

Molecular identification of *Kalicephalus* isolated from snakes in Hunan province, subtropical China

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Received 4 October 2018; received in revised form 13 November 2018; accepted 14 November 2018

Abstract. Parasite classification and identification are central to controlling parasitosis. Traditional methods for identifying parasite species are based on morphological features, but these are time-consuming and inaccurate, especially for cryptic species. The purpose of the present study was to select molecular markers to promote the development of molecular systematic for parasites. The internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) falls in between 18S, 5.8S, and 28S rDNA sequences, including ITS-1 and ITS-2 sequences. Previous studies have demonstrated that rDNA ITS sequences provide useful genetic markers for identifying parasitic nematodes. With the ultimate goal of controlling parasite transmission, we identified *Kalicephalus* belonging to three species using ITS rDNA genes. The ITS genes (750–797 bp) of 21 *Kalicephalus* belonging to 3 species were cloned and sequenced. Intra- and interspecific identities were 98.4% and 80%–89%, respectively. The phylogenetic tree reconstructed with the neighbour-joining (NJ) method revealed that congener *Kalicephalus* form the same branch, which is far apart from other branches of other nematodes. This is consistent with morphological classifications, demonstrating the accuracy of our molecular method. This is the first report stating that ITS genes can be used to classify *Kalicephalus*, and it lays the foundation for identification, molecular epidemiology, and phylogenetics of *Kalicephalus* and related parasitic nematodes.

INTRODUCTION

Kalicephalus spp. are parasites that can parasitize a broad range of snakes, as well as humans (Chai *et al.*, 2003; Santoro *et al.*, 2012). It is estimated that over 600,000 tons of snakes are consumed worldwide, and approximately 20 species of parasites are derived from animal food (Fei *et al.*, 2005), increasing the risk of infection with *Kalicephalus* and posing a threat to human health (Li, 1991). The majority of *Kalicephalus* infections cause symptoms such as anorexia, dyspnoea, and dysentery, and serious infections can be fatal (Shi, 2000; Gao, 2002; Andrei *et al.*, 2010). Thus,

Kalicephalus infections can affect snake breeding and lead to economic losses.

To date, there are only two reports on the gene sequencing of ribosomal 18S and 28S rDNA and the mitochondrial Cytochrome c Oxidase Subunit 1 of *Kalicephalus* (Chilton *et al.*, 2006; Prosser *et al.*, 2003). Surprisingly, no *Kalicephalus* ITS sequences have been reported, even though they are considered useful for parasitic nematode identification and differentiation.

Kalicephalus infections in snakes are quite common and widely distributed throughout China (Le-Van-Hoa *et al.*, 1968; Wang *et al.*, 1992; Junker *et al.*, 2009; Junker *et al.*, 2011); however, their prevalence and

species identities in snakes in China remain unclear. The objective of the present study was to sequence ITS genes of *Kalicephalus* spp. collected from snakes in Hunan province, China, to establish an identification method.

MATERIALS AND METHODS

Parasites and total genomic DNA isolation

Between June 2012 and October 2013, adult *Kalicephalus* specimens were collected from the viscera of various snake species from Hunan, China. Subsequently, the adult *Kalicephalus* from each host were washed separately using physiological saline, identified morphologically, fixed in 70% ethanol (V/V) and stored at -20°C until use. Following the morphological identification of *Kalicephalus* belonging to 3 species, 21 representative samples were selected and used for ITS gene amplification and sequence analysis. Total genomic DNA was extracted from individual samples with sodium dodecyl sulphate/proteinase K treatment, column-purified (Wizard® DNA Clean-up, Promega), and eluted into 50 µL water according to the manufacturer's recommendation.

Enzymatic amplification

Partial ITS sequence was amplified by polymerase chain reaction (PCR) with primers NC5 (5'-GTAGGTGAACCTGCGG AAGGATCATT-3') and NC2 (5'-TTAGTTT CTTTTCTCCGCT-3') (Zhu *et al.*, 1999). These primers were synthesized on a Biosearch Model 8700 DNA synthesizer (Shanghai, China). PCR was executed in a 25-µL system composed of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM MgCl₂, 200 mM of each deoxynucleoside triphosphate, 50 pmol of each primer, and 2 U Taq polymerase (Takara) in a thermocycler (Biometra) under the following conditions: an initial denaturation at 94°C for 5 min, then 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 1 min (extension) for 36 cycles, followed by a final extension at 72°C for 5 min. These optimized cycling conditions were obtained after testing different annealing and extension temperatures. Next, 10µL

(5–10 ng) of genomic DNA was added to each PCR reaction. Negative sample (no-DNA controls) was included in each amplification run. Five-microliter samples of each amplicon were examined by 1% (w/v) agarose gel electrophoresis to validate amplification efficiency. Positive PCR products were immediately sent to Sangon Company (Shanghai, China) for sequencing from both directions.

Sequence analysis and phylogenetic reconstruction

ITS sequences were separately aligned using the computer program Clustal X 1.83 (Thompson *et al.*, 1997). Pairwise comparisons were made of the level of sequence differences (D) among and within the species 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).

Representative samples with available ITS sequences were used for phylogenetic analyses. The neighbor-joining (NJ) method was used for phylogenetic reconstructions (Felsenstein, 1995). NJ analysis was carried out using the Dayhoff matrix model implemented by MEGA 4.0 (Tamura *et al.*, 2007), and maximum likelihood (ML) analysis was performed using PUZZLE 4.1 under the default setting (Strimmer & Haeseler, 1996). The consensus tree was obtained after bootstrap analysis, with 1,000 replications for NJ and maximum parsimony (MP) trees, and 100 for the ML tree, with values above 50% reported. Standard unweighted MP was performed with the Phylip 3.67 package (Felsenstein, 1995). The phylogenetic relationship among cestodes was performed using the sequences of seven nematode species (Table 1) as the ingroup plus the three mtDNA sequences obtained in the present study, using one tapeworm species (*Dipylidium caninum*, GenBank accession number, AM491339.1) as the outgroup based on ITS sequences. Phylograms were drawn using version 1.65 of the Tree View program version (Page, 1996).

Table 1. Nematode ITS genes compared with *Kalicephalus*

Species (abbreviation)	GenBank accession no.	Host	Reference
<i>A. caninum</i>	JQ812694.1	Dog	Lucio-Forster <i>et al.</i> (2012)
<i>A. duodenale</i>	EU344797.1	Human	Wang <i>et al.</i> (2007)
<i>A. braziliense</i>	JQ812692.1	Cat	Liotta <i>et al.</i> (2012)
<i>U. stenocephala</i>	HQ262055.1	Island fox	Nadler <i>et al.</i> (2013)
<i>Oesophagostomum</i> sp.	HQ844232.1	Sheep	Yang <i>et al.</i> (2010)
<i>Uncinaria</i> cf.	HE962184.1	Elephant seal	Ramos <i>et al.</i> (2013)
<i>A. tubaeforme</i>	JQ812691.1	Cat	Lucio-Forster <i>et al.</i> (2012)

RESULTS AND DISCUSSION

Genomic DNA was prepared from 21 individual *Kalicephalus* obtained from 3 species (*K. indicus*, *K. bungari* and *K. brachycephalus*; see Wu *et al.*, 2001). Partial sequences of the flanking regions of the ribosomal spacers and complete 5.8S rDNA were identical for all individuals. ITS genes were amplified individually and subjected to agarose gel electrophoresis. The amplicons of all samples appeared as single bands approximately 850 bp in length (Fig. 1).

To compare ITS sequence differences among nematode species and assess the magnitude of nucleotide variation of ITS within species, ITS amplicons representing different species were sequenced. The obtained ITS rDNA sequences of *Kalicephalus* samples were 750–797 bp in size. These sequences contained *K. indicus* (KI1–KI7) ITS rDNA sequences 764–773 bp, *K. bungari* (KBU1–KBU7) ITS rDNA sequences 750–765 bp, and *K. brachycephalus* (KBR1–KBR7) ITS rDNA sequences 793–797 bp. The contents of each of the four nucleotides varied, with A of 22.5%–24.53%, G of 24.53%–

25.55%, T of 29.18%–30.93%, C of 20.88%–22.00%, and the same for A+T and G+C (53.14%–53.86%).

Dendrograms based on ITS gene sequences representing different isolates aligned on an accordant length of 700 bp and constructed using NJ with building strategies and/or distance models were identical or similar, with only small discrepancies in bootstrap values (Fig. 1). The phylogenetic tree consisted of a large clade: *K. bungari* was sister to *K. indicus* and *K. brachycephalus*, and they were far apart from other branches of other parasitic nematodes. The *Oesophagostomum* sp. ITS gene sequence of was less similar to other nematodes, suggesting that the ITS gene could be an appropriate marker for the molecular identification of nematodes species.

It is widely acknowledged that rDNA ITS could be a useful genetic marker for variation in taxa at the level of phylogeny because its interspecies variation is much higher than intraspecies variation (Liu *et al.*, 2012; Ivica Králová-Hromadová *et al.*, 2011). The rapid evolution of rDNA ITS regions made it suitable to employ for phylogenetic

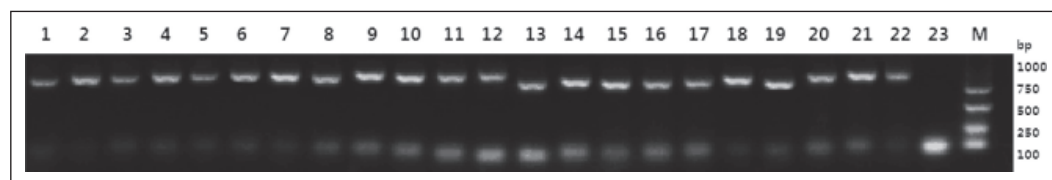


Fig. 1. Representative PCR products for a subset of the ITS rDNA genes of *Kalicephalus* from Hunan, China.

Lanes 1–23 represent samples KI1, KI2, KI3, KI4, KI5, KI6, KI7, KBU1, KBU2, KBU3, KBU4, KBU5, KBU6, KBU7, KBR1, KBR2, KBR3, KBR4, KBR5, KBR6, KBR7, positive control, and negative control, respectively. The marker lane (M) contains a DL-2000 molecular weight standard (ordinate values in bp).

		Percent Identity																													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		
1	KI7	100	78.6	76.2	77.1	76.7	78.4	78.5	78.4	87.6	86.7	87.5	88.4	87.2	87.3	87.4	80.3	80.1	80.2	79.8	80.0	79.9	80.0	99.6	99.1	99.2	98.4	99.1	99.0	1	
2	EU344797.1	78.6	100	92.7	93.9	83.7	97.9	92.2	98.0	75.7	74.6	75.6	76.7	75.4	75.4	79.3	79.1	79.1	78.8	78.8	79.0	79.1	79.0	78.8	79.0	78.8	79.0	78.5	78.7	78.4	2
3	HE962184.1	76.2	92.7	100	94.6	83.8	91.8	88.1	92.1	74.1	73.1	74.0	75.1	73.8	73.8	78.4	78.3	78.3	77.9	77.9	78.1	78.2	76.6	76.3	76.7	76.1	76.2	75.9	3		
4	HQ262055.1	76.4	76.4	5.6	100	83.5	93.4	89.4	93.1	75.3	74.3	75.1	76.4	75.0	74.9	75.0	80.2	79.9	80.1	79.7	79.7	79.9	79.9	77.5	77.1	77.7	77.0	77.2	76.9	4	
5	HQ844232.1	76.2	76.2	18.3	100	83.0	80.6	83.4	74.5	73.4	74.4	75.4	74.2	74.5	74.4	76.4	76.1	76.2	75.9	75.8	76.1	76.3	77.1	76.8	76.8	76.5	76.8	76.5	5		
6	J0812691.1	76.2	76.2	2.8	76.2	100	92.1	97.2	75.7	74.6	75.6	76.7	75.4	75.4	79.0	78.9	78.8	78.5	78.5	78.7	78.8	78.8	78.8	78.5	78.8	78.2	78.4	78.1	6		
7	J0812692.1	76.2	76.2	13.1	76.2	76.2	100	91.3	75.5	74.5	75.3	76.4	75.2	75.1	75.2	78.5	78.2	78.3	78.0	78.0	78.2	78.3	78.9	78.5	78.7	78.1	78.6	78.3	7		
8	J0812694.1	76.2	76.2	2.0	76.2	76.2	93.3	100	93.3	75.4	74.4	75.3	76.6	75.1	75.1	79.0	78.9	78.8	78.5	78.5	78.7	78.8	78.8	78.5	78.8	78.2	78.4	78.1	8		
9	KBR1	76.2	76.2	29.6	32.1	30.3	31.4	29.5	29.9	30.0	99.1	99.9	97.9	99.6	99.7	99.6	79.6	79.5	79.5	79.3	79.3	79.2	79.7	88.0	87.4	87.9	87.1	87.4	87.4	9	
10	KBR2	76.2	76.2	31.2	33.6	31.7	33.1	31.1	31.3	31.6	99.0	97.0	98.7	99.0	98.7	78.8	78.7	78.7	78.5	78.5	78.4	78.9	87.1	86.4	86.9	86.1	86.6	86.4	10		
11	KBR3	76.2	76.2	29.8	32.3	30.5	31.7	29.7	30.1	30.2	1.0	97.7	99.5	99.6	99.5	79.6	79.4	79.4	79.2	79.2	79.1	79.6	87.8	87.2	87.7	86.9	87.2	87.2	11		
12	KBR4	76.2	76.2	28.1	30.6	28.6	30.1	28.1	28.6	28.3	2.2	3.1	97.5	97.6	97.5	80.3	80.2	80.2	79.8	79.8	79.9	80.4	88.8	88.2	88.8	87.9	88.2	88.2	12		
13	KBR5	76.2	76.2	30.0	32.6	30.7	31.9	29.9	30.3	30.4	0.4	1.3	99.4	99.2	99.2	79.5	79.4	79.3	79.1	79.1	79.1	79.6	87.6	87.0	87.5	86.7	87.0	87.0	13		
14	KBR6	76.2	76.2	30.1	32.6	30.8	31.6	30.0	30.4	30.4	0.3	1.0	99.4	99.4	99.4	79.6	79.5	79.4	79.3	79.3	79.2	79.5	87.7	87.1	87.6	86.8	87.1	87.1	14		
15	KBR7	76.2	76.2	30.0	32.6	30.7	31.7	30.0	30.3	30.4	0.4	1.3	99.4	99.4	99.4	79.3	79.2	79.2	79.0	79.0	78.9	79.4	87.8	87.2	87.6	86.8	87.1	87.1	15		
16	KBU1	76.2	76.2	24.6	25.7	23.2	28.8	24.9	25.7	24.9	23.8	24.9	23.9	22.9	24.0	23.9	24.2	99.5	99.9	99.6	99.5	99.6	99.5	80.7	80.2	80.6	80.2	80.2	80.0	16	
17	KBU2	76.2	76.2	24.7	25.9	23.5	29.2	25.1	26.0	25.1	24.0	25.1	24.1	23.1	24.2	24.0	24.4	99.1	98.9	99.1	98.9	99.1	80.5	80.1	80.3	79.9	79.9	79.8	17		
18	KBU3	76.2	76.2	24.8	25.9	23.4	29.0	25.1	25.9	25.1	24.0	25.1	24.1	23.1	24.2	24.1	24.4	99.5	99.3	99.5	99.3	80.6	80.0	80.4	80.0	80.1	79.9	18			
19	KBU4	76.2	76.2	25.2	26.4	23.8	29.5	25.6	26.3	25.6	24.3	25.4	24.4	23.6	24.5	24.4	24.7	99.1	99.2	99.3	99.2	80.2	79.7	80.1	79.7	79.7	79.8	19			
20	KBU5	76.2	76.2	25.2	26.4	23.8	29.7	25.6	26.4	25.6	24.3	25.4	24.4	23.6	24.5	24.4	24.7	1.1	0.7	0.9	99.1	98.9	80.4	79.8	80.2	79.8	79.9	79.7	20		
21	KBU6	76.2	76.2	24.9	26.1	23.5	29.1	25.3	26.0	25.3	24.4	25.5	24.5	23.5	24.6	24.5	24.8	0.9	0.5	0.8	99.1	80.3	79.8	80.2	79.8	79.8	79.6	21			
22	KBU7	76.2	76.2	24.8	26.0	23.6	28.8	25.2	25.9	25.2	23.7	24.8	23.8	22.8	23.9	24.0	24.1	1.1	0.7	0.7	1.1	0.9	80.4	80.0	80.2	79.8	79.9	79.7	22		
23	KI1	76.2	76.2	24.8	28.5	27.1	27.7	25.2	24.9	25.2	13.1	14.3	13.3	12.2	13.6	13.5	13.4	22.7	22.6	23.0	22.9	23.0	22.8	99.2	99.6	99.1	99.5	99.3	23		
24	KI2	76.2	76.2	25.2	28.8	27.6	28.1	25.5	25.4	25.5	13.8	15.0	14.0	12.9	14.3	14.2	14.1	23.3	23.8	23.6	23.7	23.4	0.8	98.8	98.4	98.7	98.6	24			
25	KI3	76.2	76.2	24.9	28.3	26.9	28.1	25.2	25.3	25.2	13.2	14.4	13.5	12.1	13.7	13.6	13.5	22.9	22.8	23.1	23.1	23.0	0.4	1.2	98.7	99.0	99.0	25			
26	KI4	76.2	76.2	25.6	29.3	27.8	28.5	25.9	26.0	25.9	14.2	15.4	14.4	13.3	14.7	14.6	14.5	23.4	23.3	23.6	23.7	23.6	0.9	1.6	1.3	98.6	98.4	26			
27	KI5	76.2	76.2	25.3	29.0	27.6	28.0	25.7	25.4	25.7	13.8	14.9	14.1	12.9	14.3	14.2	14.2	23.4	23.3	23.7	23.6	23.7	0.5	1.3	1.0	1.4	98.8	27			
28	KI6	76.2	76.2	25.8	29.4	28.0	28.5	26.1	25.8	26.1	13.9	15.1	14.1	12.9	14.4	14.2	14.2	23.6	23.5	23.6	23.8	23.8	0.7	1.5	1.0	1.6	1.2	28			

Fig. 2. Pairwise comparison of ITS gene sequences between different *Kalichephalus* samples (%). Accession numbers KBR1 to KBR6 represent *K. brachycephalus*, KBU1 to KBU7 represents *K. bangari*, and KI1 to KI6 represent *K. indicus*.

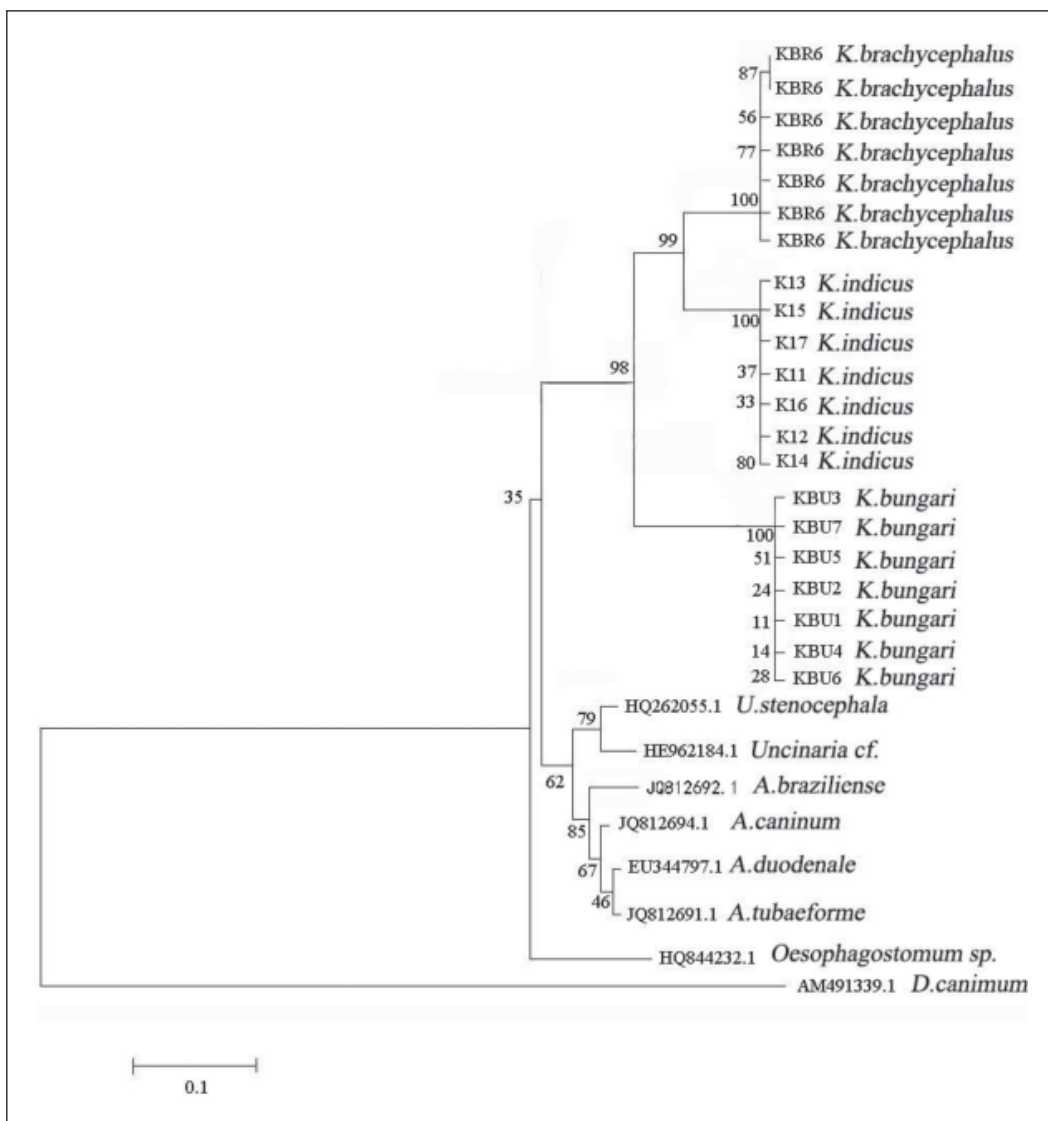


Fig. 3. Phylogenetic tree based on ITS gene sequences using the neighbour-joining method. Numbers above, below, or next to the branches represent bootstrap values. The outgroup is AM491339.1 (*D. caninum*).

reconstructions at nematode species and genus levels (Lucio-Forster *et al.*, 2012; Nadler *et al.*, 2013). In view of this, the present study characterized *Kalicephalus* ITS rDNA sequences from snakes collected in Hunan, China. Sequence comparison revealed that the intraspecific sequence identities among the three *Kalicephalus* species were significantly higher than interspecific sequence identities. These results clearly demonstrated that *K.*

indicus, *K. bungari*, and *K. brachycephalus* represent distinct species. Therefore, our study of ITS rDNA sequences of *Kalicephalus* lays a foundation for the classification, identification, diagnostics, molecular epidemiology, and phylogenetics of *Kalicephalus* and related parasitic nematodes.

Conflict of Interest.

The authors declare no conflict of interest.

Acknowledgement. This project was supported in part by grants from the Bureau of Animal Science and Aquaculture, Hunan Province and the Department of Science and Technology, Hunan Province (Grant No. 2014NK3049).

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