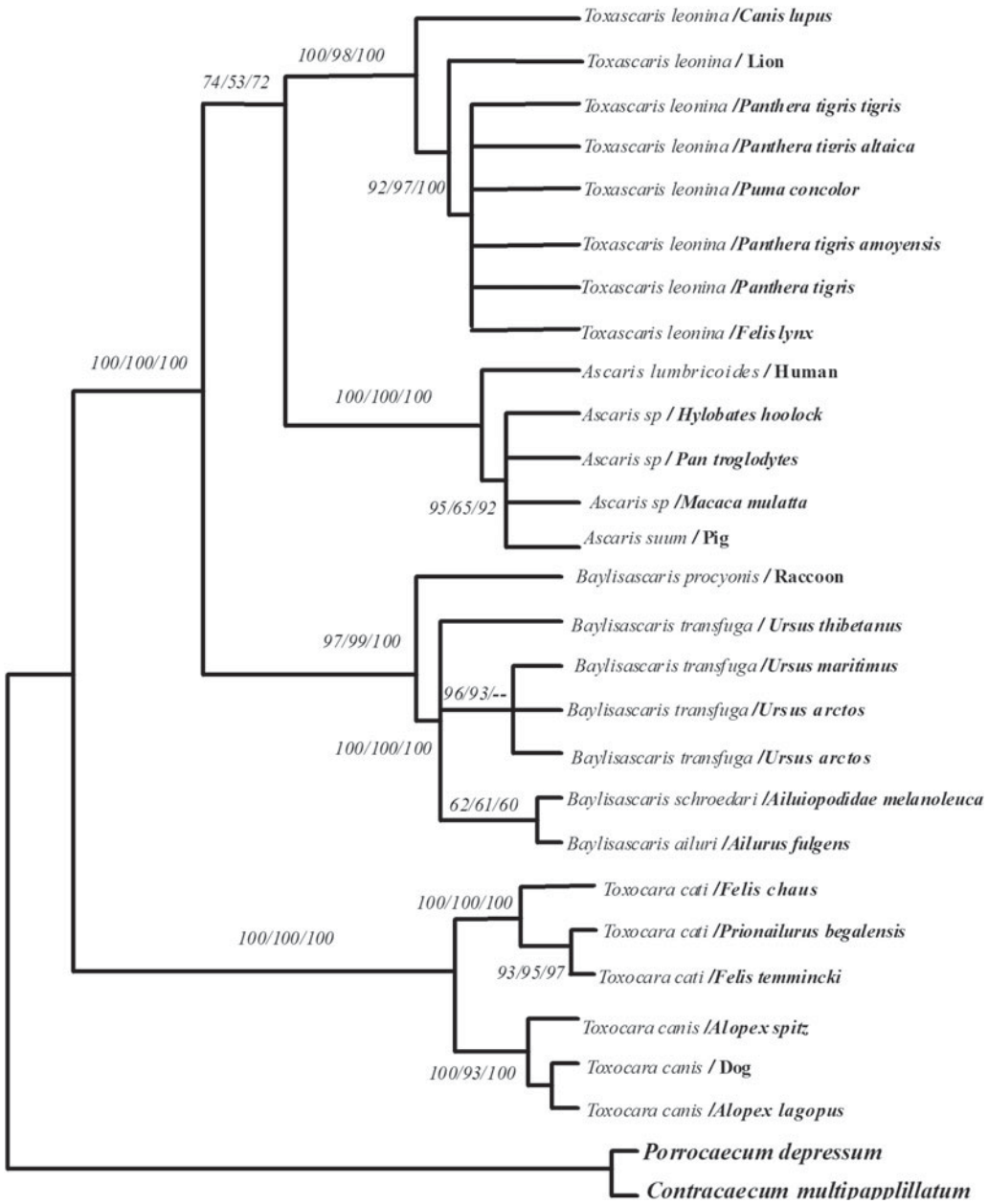


Fig. 2. Phylogenetic relationships among 21 *Ascaris*-infected species of wild animals. Trees are inferred from NJ/MP/ML methods based on rDNA (18S and 28S) gene data. Bootstrap values are shown above nodes on the trees (only values $\geqslant$50% are shown).

and accumulates substitutions more quickly than rDNA in nematodes (Li *et al*. 2010). In our study, it appeared that the differences between 12S data are most striking in many closely related species pairs, which implies that it would be a good species-specific marker for population genetic analysis.

## *Population characters*

### Baylisascaris

The roundworm from giant pandas was first named as *Ascaris schroederi*, while a new genus (*Baylisascaris*) was defined and *Ascaris schroederi* re-named as *Baylisascaris schroederi* (Sprent, 1968). The Ascarid from the red panda was described as a new species (*Toxascaris ailuri*) by Wu *et al*. (1987), but was also identified as *Toxascaris transfuga* (Shi and Zhou, 1993). Roundworms from Ursidae were identified as *Toxascaris selenarctis* or *Toxascaris transfuga* (Hu *et al*. 1993). Additionally, *Baylisascaris transfuga* was described by Foster *et al*. (2009) as an ascarid parasite in giant panda, red panda and in several bears with worldwide distribution It is therefore clear that the classification of *Baylisascaris* from different host species is still controversial, due to a lack of sufficient morphological features. Compared with morphological characters, molecular markers are more useful and are utilized to identify eggs, larvae and adults of

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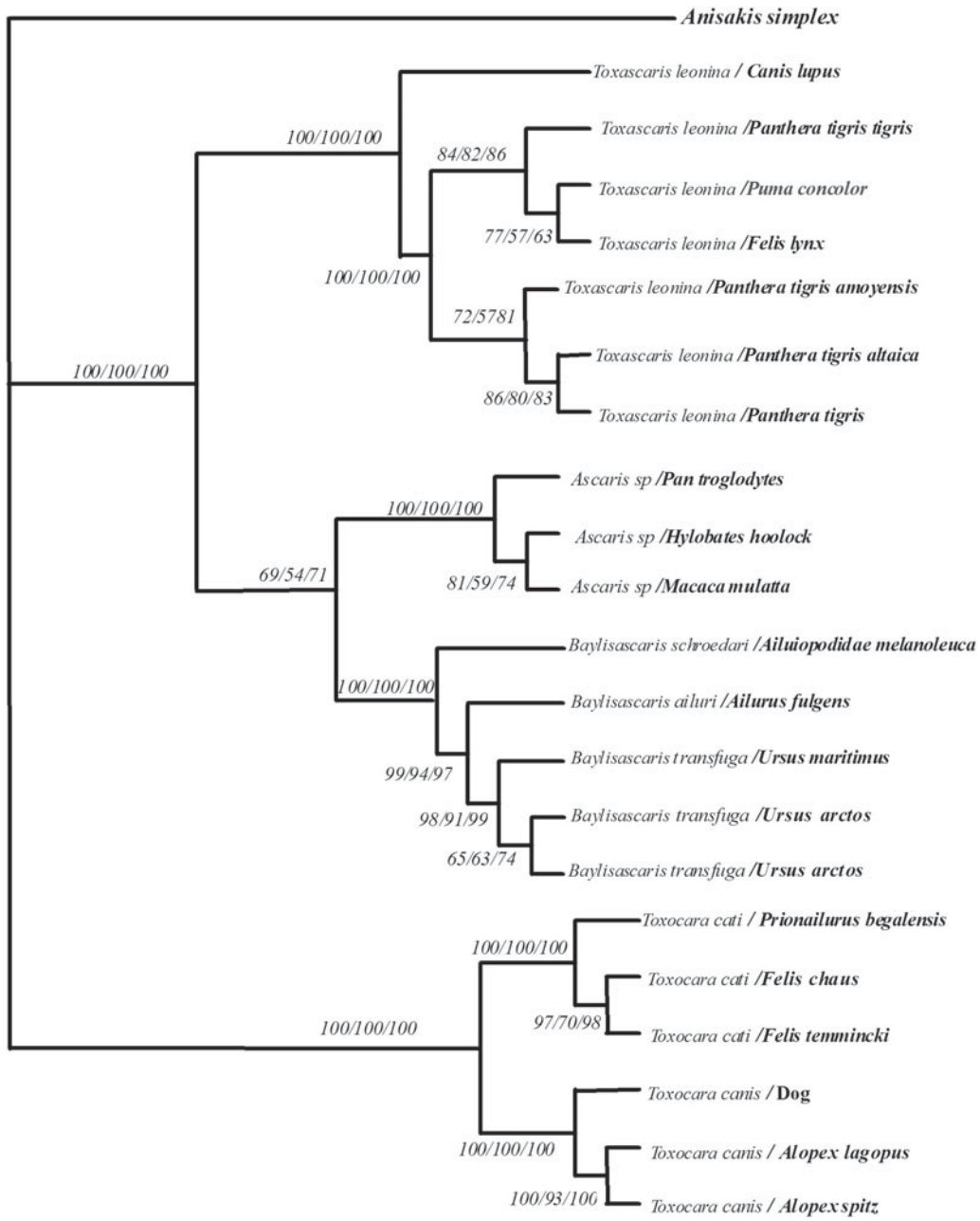


Fig. 3. Phylogenetic relationships among 21 *Ascaris*-infected species of wild animals. Trees are inferred from NJ/MP/ML methods based on the combined data (18S, 28S and 12S). Bootstrap values are shown above nodes on the trees (only values ⩾50% are shown).

ascaridoid nematodes from wild animals at species level. The partial sequences of ITS regions confirmed roundworms from red panda and 2 kinds of bears (black bear and brown bear) to be *Baylisascaris* spp. (He *et al*. 2008). Additionally, another molecular characterization of *Baylisascaris transfuga* supplemented the most important morphological characters of this little known parasitic species (Testini *et al*. 2011). In this study, the divergence displayed in alternative genes (18S, 28S and 12S) revealed that roundworms from *Ailuropoda melanoleuca*, *Ailurus fulgens* and 4 kinds of bears all belong to *Baylisascaris*, with the divergence in 12S under 5·2%. Based on the divergence in 12S, it seems that 12S can be utilized as a molecular marker to discriminate between other groups.

## Ascaris

Cases of captive non-human primates infected with *Ascaris* have been reported frequently (Nejsum *et al*. 2005, 2006). However, due to the similarity in morphological characteristics between *Ascaris* from humans and pigs (Crompton, 2001), most *Ascaris* infections were considered to be of human origin (*Ascaris lumbricoides*) (Okulewicz *et al*. 2002). Interestingly, PCR-AFLP and PCR-RFLP were recently used to confirm the possibility of

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transmission of zoonotic *Ascaris suum* among zoo chimpanzees (Nejsum *et al*. 2010). These results showed that not all infections in chimpanzees could be attributed to human *Ascaris*. Further molecular methods (e.g. systematic, population genetic and evolutionary biological studies) should be used to solve this problem. In the present study, due to the limitations in obtaining enough info-sites, the species of roundworms from the 3 primates (*Pan troglodytes, Hylobates hoolock, Macaca mulatta*) could not be identified and differentiated effectively. Further studies should be carried out to investigate whether wild primates possess their own ascarid species or whether cross-infections from humans or pigs are a common situation for *Ascaris*-infected primates living in zoos.

### Toxascaris *and* Toxocara

Both the genera *Toxascaris* and *Toxocara* species are worldwide parasites that occur in canids and felid and can cause diseases referred to as visceral or ocular larva migrans in humans (González *et al*. 2007). Accurate identification of species from these two genera is of great importance in preventing toxoascariasis. However, currently most diagnosis of the species infection is topically based on morphological characters, which might lead to false identifications. In this study, homology analysis of 3 genes indicated that roundworms from 3 felids and 2 canids were *Toxocara*, while parasites from the other 6 felids and from wolf were *Toxascaris*. A high degree of divergence was found both between and within species for the 12S gene, which indicates that 12S would be a good marker to identify these two nematode genera.

### *Phylogenetic relationships*

Phylogenetic trees reconstructed based on different approaches (NJ, MP and ML) indicated that 4 clades in the alternative topologies were monophyletic groups in the Ascarididae and Toxocaridae with 100% bootstrap support.

### Ascarididae

Maximum bootstrap support (100%) was observed for the Ascarididae, and within this clade, monophyletic groups of the genera *Ascaris*, *Baylisascaris* and *Toxascaris* received equally high support (100%). The *Baylisascaris* formed a single group consistent with former combined evidence trees analysis based on rDNA and the *cox2* gene (Nadler and Hudspeth, 2000), but was in contrast to trees based on other regions of rDNA (Nadler, 1992). Within the *Baylisascaris*, the placement of *B. schroederi* from *Ailuropoda melanoleuca* was more closely related to *B. ailur* from *Ailurus fulgens* than to *B. transfuga* from 4 bear species (*U. maritimus*, *U. arctos pruinosus*, *U. thibetanus mupinensis* and *U. arctos lasiotus*) with moderate support (65%) in the rDNA trees. However, both in the 12S and in the combined trees, *B. ailur* was more closely related to *B. transfuga* than to *B. schroederi* with higher support (95%), which was consistent with a recent study of amino acid sequences from 12 mitochondrial protein-coding genes (Xie *et al*. 2011).

Within the *Ascaris*, roundworms from 3 primates (*Pan troglodytes*, *Macaca mulatta* and *Hylobates hoolock*) were grouped together. In the trees inferred from 12S, the roundworms from *P. troglodytes* clustered together with the pig cluster, which is consistent with the results proposed by Nejsum *et al*. (2006).

*Toxascaris* species from canids and felids were sister groups in the Ascarididae clustered with *Ascaris* and *Baylisascaris* with moderate support (75%) as described by Nadler and Hudspeth (1998). Among this genus, the roundworms from 6 Felidae hosts clustered firstly, and were then grouped with roundworms from wolf, thus indicating that each sister clade of the *Toxascaris* group represents a different definitive host.

### Toxocaridae

*Toxocara* is the main genus of the Toxocaridae and is represented by *T. cati* and *T. canis*. In a previous study, the molecular positions of *T. canis* and *T. vitulorum* were investigated using mt (*atp6* and 12S) and nuclear genes (ITS2 and 28S). The results revealed that *T. vitulorum* was more closely related to *T. cati* than to *T. canis* (Wickramasinghe *et al*. 2009). A similar result was also observed in this study, wherein *T. cati*, the common ascarid of felids, was more closely related to *T. vitulorum* than to *T. canis*, the ubiquitous ascarid of canids.

## CONCLUSIONS

This analysis of partial 18S, 28S and 12S regions demonstrated that these 3 genes are suitable molecular markers for inferring phylogenetic relationships among roundworms from wild animals, and that the 12S gene is also useful for distinguishing ascarid species from different hosts. The reconstructed phylogenetic relationships showed 4 monophyletic groups with high bootstrap support. Furthermore, the high divergence of 12S in all species indicates that 12S could be used for the discrimination of species and for molecular phylogenetic studies.

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