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Sequence analyses of mitochondrial gene may support the existence of cryptic species within *Ascaridia galli*

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Short Communication

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Abstract

Ascaridia galli (Nematoda: Ascaridiidae) is the most common intestinal roundworm of chickens and other birds with a worldwide distribution. Although *A. galli* has been extensively studied, knowledge of the genetic variation of this parasite in detail is still insufficient. The present study examined genetic variation in the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene among *A. galli* isolates ($n = 26$) from domestic chickens in Hunan Province, China. A portion of the *cox*1 (p*cox*1) gene was amplified by polymerase chain reaction separately from adult *A. galli* individuals and the amplicons were subjected to sequencing from both directions. The length of the sequences of p*cox*1 is 441 bp. Although the intra-specific sequence variation within *A. galli* is 0–7.7%, the inter-specific sequence differences among other members of the infraorder Ascaridomorpha were 11.4–18.9%. Phylogenetic analyses based on the maximum likelihood method using the sequences of p*cox*1 confirmed that all of the *Ascaridia* isolates were *A. galli*, and also resolved three distinct clades. Taken together, the findings suggest that *A. galli* may represent a complex of cryptic species. Our results provide an additional genetic marker for the management of *A. galli* in chickens and other birds.

Introduction

Ascaridia galli (Schrank, 1788) is one of the most common nematodes affecting chickens and other birds that have a worldwide distribution. *Ascaridia galli* infection has been associated with retarded growth, weight loss, reductions in egg production, intestinal blockage and death in severe cases, causing economic losses to the poultry industry (Daş & Gauly, 2014). The prevalence of *A. galli* is very high (varies from 22% to 84%) in chickens from free range and organic production in some countries (Sherwin *et al.*, 2013; Thapa *et al.*, 2015). *Ascaridia galli* may be a problem in birds that come into contact with the open environment with infectious eggs of *A. galli* (such as free range and organic farms). In a recent study, neither artificial nor natural infection with *A. galli* was found to influence external and internal egg quality, irrespective of infection intensity (Sharma *et al.*, 2018, 2019). In China, *A. galli* is also considered a predominant parasite in domestic chickens (Tian *et al.*, 2015).

Due to its maternal inheritance, fast evolutionary rate and lack of recombination (Boore, 1999), mitochondrial DNA (mtDNA) has been extensively used for studies on genetic diversity and phylogenetic analyses at various taxonomic levels of different organisms, including nematodes (Goswami *et al.*, 2015; Yong *et al.*, 2015; Aguado *et al.*, 2016; Bastos Gomes *et al.*, 2017). For example, mitochondrial (mt) cytochrome *c* oxidase subunit 1 (*cox*1) sequences are useful genetic markers for the identification and differentiation of *Dirofilaria immitis* (Heidari *et al.*, 2015). Furthermore, mtDNA is a useful and reliable marker for the identification of cryptic nematode species (Blouin, 2002). A recent study showed that mt *cox*1 sequences can provide a rich source of genetic markers to assess the genetic diversity and cryptic species of *Dictyocaulus* lungworms (Ács *et al.*, 2016). *Trichuris* infecting primates represents a complex of cryptic species, with some species being able to infect both humans and non-human primates based on mt genome datasets (Hawash *et al.*, 2015). Although the mt *cox*1 gene has been studied in *A. galli* in South Africa (Malatji *et al.*, 2016), no information is available about genetic variation and cryptic species among *A. galli* isolates using mt *cox*1 polymorphisms.

The objectives of the present study were to investigate the genetic variation in mt *cox*1 genes among *A. galli* isolates from domestic chickens in Hunan Province, China, to evaluate further the claim that *A. galli* may include cryptic species and to assess the utility of the *cox*1 gene as a potential genetic marker for the management of *A. galli* in chickens and other birds.

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Materials and methods

All adult roundworms of *A. galli* ($n = 26$) were obtained from free-range adult domestic chickens, which were naturally infected with *A. galli* and that were from one flock of the same geographical origin in Hunan Province, China (table 1). These *A. galli* specimens were obtained from the gastrointestinal tracts of chickens in the slaughterhouse, washed in physiological saline, identified to species level primarily based on morphological characters, fixed in 70% (v/v) ethanol and stored at −20°C until use. Total genomic DNA was extracted from individual *A. galli* samples by sodium dodecyl sulphate/proteinase K treatment, column-purified (Wizard™ DNA Clean-Up, Promega, Madison, Wisconsin, USA) and eluted into 30 µl water according to the manufacturer's recommendations.

The *cox*1 gene was amplified with primers JB3 (5′-TTTTTT GGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAA GAACATAATGAAAATG-3′) (Bowles & McManus, 1994). Polymerase chain reactions (PCRs) (25 µl) were performed in 3.0 µl of magnesium chloride (25 mM), 0.25 µl of each primer (50 pmol/µl), 2.5 µl 10 × rTaq buffer (100 mM Tris-hydrochloride and 500 mM potassium chloride), 2 µl of deoxy-ribonucleoside triphosphate mixture (2.5 mM each), 0.25 µl of rTaq (5 U/µl) DNA polymerase (TaKaRa Biotechnology, Dalian, China), 2 µl of DNA sample and 14.75 µl water in a thermocycler (Biometra, Göttingen, German). The cycling conditions were 94°C for 5 min (initial denaturation), followed by 35 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 1 min (extension) and then 72°C for 5 min (final extension). Negative control (without DNA template) was included in each amplification run. Each amplicon (5 µl) was examined by 1% (w/v) agarose gel electrophoresis to validate amplification efficiency. PCR products were sent to Life Technology (Beijing, China) for sequencing from both directions.

Sequences of the mt *cox*1 gene were separately aligned using the software MAFFT 7.263 (Katoh & Standley, 2016). The level of sequence differences (D) among *A. galli* isolates were calculated by pairwise comparisons using the formula $D = 1 - (M/L)$, where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton *et al.*, 1995).

To study the phylogenetic relationships with representative roundworm species, *A. galli* (JX624728), *Ascaridia columbae* (JX624729), *Ascaris suum* (HQ704901), *Baylisascaris ailuri* (NC_015925), *Contracaecum rudolphii* B (FJ905109), *Baylisascaris procyonis* (NC_016200), *Baylisascaris schroederi* (NC_015927), *Baylisascaris transfuga* (NC_015924), *Heterakis beramporia* (KU529972), *Heterakis gallinae* (KU529973), *Toxascaris leonina* (NC_023504), *Toxocara canis* (NC_010690), *Toxocara cati* (NC_010773), *Toxascaris leonina* (KC902750) and *Toxocara malaysiensis* (NC_010527) were considered into the present study. Sequences of a portion of the *cox*1 (p*cox*1) with consensus lengths (441 bp) were aligned using the MAFFT 7.263 program. Maximum likelihood (ML) analyses were performed in PhyML 3.0 (Guindon *et al.*, 2010) using the subtree pruning and regrafting method with a BioNJ starting tree, and the GTR + I + G model with its parameter for the DNA dataset determined for the ML analysis using JModeltest (Posada, 2008) based on the Akaike information criterion. Bootstrap support (Bp) for ML trees was calculated using 100 bootstrap replicates. Phylograms were drawn using FigTree v.1.31 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 1. Geographical origins in China of *Ascaridia galli* samples used in the present study, as well as their GenBank accession numbers for sequences of the p*cox*1 gene.

Sample codes	Geographical origin	GenBank accession number
AGCS1	Hunan (Changsha)	KX266841
AGCS2	Hunan (Changsha)	KX266842
AGCS3	Hunan (Changsha)	KX266843
AGCS4	Hunan (Changsha)	KX266844
AGCS5	Hunan (Changsha)	KX266845
AGCS6	Hunan (Changsha)	KX266846
AGCS7	Hunan (Changsha)	KX266847
AGCS8	Hunan (Ningxiang)	KX266848
AGCS9	Hunan (Ningxiang)	KX266849
AGCS10	Hunan (Ningxiang)	KX266850
AGCS11	Hunan (Wangcheng)	KX266851
AGCS12	Hunan (Wangcheng)	KX266852
AGCS13	Hunan (Wangcheng)	KX266853
AGCS14	Hunan (Xiangtan)	KX266854
AGCS15	Hunan (Xiangtan)	KX266855
AGCS16	Hunan (Xiangtan)	KX266856
AGCS17	Hunan (Xiangtan)	KX266857
AGCS18	Hunan (Zhuzhou)	KX266858
AGCS19	Hunan (Zhuzhou)	KX266859
AGCS20	Hunan (Zhuzhou)	KX266860
AGCS21	Hunan (Shaoyang)	OM004022
AGCS22	Hunan (Shaoyang)	OM004023
AGCS23	Hunan (Shaoyang)	OM004024
AGCS24	Hunan (Shaoyang)	OM004025
AGCS25	Hunan (Shaoyang)	OM004026
AGCS26	Hunan (Shaoyang)	OM004027

Results and discussion

Genomic DNA was isolated from 26 individual adult *A. galli* samples. To examine sequence difference in the mt *cox*1 gene sequences and to assess the magnitude of genetic diversity in these sequences within *A. galli*, amplicons of p*cox*1 (approximately 450 bp) were amplified individually and subjected to agarose gel electrophoresis. The mt *cox*1 sequences were deposited in GenBank under the accession numbers KX266841–KX266860 and OM004022–OM004027 (table 1).

The sequences of p*cox*1 (441 bp) were obtained from the 26 samples, and the A + T contents of the sequences were 65.6–66.9%, consistent with that of a previous study from mt *cox*3, *nad*1 and *nad*4 genes among *A. galli* samples in China (Li *et al.*, 2013). A substantial level of nucleotide difference was detected among the mt *cox*1 of *A. galli* samples. The intra-specific sequence variations among different populations of *A. galli* isolates were 0–7.7% for p*cox*1. The inter-specific sequence differences among other members of the infraorder Ascaridomorpha were 11.4–18.9%. Comparison of the p*cox*1 sequences among

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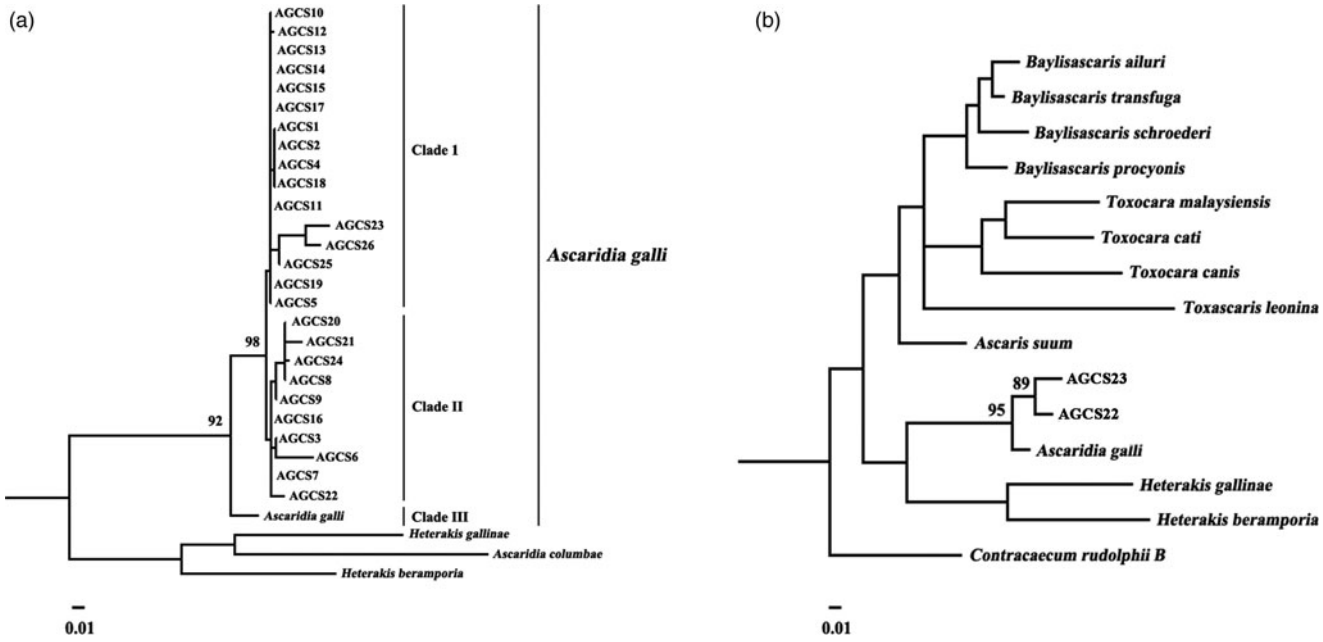

Fig. 1. Phylogenetic relationship among *Ascaridia galli* isolates in China with other nematodes inferred by ML using the *cox*1 dataset. Bp values are indicated at nodes. The scale bars show the number of subsitutions per site. All of the *A. galli* isolates in the present study were grouped together with moderate statistical support (Bp = 92), indicating that all of the *Ascaridia* isolates were *A. galli*, and also resolved three distinct clades that, at present, do not seem to be geographically isolated (a). Phylogenetic analysis of the mt *cox*1 sequences provided further support that AGCS22, AGCS23 and *A. galli* samples (JX624728) represent close but distinct taxa (b). The differences among the three *Ascaridia* isolates are about the same (looking at branch lengths) as between *Baylisascaris ailuri* and *Baylisascaris transfuga* (b). The representative roundworm species were used as follows: *A. galli* (JX624728), *Ascaridia columbae* (JX624729), *Ascaris suum* (HQ704901), *B. ailuri* (NC_015925), *Contracaecum rudolphii* B (FJ905109), *Baylisascaris procyonis* (NC_016200), *Baylisascaris schroederi* (NC_015927), *B. transfuga* (NC_015924), *Heterakis beramporia* (KU529972), *Heterakis gallinae* (KU529973), *Toxascaris leonina* (NC_023504), *Toxocara canis* (NC_010690), *Toxocara cati* (NC_010773), *Toxascaris leonina* (KC902750) and *Toxocara malaysiensis* (NC_010527).

four *Baylisascaris* species revealed a sequence difference of 2.7–6.1% (Xie *et al*., 2011a, b). In addition, sequence diversity (7.9–12.9%) was also detected in five *Toxocara* species by analysis of mt *cox*1 gene sequences (Li *et al*., 2008). Hence, the present findings provide additional genetic evidence for the existence of cryptic species within *A. galli*.

Mitochondrial gene sequences may provide reliable genetic markers in examining the taxonomic status of nematodes (Blouin, 2002). In the present study, all of the *A. galli* isolates grouped together with moderate statistical support (Bp = 92), indicating that all of the *Ascaridia* isolates were *A. galli*, and also resolved three distinct clades that, at present, do not seem to be geographically isolated (fig. 1a). In addition, phylogenetic analysis of the mt *cox*1 sequences also provided further support that AGCS22, AGCS23 and *A. galli* samples (JX624728) represent close but distinct taxa (fig. 1b). The differences among the three *Ascaridia* isolates are about the same (looking at branch lengths) as between *B. ailuri* and *B. transfuga* (fig. 1b). Taken together, the molecular evidence presented here supports the hypothesis that the gene pools of *A. galli* from domestic chickens have been isolated for a substantial period of time and that they represent a complex of cryptic species.

The present study provides additional genetic support for the existence of cryptic species within *A. galli*, but we believe it is still necessary to carry out more experimental research. Future studies could (1) examine population structure using a larger number of samples from different hosts and geographical locations, (2) implement the analysis of molecular variance and gene flow among different provinces in China; (3) characterize the complete mt genomes of these *A. galli* samples.

In conclusion, genetic diversity among *A. galli* isolates from Hunan Province, China, were revealed by sequence analyses of the mt *cox*1 gene. These results provide additional genetic evidence for the existence of cryptic species within *A. galli*. The results of the present study have implications for studying molecular epidemiology and population genetics of *A. galli*, and provide an additional genetic marker for the management of *A. galli* in chickens and other birds.

Author contributions. Y.Z. and S.-F.L. performed the experiments; Y.Z. and J.L. participated in the data analysis; Y.Z., S.-F.L. and J.L. edited the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest. None.

Ethical standards. All experiments were supervised by the Animal Ethics Committee of Hunan Agricultural University (no. 43321503) and performed in accordance with the regulations and guidelines of this committee.

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