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GENETIC DATA OF THE ITS1-5.8S rDNA SEQUENCES OF SMALL LIVER FLUKES (OPISTHORCHIIDAE) FROM CATS IN NORTHERN VIETNAM

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SUMMARY

Domestic cats and humans are favorite final hosts for some hepatic trematodes of Opisthorchiidae. The present study examined the sequence variation in the nuclear ribosomal DNA (rDNA) region, including the internal transcribed spacer (ITS1) and the 5.8S gene, of two human opisthorchiid species *Clonorchis sinensis* (Cobbold, 1875) Looss, 1907 and *Opisthorchis viverrini* (Poirier, 1886) Stiles & Hassal, 1896, in cats sampled in Northern Vietnam. The length of 30 partial ITS1–5.8S rDNA sequences of *C. sinensis* from different hosts and localities varied from 761 to 766 bp. The results showed completely identity for all investigated 5.8S rDNA sequences of *C. sinensis*. In contrast, the variations were revealed in the complete ITS1 sequences of *C. sinensis*. Three polymorphic sites, indel and new sites with intragenomic polymorphism were detected. The length of the complete ITS1 rDNA region ranged from 656 to 661 bp. There are no substitutions downstream of the 5 bp insertion, and the different ratio of nucleotide in polymorphic sites with presence/absence of insertion leads to the existence of 6 genotypes in the investigated samples. The major genotype was obtained in 70% of sequences, other genotypes were presented by 1, 2 or 3 sequences. Nucleotide diversity in whole sample of *C. sinensis* was 0.00122 ± 0.00073. Partial sequences of ITS1–5.8S rDNA of *O. viverrini* were 745 bp in length, and no difference was observed. Phylogenetic analyses by neighbor joining phylogram inferred from ITS1–5.8S rDNA of 3 sequences of *O. viverrini* and 30 sequences of *C. sinensis* were formed two separately monophyletic clades.

Keywords: *Clonorchis sinensis*; *Opisthorchis viverrini*; small liver flukes; ITS1; Vietnam

INTRODUCTION

Fish-borne zoonotic trematodes, especially species of the family Opisthorchiidae are important emerging and re-emerging pathogens causing bile-duct and liver diseases in humans (Chai *et al.*, 2005; Keiser, Utzinger, 2005; 2009). In Vietnam, two opisthorchiid infecting human species, ie. *Clonorchis sinensis* and *Opisthorchis viverrini*, are present (Nguyen, 2000; Nguyen *et al.*, 2013). *Clonorchis sinensis* is distributed in most of Northern and in some provinces in Central Vietnam (Nguyen, 2000; De *et al.*, 2003; Doanh, Nawa, 2016), while *O. viverrini* is distributed in the central and south of Vietnam (De, 2004; Thu *et al.*, 2007). De *et al.* (2003) estimated that more than one million people were infected with small liver flukes in the whole country, however Doanh, Nawa (2016) claimed that the human cases were over-estimated because of mis-identification of parasites in copro-parasitological examination. Nevertheless, both species constitute an important problem to human and animal health in Vietnam (Nguyen *et al.*, 2015).

In Vietnam, Nguyen *et al*. (2018) found three liver fluke species from cats from slaughterhouses in Northern Vietnam, e.g. *C. sinensis*, *O. viverrini* and *Platynosomum fastosum*, and both prevalence and intensity of infection in non-local cats were significantly higher than in local cats. The transportation of cats, therefore, could result in spread of *O. viverrini* and other parasites. The finding of common hepatic trematodes and also the

present of *O. viverrini* in these cats as an evident to the public health administration, therefore the transportation of cats for human consumption should be under veterinary control.

In addition, hepatic trematodes from different geographic regions might display molecular divergence which might be used to determine the origin of the final host (cat). Among all target sequences used for phylogenetic and diagnostic studies to elucidate interspecies and intraspecies relationship among closely related genera in many trematodes, the ITS1 rDNA is a useful genetic marker for examining taxonomic status and genetic diversity of *C. sinensis* (Park, Yong, 2001; Lee, Huh, 2004; Liu *et al.*, 2007; Sun *et al.* 2011; Tatonova *et al.*, 2012). In addition, previous investigations of *C. sinensis* have revealed that despite the lower variability compared with mitochondrial markers, the distribution of nucleotide substitutions along the ITS1 region reflects the geographic expansion of this parasite (Sun *et al.* 2011; Tatonova *et al.*, 2012). There are ITS1 rDNA data for *C. sinensis* from different areas (Tatonova *et al.*, 2012; Sun *et al.*, 2013). Vietnam is the southernmost area for this parasite and previously, *C. sinensis* from Vietnam has been investigated using mitochondrial *cox1* gene (Chelomina *et al.*, 2014), while there are no data on the ITS1 marker in this area. Also, *O. viverrini* from Vietnam has not been analysed using the ITS1 rDNA marker.

This study attempts to determine the genetic features of two human opisthorchiid species in cats sampled in Northern Vietnam.

MATERIALS AND METHODS

Sample collection

Most of trematode specimens in this study were taken from Department of Parasitology, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. One trematode specimen was randomly selected from different cats that were positive for small liver flukes. These were 26 *C. sinensis* samples (5 from local and 21 from non-local cats), and 3 *O. viverrini*. In addition, one specimen of *C. sinensis* obtained from a human patient in Nam Dinh province (provided by Dr. Do Trung Dung, Head of Parasitology Department, National Institute of Malariology, Parasitology and Entomology, Hanoi, Vietnam) was analysed.

DNA extraction, PCR, and sequencing

Trematode DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Germany). Partial fragments of ITS1–5.8S rDNA were amplified using the following primer pair 18SF: 5'–CCT GGT AAG TGC AAG TCA GAT GC–3' and 5.8SR: 5'–CAT GGC CGC AAT ATG CTT GCA–3'(designed in program Primer-BLAST). Amplification of the partial sequence of ITS1–5.8S was performed in a total volume of 20μL and contained 0.25 mM of each primer, 1μL of DNA, 2x Taq PCR Mastermix (Promega, USA) on an Eppendorf Mastercycle. Negative and positive controls were used. The PCR products of all samples were sent to First Base company (www.base.asia.com) for sequencing using BD1primer 5'–TCGTAACAAGGTTTCCGTA–3' (Morgan, Blair, 1995). The results of sequencing were checked by eye using Chromas software.

When more than one variant of the ribosomal sequence found, the purified PCR products were cloned with the InsT/Aclone PCR Product Cloning Kit (Fermentas) according to the manufacturer's protocol. The clones were amplified and sequenced with the universal M13-F (−20) (5′-GTAAAACGACGGCCAGT-3′) and M13-R(−24) (5′-AACAGCTATGACCATG-3′) primers. All newly obtained ITS1–5.8S partial sequences of small liver fluke were deposited in GenBank database (Table 1).

Analyses of genetic data

The ITS1 rDNA sequences of *O. viverrini* from GenBank (Laos: EU038141–EU038143, EU038148, EU038149, EU038153; Russia: KC427198, KC427199), were used for comparisons with own data. Estimates of evolutionary divergence and genetic diversity were calculated by MEGA 6.0.6 (Tamura *et al.*, 2013) using maximum composite likelihood model.

RESULTS

Sequence analyses

Among 30 partial sequences of ITS1–5.8S rDNA from 27 *C. sinensis* specimens, the length varied from 761 to 766 bp. The length of the complete ITS1 rDNA region ranged from 656–661 bp. The different length of the ITS1 regions was caused by additional repeat of 5 nucleotides, GCCTG. This insertion was present in six ITS1

sequences (20%). Two of three specimens with double peak had simultaneously clones with 5 bp insertion and without it. There are no substitutions downstream of the 5 bp insertion. A total of 3 polymorphic sites were detected for ITS1 sequences of *C. sinensis*: 1 transition C → T in the position 114 bp, 1 transition T → C in the position 339 bp, and 1 transversion G ↔ C in the position 507 bp.

Double peaks were obtained for all polymorphic sites. Clones of one specimen (Ci14-1 and Ci14-2) differed between all three sites. The most of direct and cloning sequences (77%) had in 114 bp position cytosine, while the 339 bp position was presented by Thymine (T) in 90% of the sequences. 87% of all sequences had Guanine (G) in 507 bp position. All sequences with cytosine in this position had also 5 bp-insertion, however, for two another sequences with insertion Cytosine (C) in 507 bp position was not detected (Fig. 1A). Different ratio of nucleotide in polymorphic sites with presence/absence of insertion leads to the existence of six genotypes in the investigated sample (Fig. 1B). The major genotype was obtained in 70% of sequences, other genotypes were presented by 1–3 sequences. Nucleotide diversity in whole sample of *C. sinensis* was 0.00122±0.00073.

Partial sequences of ITS1–5.8S rDNA of *O. viverrini* were 745 bp in length, and no differences were observed within three sequences. Differences were not either obtained between ITS1 rDNA sequences of *O. viverrini* samples in this study and sequences from Russia. The representatives of *O. viverrini* from Laos slightly differ from both Russian and Vietnamese (this study) samples (0.3%), but only one substitution was parsimony informative and no fixed substitutions were between different localities of parasite.

The neighbor-joining phylogram inferred from ITS1 comprised two lineages of dicrocoeliid (outgroup) and opisthorchiid species (Fig. 2). In the lineage of opisthorchiids, 3 sequences of *O. viverrini* and 30 sequences of *C. sinensis,* respectively, formed two separately monophyletic clades.

Table 1. The partial ITS1–5.8S rDNA sequences of hepatic trematodes analysed in the present study.

	Notation	Host	Origin (province)	GenBank accession number
C. sinensis	Csh	Human	Nam Dinh	KX378009
C. sinensis	Csd1	Cat	Thai Binh	KX377983
C. sinensis	Csd2	Cat	Thai Binh	KX377982
C. sinensis	[a]Csd3-1	Cat	Nam Dinh	KX378005
C. sinensis	[a]Csd3-2	Cat	Nam Dinh	KX378006
C. sinensis	Csd5	Cat	Nam Dinh	KX377989
C. sinensis	Csd6	Cat	Nam Dinh	KX377990
C. sinensis	Ci1	Cat	unknown	KX377991
C. sinensis	Ci2	Cat	unknown	KX377986
C. sinensis	Ci4	Cat	unknown	KX377988
C. sinensis	Ci5	Cat	unknown	KX377992
C. sinensis	Ci11	Cat	unknown	KX377987
C. sinensis	Ci12	Cat	unknown	KX377985
C. sinensis	Ci13	Cat	unknown	KX377980
C. sinensis	[a]Ci14-1	Cat	unknown	KX378012
C. sinensis	[a]Ci14-2	Cat	unknown	KX378007
C. sinensis	Ci16	Cat	unknown	KX377984
C. sinensis	Ci18	Cat	unknown	KX377993
C. sinensis	Ci19	Cat	unknown	KX377994
C. sinensis	[a]Ci23-1	Cat	unknown	KX377981
C. sinensis	[a]Ci23-2	Cat	unknown	KX378004
C. sinensis	Ci24	Cat	unknown	KX377995
C. sinensis	Ci32	Cat	unknown	KX377996
C. sinensis	Ci72	Cat	unknown	KX377997
C. sinensis	Ci102	Cat	unknown	KX377998
C. sinensis	Ci111	Cat	unknown	KX377999
C. sinensis	Ci121	Cat	unknown	KX378000
C. sinensis	Ci122	Cat	unknown	KX378001
C. sinensis	Ci131	Cat	unknown	KX378002
C. sinensis	Ci132	Cat	unknown	KX378003
O. viverrini	OP1	Cat	unknown	KX378003
O. viverrini	OP2	Cat	unknown	KX378011
O. viverrini	OP3	Cat	unknown	KX378012

[a]The clone sequence

A

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        13555555
        13011111
        49756789
Csh     CTG-----  R1
Csd1    ...-----  R1
Csd2    ...-----  R1
Csd3-1  ...-----  R1
Csd3-2  T.CGCCTG  R2
Csd6    ...-----  R1
Csd5    ...-----  R1
Ci1     ...-----  R1
Ci2     T..-----  R3
Ci4     ...-----  R1
Ci5     ...-----  R1
Ci11    ...-----  R1
Ci12    ...-----  R1
Ci13    ...-----  R1
Ci14-1  ...-----  R1
Ci14-2  TCCGCCTG  R4
Ci16    ...-----  R1
Ci18    ...-----  R1
Ci19    T.CGCCTG  R2
Ci23-1  TCCGCCTG  R4
Ci23-2  .C.GCCTG  R5
Ci24    ...-----  R1
Ci32    ...-----  R1
Ci72    T..-----  R3
Ci102   ...GCCTG  R6
Ci111   ...-----  R1
Ci121   ...-----  R1
Ci122   ...-----  R1
Ci131   ...-----  R1
Ci132   T..-----  R3
```

B

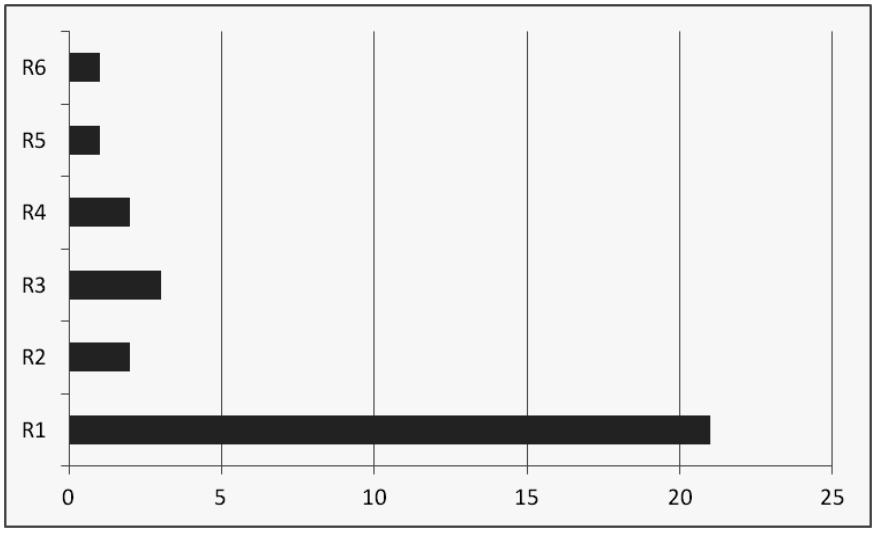

Figure 1. Variable sites (A) and ribotypes (B) for complete ITS1 sequences of *C. sinensis*; the notations of sequences are listed in Table 1; Rn is number of ribotype; *n* is number of sequences in rybotype; the sequences of clones are in bold.

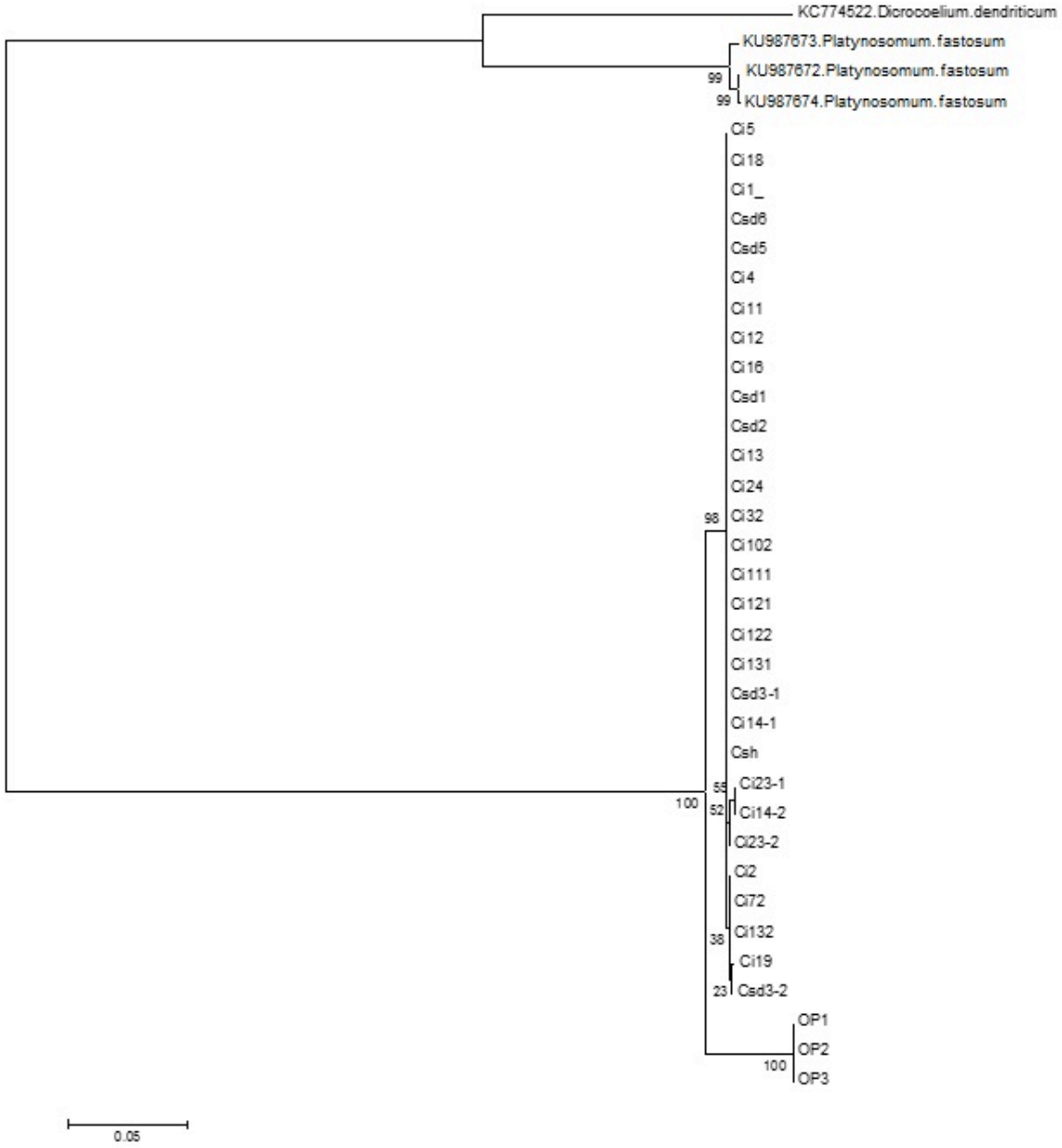

Figure 2. A neighbor-joining phylogram inferred from ITS1 rDNA sequences of hepatic trematodes in cats from slaughter houses and in human.

DISCUSSION

Although 30 ITS1 sequences were analysed for *C. sinensis* from different host species or origins of hosts, only 3 polymorphic sites were found. This result is supported by Park, Young (2001) when they found the high homogeneity in 18S rDNA, ITS2 and mitochondrial CO1 genes of three populations of *C. sinensis* from Korea and China. Nevertheless, we obtained new data about intragenomic polymorphism in ITS1 region. Tatonova *et al.* (2012) described for *C. sinensis* the intragenomic polymorphism in 114 bp position of ITS1 sequences, but double peaks in 339 and 507 bp were detected for the first time.

Moreover, by cloning the whole length, polymorphism was revealed for two samples. This can be explained by the fact that ribosomal DNA sequences are clusters of tandem repeats. Although these repeats are usually conservative in one individual organism in consequence of homogenization and fixation processes in this multigene family (Tatonova *et al.*, 2012), the variation in ITS regions were found for some trematodes, such as *Paragonimus westermani* or *Schistosoma japonicum* (van Herwerden *et al.*, 1998; 1999). Some variants of internal transcribed spacers can positively influence on the adaption of parasites, particularly, 5-bp repeat reduces the folding energy for ITS1 secondary structure of ITS1 (Tatonova *et al.*, 2012). Presence of both length variants in one genome obtained in our study increases the plasticity of the parasite. In addition, the ITS1 sequences from Southern China usually have substitutions downstream of the 5 bp insertion (Tatonova *et al.*, 2012), while in our study we obtained substitution only upstream of the additional five-nucleotide repeat.

Sun *et al.* (2013) found low divergence of *C. sinensis* based on multilocus analysis, although 271 ITS1 sequences were divided into three clusters; cluster I isolated from 17 northern provinces from China, and from Japan, Korea; cluster II isolated from 2 central provinces, Henan and Hubei; cluster III isolated from central and southern provinces near Vietnam. Unfortunately, these clusters on the phylogenetic tree were not statistically supported. Interestingly we found that nucleotide diversity of the Vietnamese ITS1 sequences was lower than in Chinese and Korean populations of *C. sinensis* (Sun *et al.*, 2013) and slightly higher than that in *C. sinensis* populations from Russia (Tatonova *et al.*, 2012). As to the zoogeographical origin of *C. sinensis* in "local cat", we propose that this fluke strain in Vietnam belongs to the "northern" group of the Chinese sub-region according to the arrangement of substitutions in ITS1. The "northern" group of *C. sinensis* includes only substitutions upstream of the 5bp-insertion (Tatonova *et al.*, 2012). Sun *et al.* (2013) did not describe genetic diversity of *C. sinensis* from samples in the Yunnan province (the Sino–Vietnamese border). In our opinion, the cats infected by *C. sinensis* can be transported from a yet uninvestigated area of China. It is possible this province has ITS1 sequences from "northern" group of China. In local origins of Northern Vietnam, the natural distribution of *C. sinensis* from neighboring south part of China (Guangxi province) can be limited by Hoang Lien Son Mountain.

We did not find any genetic variation among the few samples of *O. viverrini*, while Kang *et al.* (2008) mentioned that the ITS1 sequences of *O. viverrini* are more variable than that of *C. sinensis*. These authors have revealed six genotypes in their analyses of 13 ITS1 sequences of *O. viverrini* from Laos. It is interesting that sequences for samples from Russia (Brusentsov, Katokhin, 2017) and from this study were the same, but all these sequences belong to the most common genotype in Laos. This is possibly due to the spread of this common genotype to neighboring territories. In Vietnam, the ITS2 and *cox1* gene were used for investigating *O. viverrini* (Vo *et al.*, 2014; Dao *et al.*, 2016), this is the first time the ITS1 rDNA fragment has been used to describe the genetic characters of this fluke species.

As Nguyen *et al.* (2018) mentioned that the origin of the cats are unable to get detailed information, so we recommend further genetic studies to clarify their origin.

In conclusion, we detected 3 polymorphic sites, indel and new sites with intragenomic polymorphism from the ITS1–5.8S rDNA sequences of *C. sinensis*in different hosts and localities. The length of partial ITS1–5.8S rDNA sequences of *C. sinensis* varied from 761 to 766 bp. Partial sequences of ITS1–5.8S rDNA of *O. viverrini* were 745 bp in length. The nucleotide diversity in the ITS1–5.8S rDNA sequences varied significantly among the Vietnamese, Chinese, Korean and Russian populations of *C. sinensis* up-to-date investigated.

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DỮ LIỆU VỀ ĐOẠN GEN NHÂN ITS1-5.8S rDNA CỦA SÁN LÁ GAN NHỎ (OPISTHORCHIIDAE) Ở MÈO TẠI MIỀN BẮC VIỆT NAM

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TÓM TẮT

Mèo nhà và người đều là vật chủ chính của nhiều loài sán lá gan nhỏ thuộc họ Opisthorchiidae. Ở Việt Nam, có hai loài sán lá gan nhỏ ký sinh ở người được ghi nhận là *Clonorchis sinensis* (Cobbold, 1875) Looss, 1907 và *Opisthorchis viverrini* (Poirier, 1886) Stiles & Hassal, 1896. Nghiên cứu được thực hiện nhằm tìm hiểu mức độ biến đổi của vùng gen nhân ri bô xôm (rDNA) của *C. sinensis* và *O. viverrini* mẫu thu từ mèo nhà ở miền Bắc Việt Nam. Chiều dài đoạn gen ITS1-5.8S rDNA của loài *C. sinensis* thu từ các điểm khác nhau và các vật chủ khác nhau dao động trong khoảng 761-766 bp. Kết quả phân tích dữ liệu gen cho thấy sự đồng nhất 100% của đoạn 5.8S rDNA ở tất cả các mẫu *C. sineneis*. Ngược lại, sự biến đổi về di truyền chỉ thể hiện trong đoạn ITS1, gồm có 3 vị trí đa hình, thêm hay bớt nucleotide ở ngay trong đoạn gen được phát hiện. Chiều dài của đoạn ITS1 dao động từ 656-661 bp, không có biến đổi nào phía sau của đoạn gen tính từ điểm thêm 5 nucleotit. Có 6 kiểu gen ở các mẫu nghiên cứu, được hình thành do có hay không 5 nucleotit thêm vào và do sự khác biệt về trật tự nucleotit ở các điểm đa hình trên đoạn gen. Kiểu gen phổ biến nhất chiếm 70% tổng số mẫu nghiên cứu, các kiểu gen còn lại chỉ phát hiện ở 1-3 chuỗi gen. Sự đa dạng nucleotit trong toàn bộ mẫu *C. sinensis* là 0.00122 ± 0.00073. Chiều dài đoạn ITS1–5.8S rDNA của *O. viverrini* là 745 bp, và không có sự khác biệt nào giữa các chuỗi. Ba đoạn gen của *O. viverrini* và 30 đoạn gen của *C. sinensis* tạo thành hai nhánh tách biệt có cùng gốc trong cây chủng loại phát sinh.

Từ khóa: *Clonorchis sinensis*; *Opisthorchis viverrini*; sán lá gan nhỏ; ITS1; Việt Nam