Short Communication

Molecular characterization of *Trichuris ovis* from goats in hunan province of China

Jia-Min Tang[1] Lu-Lin Song[1] Lin-Feng Nie[1] Fen Li[1,2*]

Abstract

Trichuris ovis (Nematoda: Trichuridae) is a widespread nematode that can infect a broad range of animal hosts, such as goats, sheep and oryx. This parasite is of vital importance as a pathogen, but its population genetics, molecular epidemiology and biology are still not well understood. In this study, *T. ovis* isolates were collected from goats in Hunan province, China, and sequences variation in *cox*1 gene were examined. A portion of *cox*1 gene from each collected adult *T. ovis* individual was separately amplified using the PCR method, and the amplicons were sequenced from both directions. The sequences of p*cox*1 gene were 382 bp long with A+T contents of between 59.7% and 60.2%. Although there were 0-1% differences in internal specific sequences, the interspecific variations were from 23.3% to 27.2%. Phylogenetic analysis based on Bayesian inference (BI) method using the p*cox*1 gene sequences confirmed that all isolates of *Trichuris* from the present study were *Trichuris ovis*. Phylogenetic tree revealed that within the comparation of members of *Trichuris*, *T. ovis* and *T. discolor* were more closely related than to others. Our results have improved the molecular epidemiology and population genetics comprehension of *T. ovis* species.

Keywords: *Trichuris ovis*, Mitochondrial DNA, Phylogenetic analysis, China

[1]*Hunan Engineering Research Center of Veterinary Drug, College of Veterinary Medicine, Hunan Agricultural University, Changsha, Hunan Province 410128, PR China*

[2]*Hunan Co-Innovation Center of Animal Production Safety, Changsha, Hunan Province 410128, PR China*

[*]***Correspondence:*** *loislf@qq.com*

Introduction

Whipworms are common soil-borne helminths that play an important role as pathogens causing trichuriasis in humans. The estimated population of whipworm infections in 2011 was 604-795 million (Bethony et al., 2011). This parasite usually causes entero-typhlocolitis and some other clinical signs such as dysentery, bloody diarrhoea and/or rectal prolapse (Hotez, 2009). In addition, other whipworms of this species (e.g. *Trichuris ovis*, *T. discolor*, *T. suis* and *T. vulpis*) can infect many animals (such as sheep, goats, pigs and dogs), causing economic losses to animal owners.

Host origin and morphological features such as spicule and pericloacal papillae are the basis to be used to identify adult whipworms of *Trichuris* species (Spakulová, 1994). Many *Trichuris* species (e.g. *T. ovis*, *T. discolor*, *T. skrjabini*, *T. concolor*, *T. gazellae* and *T. globulosa*) have been found in goats and sheep worldwide. Nevertheless, unequivocal identification and differentiation of *Trichuris* species are difficult to conduct solely based on the morphology of adult worms. In addition, morphological differentiation of *T. ovis* from other *Trichuris* species especially *T. discolor* is confirmed to be arduous (Knight, 1971). Therefore, there is a need for suitable molecular approaches to accurately identify and distinguish closely-related *Trichuris* species from different hosts (such as sheep and goats). Mitochondrial DNA (mtDNA) sequences are presented as effective genetic markers used to specifically identify and differentiate *Trichuris* species. For example, a recent study has indicated that mt gene sequences (including *cox*1 gene) can provide a useful molecular marker for the identification of *T. trichiura* and *T. suis* (Liu et al., 2012). Furthermore, mt *cox*1 gene sequences can discriminate closely-related *Trichuris* species (*T. navonae*, *T. bainae*, and *T. pardinasi*) (Callejón et al., 2016). However, so far, little information is presented about the genetic variability in *T. ovis* from goats and sheep (Wang et al., 2013). Therefore, investigation into the genetic variation in mt *cox*1 among *T. ovis* isolates has important implications for the prevention and control of *T. ovis* in goats and other animals.

The objectives of the present study were to investigate the sequence variation in mt *cox*1 gene among *T. ovis* isolates collected from goats in Hunan province, China, and to evaluate the phylogenetic relationship of *T. ovis* isolates and other whipworms.

Materials and Methods

Collection of worms and DNA extraction: Whipworm samples of the present study were collected from goats, and the collection was consented by the owners. All related animals were handled strictly in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Thirty-three adult whipworms of *T. ovis* were collected from the caecum of goats in Hunan province, China (Table 1). Each whipworm was extensively washed in physiological saline, previously identified to genus level according to their predilection site and morphological features using existing keys and descriptions (Cutillas et al., 1995), and then fixed in 70% ethanol until extraction of genomic DNA. DNA was extracted from individual samples using sodium SDS/proteinase K treatment, followed by spin column purification (Wizard™ DNA Clean-Up, Promega, Madison, Wisconsin, USA) and eluted into 50 µL H_2O according to the manufacturer's recommendations. Each DNA sample was identified to species based on its sequence of small subunit of nuclear ribosomal RNA gene. The sequences of these *T. ovis* samples were 99% similar to that of in Spain (GenBank accession number HF586911). The DNA samples were stored at -20°C until further use.

PCR amplification: *cox*1 was amplified with primers T-COX1F (5'- TTGCCCGCATTTGGCGCAATTTC-3') and T-COX1R (5'- TCACGACACTTACTATGTAGT GG -3'). The primers were designed in the conserved regions to amplify the complete mt genome of *T. ovis* (JQ996232). PCR reactions (25 µL) were performed in 2.5 µL of $MgCl_2$ (25 mM), 0.5 µL of each primer (50 pmol/µL), 2.5 µL 10×rTaq buffer (100 mM Tris-HCl and 500 mM KCl), 2 µL of dNTP mixture (2.5 mM each), 0.25 µL of r*Taq* (5 U/µL) DNA polymerase (TaKaRa Biotechnology, Dalian, China) and 1 µL of DNA sample 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). Each amplification run included a negative control (without DNA template). One percent (w/v) agarose gel electrophoresis examination was used on each amplicon (5 µL) to validate amplification efficiency. Sangon Company (Shanghai, China) was mandatorily selected for sequencing these PCR products from both directions.

Phylogenetic and population genetic analysis: To conclude the genetic diversity of *T. ovis* and to examine processes of the formation of the present distributions, analyses of p*cox*1 sequences were conducted. Clustal X 1.81 (Thompson et al., 1997) was used to align *cox*1 sequences of different samples in this study, and then visually modified the sequences. DnaSP 5.0 (Librado and Rozas, 2009) was adopted to calculate haplotype numbers, nucleotide diversity (Pi) and haplotype diversity (Hd) of each species based on p*cox*1 genes. Median-joining networks were also inferred the relationship of these selected species using Network software version 5.0.0.1.

Other representative whipworm species as *T. discolor* (JQ996231), *T. ovis* (JQ996232), *T. suis* (GU070737), *T. trichiura* (GU070738), *T. muris* (LC050561) and *Trichuris* sp. (KC461179) were selected for the study of phylogenetic relationships, while *Trichinella spiralis* (NC_002681) was used as the outgroup. Consensus lengths of 382 bp of p*cox*1 sequences were aligned using MAFFT 7.263 (Katoh and Standley, 2013). Phylogenetic analysis was performed with Bayesian inference (BI) method. Model of evolution was calculated based on the Akaike information criterion (AIC) as implemented in JModeltest (Posada, 2008). BI analysis was conducted in software MrBayes using the GTR + I + G model of evolution, with BI two runs set, each of four

simultaneous chains for the Monte Carlo Markov Chain (MCMC). In each of the two runs, 1,000,000 generations were set and every 100 generations resulted in a tree in MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). The average standard deviation of split frequencies (ASDSF) was less than 0.01 and the potential scale reduction factor (PSRF) approaching 1 was confirmed to ensure convergence of the two runs. The first 25% trees were discarded as burn-in. A 50% majority rule consensus tree was used to calculate Bayesian posterior probabilities (Bpp). Phylograms were drawn using FigTree v.1.31 (http://tree.bio.ed.ac.uk/software/figtree/).

Results and Discussion

MtDNA was separated from 33 individual adult *T. ovis* samples. To examine sequence difference in the mt *cox*1 gene sequences and to assess the magnitude of genetic diversity in these sequences within *T. ovis*, each p*cox*1 amplicon (approximately 400 bp) was amplified individually and then subjected to agarose gel electrophoresis. For this region, in no case was product amplified from no DNA sample or host DNA control (not shown). The mt *cox*1 sequences in the present study were deposited in GenBank (accession numbers: KY656485 - KY656517) (Table 1).

Table 1 Sample codes, geographical origins and GenBank accession numbers of *Trichuris ovis* samples used in the present study

Sample codes	Geographical origin	GenBank accession number
TOCS1	Hunan (Changsha)	KY656485
TOCS2	Hunan (Changsha)	KY656486
TOCS3	Hunan (Changsha)	KY656487
TOCS4	Hunan (Changsha)	KY656488
TOCS5	Hunan (Changsha)	KY656489
TOXP1	Hunan (Xupu)	KY656490
TOXP2	Hunan (Xupu)	KY656491
TOXP3	Hunan (Xupu)	KY656492
TOXP4	Hunan (Xupu)	KY656493
TOXP5	Hunan (Xupu)	KY656494
TONY1	Hunan (Ningyuan)	KY656495
TOLY1	Hunan (Liuyang)	KY656496
TOLY2	Hunan (Liuyang)	KY656497
TOLY3	Hunan (Liuyang)	KY656498
TOLY4	Hunan (Liuyang)	KY656499
TOLY5	Hunan (Liuyang)	KY656500
TOLY6	Hunan (Liuyang)	KY656501
TOMY1	Hunan (Mayang)	KY656502
TOMY2	Hunan (Mayang)	KY656503
TOMY3	Hunan (Mayang)	KY656504
TOMY4	Hunan (Mayang)	KY656505
TOMY5	Hunan (Mayang)	KY656506
TOJS1	Hunan (Jishou)	KY656507
TOJS2	Hunan (Jishou)	KY656508
TOJS3	Hunan (Jishou)	KY656509
TOJS4	Hunan (Jishou)	KY656510
TOJS5	Hunan (Jishou)	KY656511
TOJS6	Hunan (Jishou)	KY656512
TOJS7	Hunan (Jishou)	KY656513
TOCZ1	Hunan (Chenzhou)	KY656514
TOCZ2	Hunan (Chenzhou)	KY656515
TOCZ3	Hunan (Chenzhou)	KY656516
TOCZ4	Hunan (Chenzhou)	KY656517

The 382 bp long p*cox*1 sequences were obtained from the 33 samples, and the A+T contents occupied the proportion of 59.7-60.2% of these sequences, which is consistent with that of a previous study inferred from mt *cox*3, *nad*5 and *cyt*b genes among *T. ovis* samples from *Addax nasomaculatus* and *Taurotragus derbianus* in China (Wang et al., 2013). Differences of 0-1% in internal specific sequences of *T. ovis* were observed, while the interspecific variations within the other members of *Trichuris* species were from 23.3% to 27.2%. This study clearly showed that mt *cox*1 gene sequences were reliable genetic markers for specifically identifying and differentiating *Trichuris* species.

Genetic diversity is ubiquitous in whipworm populations, and the accurate analysis of genetic diversity in whipworms is of great significance to study genetics, epidemiology and biology of whipworms. Although investigations in these areas usually use mtDNA as genetic markers, mt datasets for *T. ovis* are still little known (Wang et al., 2013). Thus, the genetic variation of *T. ovis* in goats in China was further studied with mt *cox*1 marker in this study.

Among these 33 *T. ovis* samples, five haplotypes were observed (Table 2). Within the 7 habitats of *T. ovis*, only 3 cities (Xupu, Liuyang and Jishou) presented haplotype diversity, whereas the others appeared to have no evolvement. Both haplotype diversity (Hd) and nucleotide differences (π) calculated had low values, suggesting that there has been a recent population bottleneck effect or an establishment effect of a single, small population within the isolates. Interestingly, the haplotype diversity (Hd) of the samples collected in Jishou was

relatively high but with a low nucleotide difference (π) parameter, revealing that the population has been accompanied by rapid population expansion and accumulation of variation after the bottleneck effect, which was also confirmed by the mismatch distribution analysis (Fig. 1).

The network analysis (Fig. 2) revealed that haplotype I had the highest frequencies (n=28) and was shared by all cities from which the samples were collected. Furthermore, haplotype I was surrounded by all the other isolates and formed a one-star-like pattern (Fig. 2), suggesting that haplotype I is the original haplotype of *T. ovis* isolates. Other haplotypes were restricted to Jishou, Liuyang and Xupu, respectively. Moreover, Jishou appeared to have the highest quantity of haplotypes among the seven cities with 3 kinds, compared with that of Xupu and Liuyang with 2 kinds and the other cities with just one.

Table 2 Sequence diversity indices of *cox*1 gene of mtDNA in *T. ovis* populations of the seven cities

Population	N	S	H	Hd	π	k
Changsha	5	0	1	0	0	0
Xupu	5	1	2	0.400	0.00105	0.400
Ningyuan	1	0	1	0	0	0
Liuyang	6	1	2	0.333	0.00087	0.333
Mayang	5	0	1	0	0	0
Jishou	7	2	3	0.667	0.00199	0.762
Chenzhou	4	0	1	0	0	0

N: Number of sequence used; S: Number of segregation site; H: Number of haplotypes; Hd: Haplotype diversity; π: Nucleotide diversity; k: Average number of nucleotide differences

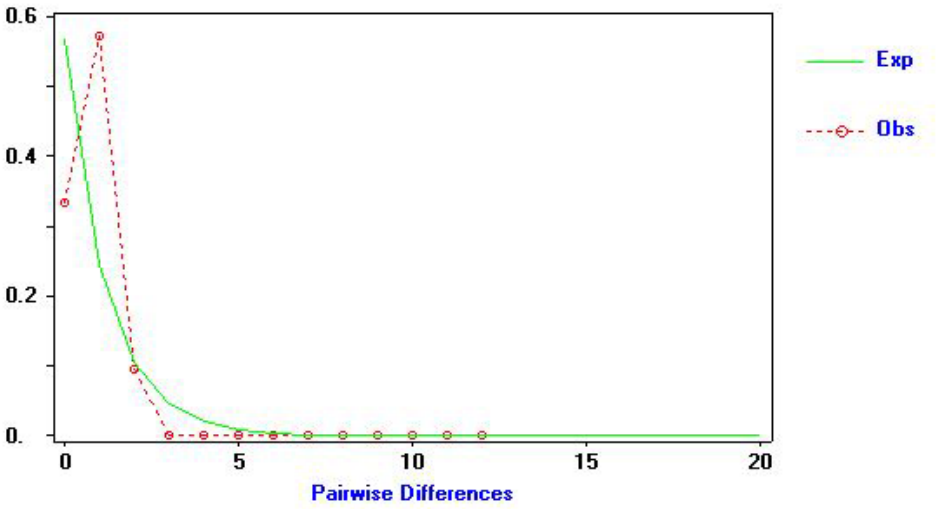

Figure 1 Mismatch-distribution to test the expansion of *cox*1 in the population of Jishou *T. ovis* isolates. The number of nucleotide differences between pairs of sequences is indicated by the x-axis, while their frequency is indicated by the y-axis.

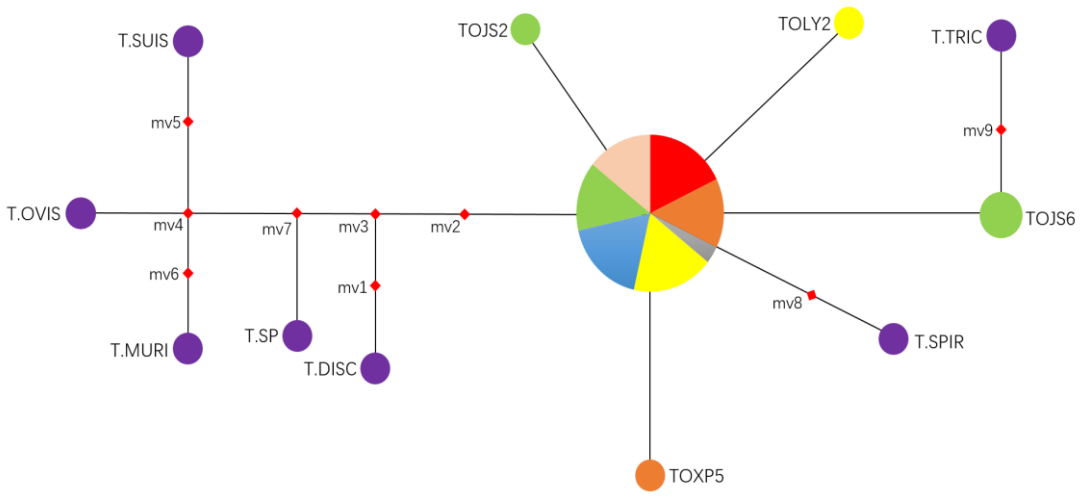

Figure 2 Median-joining networks depicting the genetic relationships among *Trichuris ovis* isolates (mtDNA). The sizes of the circles and colored segments are proportional to the haplotype frequencies in the datasets. Red denotes Changsha, orange refers to Xupu, grey stands for Ningyuan, yellow represents Liuyang, blue denotes Mayang, green refers to Jishou, and pink is on behalf of Chenzhou. GenBank sequences are represented by purple. Red diamonds are median vectors representing either extinct or un-sampled haplotypes.

Mt gene sequences may provide reliable genetic markers in examining the taxonomic status of whipworms, such as the use of protein-coding gene as markers in comparative analyses (Liu et al., 2016, b; Li et al., 2016). In the present study, strong statistical support (Bpp=1.0) showed all the *T. ovis* isolates grouped together (Fig. 3), indicating that all isolates of whipworm were *T. ovis*. The results of this study also indicated the much closer relationship between *T. ovis* and *T. discolor* isolates, and the lesser with any other whipworms (such as *T. trichiura* and *T. suis*), in accord with the results of previous studies (Liu et al., 2012; Liu et al., 2013). However, the study of Liu et al. (2012) indicated that high statistical supports were presented in the cluster of *T. ovis* + *T. discolor* and *T. suis* + *T. trichiura*. However, in the present study, the result that *T. ovis* + *T. discolor* and *T. trichiura* + *Trichuris* sp. were clustered together as moderate statistical support (Bpp=0.87) was shown (Fig. 3). Although several *Trichuris* species were analyzed with mt *cox*1 gene sequences and phylogenetic relationships among these worms were also researched, many *Trichuris* species are not fully presented in the current analyses. Thus, it is necessary to expand taxon sampling for future phylogenetic studies of *Trichuris*.

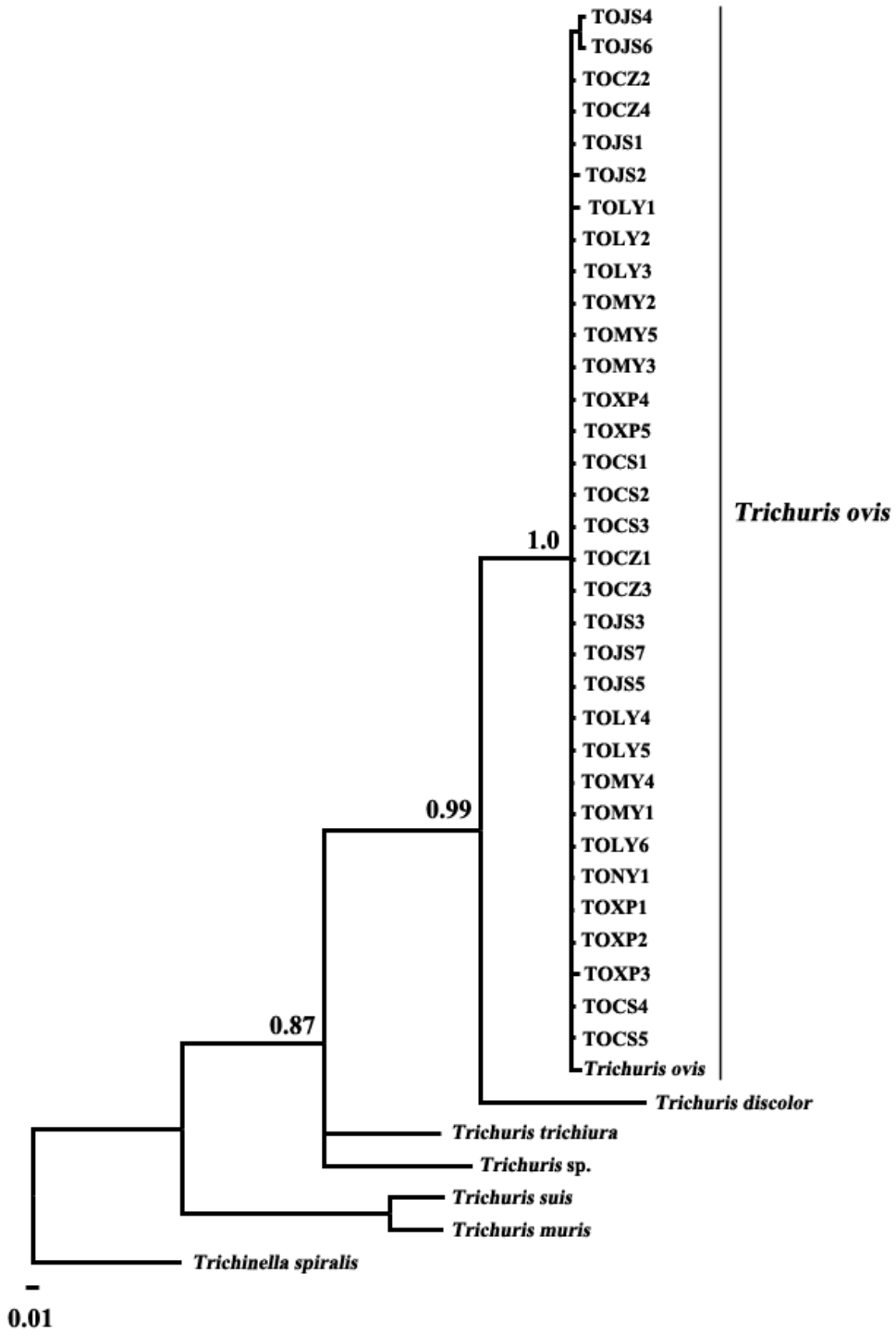

Figure 3 Phylogenetic relationship among *Trichuris ovis* isolates with other whipworms inferred by Bayesian analyses using the *cox*1 dataset, with *Trichinella spiralis* (NC_002681) as outgroup

In conclusion, the phylogenetic analysis revealed that all isolates of *Trichuris* from the present study were *Trichuris ovis*. The present study has improved the molecular epidemiology and population genetics comprehension of *T. ovis* species.

Competing interests: The authors declare that they have no competing interests.

Ethic statement: All animals were handled in strict accordance with the Good Animal Practice requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Acknowledgements

This research was supported by the Outstanding Young Scientific Research Project of the Education Department of Hunan Province (Grant No.14B092).

References

Bethony JM, Cole RN, Guo X, Kamhawi S, Lightowlers MW, Loukas A, Petri W, Reed S, Valenzuela JG and Hotez PJ 2011. Vaccines to combat the neglected tropical diseases. Immunol Rev. 239(1): 237-270.

Callejón R, Robles Mdel R, Panei CJ and Cutillas C 2016. Molecular diversification of *Trichuris* spp. from Sigmodontinae (Cricetidae) rodents from Argentina based o-n mitochondrial DNA sequences. Parasitol Res. 115(8): 2933-2945.

Cutillas C, German P, Arias P and Guevara D 1995. *Trichuris ovis* and *Trichuris globulosa*: morphological, biometrical, and genetic studies. Exp Parasitol. 81(4): 621-625.

Hotez PJ 2009. The neglected tropical diseases and their devastating health and econ-omic impact on the member nations of the Organisation of the Islamic Conference. PLoS Negl Trop Dis. 3(10): e539.

Katoh K and Standley DM 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 30(4): 772-780.

Knight RA 1971. *Redescription* of *Trichuris discolor* (von Linstow, 1906) and *T. skrjabini* (Baskakov, 1924) from domestic ruminants in the United States and comparisons with *T. ovis* (Abildgaard, 1795). J Parasitol. 57(2): 302-310.

Li K, Yang F, Abdullahi AY, Song M, Shi X, Wang M, Fu Y, Pan W, Shan F, Chen W and Li G 2016. Sequence an-alysis of mitochondrial genome of *Toxascaris leonina* from a south China tiger. Korean J Parasitol. 54(6): 803-807.

Librado P and Rozas J 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25(11): 1451-1452.

Liu GH, Gasser RB, Su A, Nejsum P, Peng L, Lin RQ, Li MW, Xu MJ and Zhu XQ 2012. Clear genetic distinctiveness between human- and pig-derived *Trichuris* based on analyses of mitochondrial datasets. PLoS Negl Trop Dis. 6(2): e1539.

Liu GH, Gasser RB, Nejsum P, Wang Y, Chen Q, Song HQ Zhu and XQ 2013. Mitochondrial and nuclear ribosomal DNA evidence supports the existence of a new *Trichuris* species in the endangered François' leaf-monkey. PLoS One. 8(6): e66249.

Liu GH, Wang Y, Xu MJ, Zhou DH, Ye YG, Li JY, Song HQ, Lin RQ and Zhu XQ 2012. Characterization of the complete mitochondrial genomes of two whipworms *Trichuris ovis* and *Trichuris discolor* (Nematoda: Trichuridae). Infect Genet Evol. 12(8): 1635-1641.

Liu GH, Nadler SA, Liu SS, Podolska M, D'Amelio S, Shao R, Gasser RB and Zhu XQ 2016. Mitochondrial phylogenomics yields strongly supported hypotheses for ascaridomorph nematodes. Sci Rep. 6: 39248.

Posada D 2008. jModelTest: phylogenetic model averaging. Mol Biol Evol. 25(7): 1253-1256.

Ronquist F and Huelsenbeck JP 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19(12): 1572-1574.

Spakulová M 1994. Discriminant analysis as a method for the numerical evaluation of taxonomic characters in male trichurid nematodes. Syst Parasitol. 29(2): 113-119.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25(25): 4876- 4882.

Wang Y, Liu GH, Li JY, Xu MJ, Ye YG, Zhou DH, Song HQ, Lin RQ and Zhu XQ 2013. Genetic variability among *Trichuris ovis* isolates from different hosts in Guangdong Province, China revealed by sequences of three mitochondrial genes. Mitochondr DNA. 24(1): 50-54.

บทคัดย่อ

การจัดจำแนกทริคูริส โอวิส ของแพะในจังหวัดฮูนานของประเทศจีนด้วยวิธีอณูชีววิทยา

เจีย-มิน ตัง[1] ลู-ลิน ซอง[1] ลิน-เฟง ไนล์[1] เฟน ลิ[1,2*]

ทริคูริส โอวิส เป็นพยาธิตัวกลมที่มีการแพร่กระจายกว้างขวางซึ่งสามารถติดต่อในสัตว์หลายชนิด เช่น แพะ แกะ และออริกซ์ ปรสิตชนิดนี้มีอันตรายต่อชีวิตแต่ยังไม่มีการศึกษาด้านพันธุกรรม ระบาดวิทยา และ ชีววิทยา ด้วยวิธีการทางอณูชีววิทยาให้เป็นที่เข้าใจ ในการศึกษานี้ทำการจำแนก ที โอวิส จากแพะในจังหวัดฮูนาน ประเทศจีนและจัดแยกด้วย ***cox1 gene*** โดยนำ ***cox1 gene*** จาก ที โอวิส ที่เก็บรวบรวมมา ทำการเพิ่มปริมาณด้วยพีซีอาร์ จากนั้นนำไปจัดลำดับทั้งสองแนวทาง ลำดับของ ***pcox1 gene*** มีค่า ***382 dp*** โดยมี ***A+T*** ระหว่างค่า 59.7**%** และ 60.2**%** ถึงแม้ว่าจะมีความแตกต่างของการจัดลำดับอย่างเฉพาะเจาะจงคือ 0-1**%** แต่มีความแตกต่างระหว่างกลุ่มคือ 23.3**%** ถึง 27.2**%** จากการจัดสายวิวัฒนาการด้วย ***Bayesian inference (BI)*** โดยใช้ ***pcox1 gene*** พบว่าทุกชนิดของเชื้อปรสิตที่นำมาวิเคราะห์เป็น ที โอวิส จากการจัดสายวิวัฒนาการพบว่า ที โอวิส และ ที ดิสคัลเลอร์ มีความใกล้ชิดมากกว่าชนิดอื่น ผลการศึกษานี้เป็นการพัฒนาระบาดวิทยาและยีนประชากรของ ที โอวิส

คำสำคัญ: ทริคูริส โอวิส ไมโตคอนเดรีย ดีเอ็นเอ Phylogenetic analysis ประเทศจีน

[1]Hunan Engineering Research Center of Veterinary Drug, College of Veterinary Medicine, Hunan Agricultural University, Changsha, Hunan Province 410128, PR China

[2]Hunan Co-Innovation Center of Animal Production Safety, Changsha, Hunan Province 410128, PR China

****ผู้รับผิดชอบบทความ E-mail:** loislf@qq.com*