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Cytochrome C Oxidase Subunit 1, Internal Transcribed Spacer 1, Nicotinamide Adenine Dinucleotide Hydrogen Dehydrogenase Subunits 2 and 5 of *Clonorchis sinensis* Ancient DNA Retrieved from Joseon Dynasty Mummy Specimens

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ABSTRACT

We analyzed *Clonorchis sinensis* ancient DNA (aDNA) acquired from the specimens of the Joseon mummies. The target regions were cytochrome C oxidase subunit 1 (CO1), internal transcribed spacer 1 (ITS1), nicotinamide adenine dinucleotide hydrogen (NADH) dehydrogenase subunits 2 (NAD2) and 5 (NAD5). The sequences of *C. sinensis* aDNA was completely or almost identical to modern *C. sinensis* sequences in GenBank. We also found that ITS1, NAD2 and NAD5 could be good markers for molecular diagnosis between *C. sinensis* and the other trematode parasite species. The current result could improve our knowledge about genetic history of *C. sinensis*.

Keywords: *Clonorchis sinensis*; Ancient DNA; Phylogenetic Analysis; Mummies; Republic of Korea

Clonorchis sinensis infects approximately 35 million people worldwide, causing various subclinical or clinical signs known as clonorchiasis.[1-5] People are infected by ingestion of undercooked or raw freshwater fish harboring metacercariae of *C. sinensis*.[6-8] In a historical context, *C. sinensis* infection was one of the most common trematode infections in Korea, especially due to the cuisine based on raw fish, which was enjoyed by the inhabitants of the country.[9-11]

To reveal the genetic characteristics of *C. sinensis*, researchers have attempted DNA analysis. Recently, parasitologists diagnosed *C. sinensis* through DNA analysis on internal transcribed

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Disclosure

The authors have no potential conflicts of interest to disclose.

Author Contributions

Conceptualization: Shin DH, Seo M.
Data curation: Hong JH, Seo M, Shin DH.
Investigation: Hong JH, Oh CS, Seo M. Writing - original draft: Hong JH, Seo M, Shin DH.
Writing - review & editing: Hong JH, Chai JY, Seo M, Shin DH.

spacer (ITS),[12-15] cytochrome C oxidase subunit (CO),[5,10] and nicotinamide adenine dinucleotide hydrogen dehydrogenase (NAD) subunits.[5,12] The molecular analyses claimed that *C. sinensis* is genetically distinct from other trematodes.[1,12-16]

Meanwhile, paleoparasitologists have also tried to reveal the genetic characteristics of ancient *C. sinensis* through research on the samples collected at archeological sites. One of such studies was carried out in Korea. Shin et al.[17] successfully analyzed ancient DNA (aDNA) sequences of *C. sinensis* eggs collected from the 17th century Korean mummy feces. They showed that amplified sequences of *C. sinensis* ITS1, ITS2 and CO1 were completely identical to modern *C. sinensis* sequences in GenBank.[17]

Although this pioneering work was to reveal genetic traits of ancient *C. sinensis*, the number of aDNA cases reported so far was too insufficient to get detailed information of ancient *C. sinensis* genetics. Fortunately, by paleoparasitological studies in Korea over the past several years, we collected a number of pre-modern Korean mummy feces or tissue specimens in which the presence of ancient *Clonorchis* eggs was microscopically confirmed.[18,19] Utilizing the ancient specimens, in this study, we analyzed CO1, ITS1, NAD2 and NAD5 of *C. sinensis* aDNA. The current report could expand the spatiotemporal scope of parasitological research about the genetic history of *C. sinensis*.

The samples used in this study were obtained from the 16th to 17th century Joseon mummies (n = 5; Andong, Cheongdo, Dalsung, Hadong1 and Mungyeong) (**Table 1**, **Supplementary Figs. 1** and **2**). The specimens were coprolites retrieved from mummy intestines (Andong, Dalsung, and Hadong1) or mummified livers (Cheongdo and Mungyeong). We followed the *Criteria of Authentication* for authentic aDNA analysis.[20]

For aDNA extraction, we followed the method in our previous report.[21] The specimens (0.3 g) were treated in a lysis buffer (1 mL) for 24 hours at 56°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then chloroform/isoamyl alcohol (24:1). DNA isolation/purification was performed by a QIAmp PCR purification kit (Qiagen, Hilden, Germany). Extract DNA (10 μL) was treated with 1 unit of uracil-DNA-glycosylase (New England Biolabs, Ipswich, MA, USA) for 30 minutes at 37°C. It (40 ng) was then mixed with a reagent premix containing 10 pmol of each primer (**Table 2**) and 1X AmpliTaq Gold® 360 Master Mix (Life Technologies, Camarillo, CA, USA). PCR conditions were as follows: pre-denaturation at 95°C for 10 minutes; 45 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C–63°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. The amplified PCR products separated on 2.5% agarose gel (Invitrogen, Waltham, MA, USA) were stained by ethidium bromide. Electrophoresis also included negative (extraction) controls.

Table 1. The archaeological information of Korean mummies in this study

Cases	Estimated date, century	Sample condition	Sample type	Gender
Andong	16	Mummy	Coprolites	M
Cheongdo	17	Mummy	Mummified liver	M
Dalsung	16–17	Mummy	Coprolites	W
Hadong1	17	Half mummified	Coprolites	W
Mungyeong	17	Mummy	Mummified liver	W

M = men, W = women.

Table 2. List of primers used for the amplification of *C. sinensis* DNA in this study

DNA region	Set	Primers	Sequence, 5′ to 3′	Annealing Temp., °C	Length, bp
CO1	CO1	CO1 F	GTG TTA ATA TTG CCG GGG TTT GG	54	207
		CO1 R	ACC TAT AAT CAT AGT AAC CG		
ITS1	ITS1-2	ITS1 F2	CTG GCA CGT GTA CCC AAT A	56	122
		ITS1 R2	TCA CCC CCA ATA TGG ACT		
	ITS1-3	ITS1 F3	TCG GTA TGC TCG CTT CCG TTG	62	151
		ITS1 R3	CGG TTT GAA ATG AAC AAC AA		
	ITS1-4	ITS1 F4	GAG TGG GCA TGA TGT GTC TC	63	215
		ITS1 R4	GGC GTT ATC AGT CGT ACC CGG		
NAD2	NAD2-1	NAD2 F1	GCT ATG TTG TTG TTT CTG GTG	56	194
		NAD2 R1	ACG ACC TCT TCA AAA TGG TT		
	NAD2-2	NAD2 F2	TGA AGT TTG GTC TTT TTC CA	54	260
		NAD2 R2	TGA TGC ACT GGA ACT AAT CA		
	NAD2-3	NAD2 F3	TGG GGG TTT AAC GTT TAT TT	56	195
		NAD2 R3	CTC AGC AAC ATA ACC ACC AT		
	NAD2-4	NAD2 F4	GAG CTT TCT CCT GAT TTG CT	56	164
		NAD2 R4	ATG GAT AAA GAC CCT GGA AA		
	NAD2-5	NAD2 F5	CCG CAG TTG GGA TAT ATT TT	54	159
		NAD2 R5	ATA AAA CTG CTC CGA AAT GC		
NAD5	NAD5-1	NAD5 F1	GAT GCC GTC CTT GAT ATT TT	54	164
		NAD5 R1	CCC AAT TCT GAA AAT GAC CT		
	NAD5-2	NAD5 F2	TGC TAA ACC TCG GAG TAT GC	58	191
		NAD5 R2	CCA CCA ACC AGG AAA TAA AT		
	NAD5-3	NAD5 F3	CAG AAT TGG GTT GGT ATG TG	56	200
		NAD5 R3	CCC CTG ATA GCA GAA TAA CG		
	NAD5-4	NAD5 F4	CCC CAG TTA GTT GTT TGG TT	56	211
		NAD5 R4	GCA ACA TTT TTG CAG GTA GA		

CO = cytochrome C oxidase subunit, ITS = internal transcribed spacer, NAD = nicotinamide adenine dinucleotide hydrogen dehydrogenase subunits.

The PCR amplicon was isolated by a QIAquick Gel Extraction Kit (Qiagen). Bacterial transformation was done using a pGEM-T Easy Vector system (Promega Corporation, Madison, WI, USA). Transformed bacteria were then grown on agar plate containing X-GAL (40 μg/μL), ampicillin (50 μg/mL) and 0.5 mM IPTG for 14 hours. After colonies were grown in LB media for 12 hours, the cultured bacteria were purified by a QIAprep® Spin Miniprep kit (Qiagen). Each amplified DNA strand was sequenced by an ABI Prism BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, MA, USA) and 3730xl Automatic Sequencer (Applied Biosystems).

To obtain consensus sequence, multiple sequence alignment was performed for each aDNA region by Clustal W implemented in MEGA7.[22] We compared the consensus sequences of ancient *C. sinensis* to GenBank taxa by NCBI/BLAST tools.[23] The evolutionary relationship of ancient *C. sinensis* and other parasites of NCBI GenBank was inferred by the *Phylogeny Reconstruction* analysis implemented in MEGA7. We used Maximum Likelihood method. Selected parameters are Tamura 3-parameter[24] (CO1), Kimura 2-parameter[25] (ITS1), Hasegawa-Kishino-Yano model[26] (NAD2 and 5) for Model/Method. We performed bootstrap test to estimate the reliability of the tree. The number of bootstrap replicates was 500.[27]

To select the specimens used for aDNA analysis, we screened all the mummy coprolite samples using PCR with *C. sinensis* primers for CO1 (206 bp), ITS1-2 (122 bp), NAD2-1 (194 bp) and NAD5-1 (164 bp). In agarose gel electrophoresis, negative (extraction) controls exhibited no amplified bands. In Andong feces, the PCR products were detected for *C. sinensis* CO1, ITS1-2, NAD2-1 and NAD5-1. Mungyeong specimen also showed positive results for *C. sinensis* CO1

and ITS1-2 (**Supplementary Fig. 3**). We thus used the Andong and Mungyeong specimens for subsequent aDNA analysis.

To get the consensus aDNA sequences of *C. sinensis* CO1, ITS1, NAD2 and NAD5, we tried to do cloning and sequencing of each specific amplicon. By these trials, 9–10 clone sequences were successfully acquired for CO1, ITS1, NAD2 and NAD5 amplicons (**Supplementary Fig. 4**). The total sizes of consensus sequences obtained by multiple sequence alignment were 162 bp (CO1), 431 bp (ITS1), 588 bp (NAD2) and 443 bp (NAD5), respectively. The *C. sinensis* consensus sequences of Andong and Mungyeong specimens were almost the same to each other, except for a little difference at a nucleotide position (transversions occurred in the positions CO1: 100 and ITS1: 167) (**Fig. 1**). Considering these results, we conjecture that genetic characteristics of ancient *C. sinensis* might not have been uniform during the Joseon period.

In BLAST searching, the *C. sinensis* consensus sequences of Andong and Mungyeong specimens were completely or almost identical to *C. sinensis* CO1, ITS1, NAD2 and NAD 5 sequences reported in GenBank (**Table 3** and **Fig. 1**). Briefly, the current ancient *C. sinensis* CO1 sequences were 100% identical to GenBank sequences of *C. sinensis* reported from Korea (KY564177.1), China (FJ965391.1; FJ965383.1; AF188122.2; AF184619.2), Russia (MF406205.1; MF406204.1), and Vietnam (MF287785.1; KJ204609.1). *C. sinensis* ITS1 sequences of Korean mummies also exhibited very high similarities (99%) to the GenBank ITS1 sequences reported from Korea (JN638318.1; JN638320.1), China (KJ137226.1; KF740425.1; HQ186255), Russia (JQ048578.1; KC987517.1) and Vietnam (MF319655.1; MF319650.1; MF319653.1). The aDNA sequences of Andong and Mungyeong specimens were also completely or almost (99%) identical to GenBank *C. sinensis* NAD2 and NAD sequences from Korea (NAD2, JF729304.1; NAD5, FJ729304.1), China (NAD2, KC170192.1; NAD5, KY564177.1), Russia (NAD2, FJ381664.2; NAD5, FJ381664.1) and Vietnam (NAD2, AY264851.1) (**Table 3** and **Fig. 1**).

In the analyses, we found that CO1 region could not be an effective marker for differential diagnosis between *C. sinensis* and other trematode species because the CO1 sequences of *Pygidiopsis summa* (AF184884.3) and *Trichinella spiralis* (AF182302.1) were not distinguishable from *C. sinensis* CO1 sequences. Meanwhile, *C. sinensis* ITS1, NAD2 and NAD5 sequences were clearly distinct from those of other trematode species (**Fig. 1**). We identified similar patterns in phylogenetic analyses (**Fig. 2**). In case of CO1, ancient Andong and Mungyeong sequences belonged to the clade not only with *C. sinensis*, but also with *P. summa* and *M. xanthosomus*. On the other hand, ITS1, NAD2 and NAD5 of *C. sinensis* and other trematode species were separately clustered into different clades (**Fig. 2**). Actually, previous studies proposed that the interspecific sequence variations within zoonotic trematodes were observed for ITS1, NAD2 and NAD5.[13] In this study, we re-confirmed the usefulness of ITS1, NAD2 and NAD5 as molecular markers for differential diagnosis of *C. sinensis* from other trematode species.

In summary, our present study about *C. sinensis* aDNA retrieved from Korean mummies is designed to uncover invaluable genetic information of *C. sinensis* prevalent among pre-20th century Korean people. Although detailed understanding of *C. sinensis* genetics require a future retrieval of ancient or modern DNA sequences in wider geo-historical scope, our current report represent a significant step to improve our knowledge about genetic history of *C. sinensis*.

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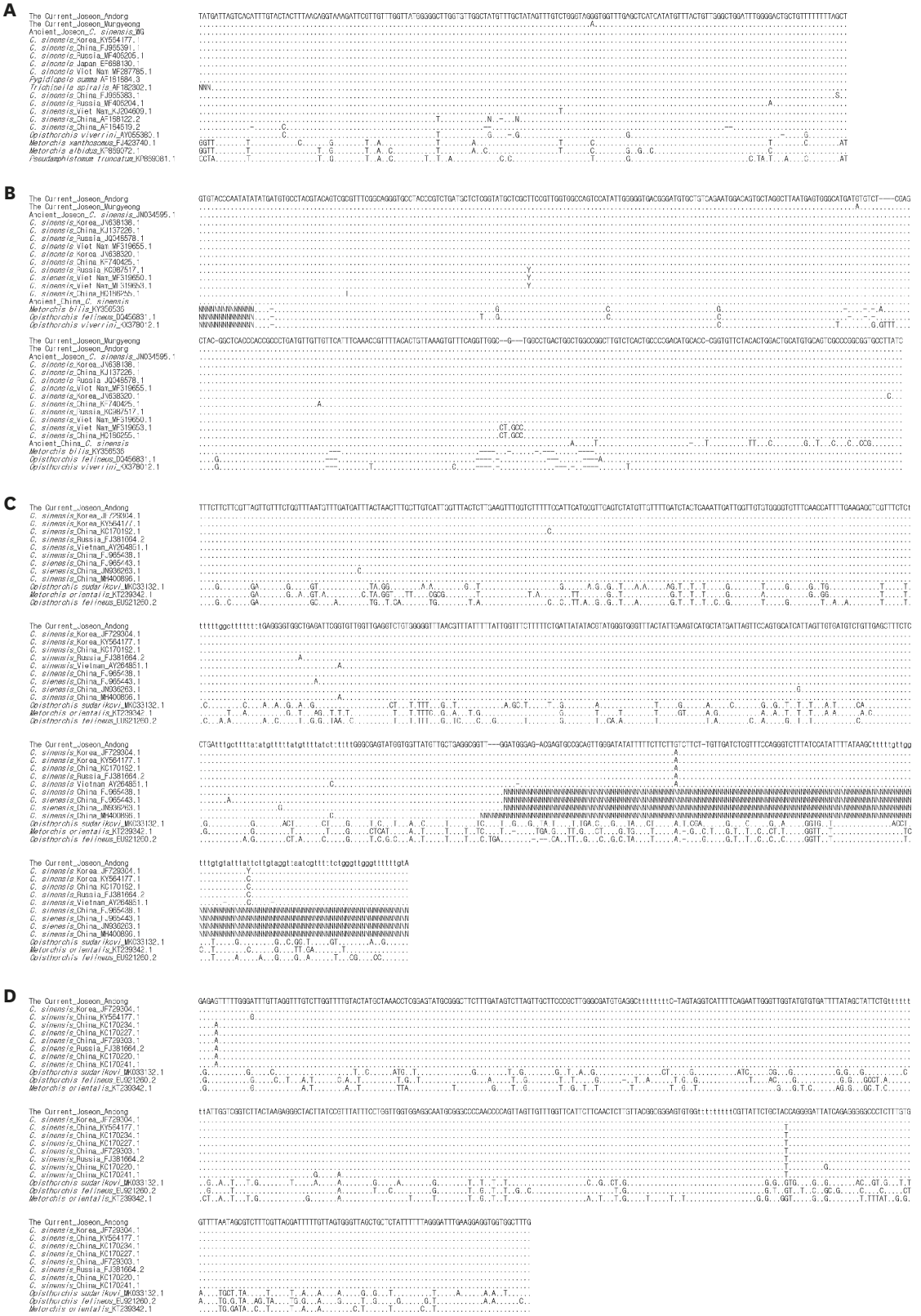

Fig. 1. BLAST analyses of the consensus DNA sequences from ancient *C. sinensis* and other sequences in GenBank. (**A**) CO1, (**B**) ITS1, (**C**) NAD2, and (**D**) NAD5 DNA regions.
CO = cytochrome C oxidase subunit, ITS = internal transcribed spacer, NAD = nicotinamide adenine dinucleotide hydrogen dehydrogenase subunits.

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Table 3. BLAST searching results of ancient *C. sinensis* CO1, ITS1, NAD2, and NAD5 consensus sequences obtained from Andong mummy

DNA region	Species	Coverage, %	Percent identity, %	Accession No.	Geographical region
CO1	*C. sinensis*	100	100	KY564177.1	Korea
		100	100	MF287785.1	Vietnam
		100	100	MF406205.1	Russia
		100	100	FJ965391.1	China
		100	100	EF688130.1	Japan
		100	99	FJ654383.1	China
		100	99	KJ204609.1	Vietnam
		100	99	JX040566.1	Russia
		100	99	JF729304.1	Korea
		100	98	MF406206.1	Russia
		100	97	AF188122.2	China
		100	96	AF184619.2	China
	P. summa	100	100	AF181884.3	Korea
	T. spiralis	98	100	AF182302.1	Unknown
	O. viverrini	100	95	AY055380.1	Laos
	M. xanthosomus	96	93	FJ423740.1	Unknown
ITS1	*C. sinensis*	100	100	JN638318.1	Korea
		100	100	MF319655.1	Vietnam
		100	100	KJ137226.1	China
		100	100	JQ048578.1	Russia
		100	99	JN638320.1	Korea
		100	99	KF740425.1	China
		100	99	MF319650.1	Vietnam
		100	99	KC987517.1	Russia
		100	98	MF319653.1	Vietnam
		100	98	HQ186255.1	China
	M. bilis	95	93	KY356536.1	Russia
	O. felineus	95	93	DQ456831.1	Russia
	O. viverrini	94	91	KX378012.1	Vietnam
NAD2	*C. sinensis*	100	99	JK729304.1	Korea
		100	99	KC170213.1	China
		100	99	FJ381664.2	Russia
		100	98	AY264851.1	Vietnam
	O. sudarikovi	100	77	MK033132.1	Pakistan
	M. orientalis	100	76	KT239342.1	China
	O. felineus	100	76	EU921260.2	Russia
NAD5	*C. sinensis*	100	100	JF729304.1	Korea
		100	99	KY564177.1	China
		100	99	FJ381664.2	Russia
	O. sudarikovi	99	81	MK033132.1	Pakistan
	M. orientalis	99	81	KT239342.1	China
	O. felineus	98	80	EU921260.2	Russia

CO = cytochrome C oxidase subunit, ITS = internal transcribed spacer, NAD = nicotinamide adenine dinucleotide hydrogen dehydrogenase subunits.

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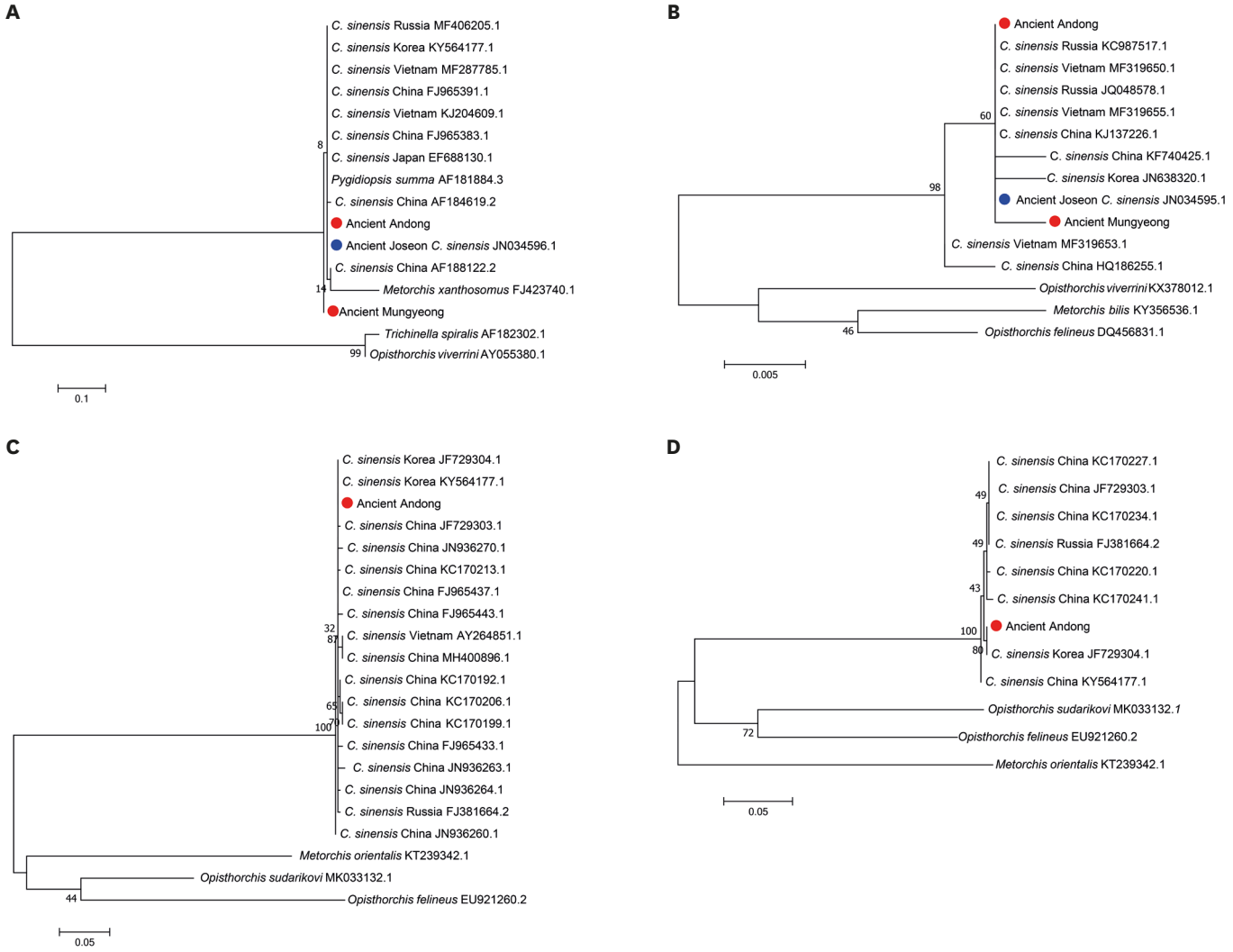

Fig. 2. Phylogenetic analyses (Maximum Likelihood method) of the current ancient *C. sinensis* (red dots) and the other trematodes in GenBank. (**A**) CO1, (**B**) ITS1, (**C**) NAD2, and (**D**) NAD5 DNA regions. Blue dots, ancient *C. sinensis* sequence previously reported by Shin et al.[17]
CO = cytochrome C oxidase subunit, ITS = internal transcribed spacer, NAD = nicotinamide adenine dinucleotide hydrogen dehydrogenase subunits.

SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

The map of Korea. Red dots represent the sites where the mummies of the current studies were found. 1 = Andong, 2 = Cheongdo, 3 = Dalsung, 4 = Hadong1, 5 = Mungyeong.

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Supplementary Fig. 2

The archaeological information of Korean mummy specimens used for this study. (**A**) The tomb of Joseon period. (**B**) A mummy (Andong) used in this study.

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Supplementary Fig. 3

Agarose gel electrophoresis of the PCR products amplified from ancient *C. sinensis* samples. Specific bands were indicated by arrows.

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Supplementary Fig. 4

Aligned clone sequences of CO1, ITS1, NAD2 and 5 DNA fragments from Joseon Dynasty mummies. (**A**) Andong mummy, (**B**) Mungyeong mummy.

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REFERENCES

1. Park GM, Yong TS. Geographical variation of the liver fluke, *Clonorchis sinensis*, from Korea and China based on the karyotypes, zymodeme and DNA sequences. *Southeast Asian J Trop Med Public Health* 2001;32 Suppl 2:12-6.
PUBMED
2. Kaewkes S. Taxonomy and biology of liver flukes. *Acta Trop* 2003;88(3):177-86.
PUBMED | CROSSREF
3. Lun ZR, Gasser RB, Lai DH, Li AX, Zhu XQ, Yu XB, et al. Clonorchiasis: a key foodborne zoonosis in China. *Lancet Infect Dis* 2005;5(1):31-41.
PUBMED | CROSSREF
4. Keiser J, Utzinger J. Emerging foodborne trematodiasis. *Emerg Infect Dis* 2005;11(10):1507-14.
PUBMED | CROSSREF
5. Sun J, Huang Y, Huang H, Liang P, Wang X, Mao Q, et al. Low divergence of *Clonorchis sinensis* in China based on multilocus analysis. *PLoS One* 2013;8(6):e67006.
PUBMED | CROSSREF
6. Kang SY, Ahn IY, Park CY, Chung YB, Hong ST, Kong Y, et al. *Clonorchis sinensis*: molecular cloning and characterization of 28-kDa glutathione S-transferase. *Exp Parasitol* 2001;97(4):186-95.
PUBMED | CROSSREF
7. Yu SH, Masanori K, Li XM, Xu LQ, Lan CG, Lin R. Epidemiological investigation on *Clonorchis sinensis* in human population in an area of South China. *Jpn J Infect Dis* 2003;56(4):168-71.
PUBMED
8. Lim JU, Joo KR, Shin HP, Cha JM, Lee JI, Lim SJ. Obstructive jaundice caused by Clonorchiasis-associated duodenal papillitis: a case report. *J Korean Med Sci* 2011;26(1):135-7.
PUBMED | CROSSREF
9. Seo M, Shin DH, Guk SM, Oh CS, Lee EJ, Shin MH, et al. *Gymnophalloides seoi* eggs from the stool of a 17th century female mummy found in Hadong, Republic of Korea. *J Parasitol* 2008;94(2):467-72.
PUBMED | CROSSREF
10. Ki HC, Shin DH, Seo M, Chai JY. Infection patterns of trematode parasites among Joseon people. *J Korean Med Assoc* 2014;57(10):866-75.
CROSSREF
11. Jeong YI, Shin HE, Lee SE, Cheun HI, Ju JW, Kim JY, et al. Prevalence of *Clonorchis sinensis* infection among residents along 5 major rivers in the Republic of Korea. *Korean J Parasitol* 2016;54(2):215-9.
PUBMED | CROSSREF
12. Lee SU, Huh S. Variation of nuclear and mitochondrial DNAs in Korean and Chinese isolates of *Clonorchis sinensis*. *Korean J Parasitol* 2004;42(3):145-8.
PUBMED | CROSSREF
13. Xiao JY, Gao JF, Cai LS, Dai Y, Yang CJ, Luo L, et al. Genetic variation among *Clonorchis sinensis* isolates from different hosts and geographical locations revealed by sequence analysis of mitochondrial and ribosomal DNA regions. *Mitochondrial DNA* 2013;24(5):559-64.
PUBMED | CROSSREF
14. Tatonova YV, Chelomina GN, Nguyen HM. Inter-individual and intragenomic variations in the ITS region of *Clonorchis sinensis* (Trematoda: Opisthorchiidae) from Russia and Vietnam. *Infect Genet Evol* 2017;55:350-7.
PUBMED | CROSSREF

15. Liu WQ, Liu J, Zhang JH, Long XC, Lei JH, Li YL. Comparison of ancient and modern *Clonorchis sinensis* based on ITS1 and ITS2 sequences. *Acta Trop* 2007;101(2):91-4.
PUBMED | CROSSREF
16. Park GM. Genetic comparison of liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, based on rDNA and mtDNA gene sequences. *Parasitol Res* 2007;100(2):351-7.
PUBMED | CROSSREF
17. Shin DH, Oh CS, Lee HJ, Chai JY, Lee SJ, Hong DW, et al. Ancient DNA analysis on *Clonorchis sinensis* eggs remained in samples from medieval Korean mummy. *J Archaeol Sci* 2013;40(1):211-6.
CROSSREF
18. Seo M, Oh CS, Chai JY, Jeong MS, Hong SW, Seo YM, et al. The changing pattern of parasitic infection among Korean populations by paleoparasitological study of Joseon Dynasty mummies. *J Parasitol* 2014;100(1):147-50.
PUBMED | CROSSREF
19. Seo M, Oh CS, Hong JH, Chai JY, Cha SC, Bang Y, et al. Estimation of parasite infection prevalence of Joseon people by paleoparasitological data updates from the ancient feces of pre-modern Korean mummies. *Anthropol Sci* 2017;125(1):9-14.
CROSSREF
20. Hofreiter M, Serre D, Poinar HN, Kuch M, Pääbo S. Ancient DNA. *Nat Rev Genet* 2001;2(5):353-9.
PUBMED | CROSSREF
21. Kim YS, Oh CS, Lee SJ, Park JB, Kim MJ, Shin DH. Sex determination of Joseon people skeletons based on anatomical, cultural and molecular biological clues. *Ann Anat* 2011;193(6):539-43.
PUBMED | CROSSREF
22. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33(7):1870-4.
PUBMED | CROSSREF
23. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25(17):3389-402.
PUBMED | CROSSREF
24. Tamura K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol Biol Evol* 1992;9(4):678-87.
PUBMED
25. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980;16(2):111-20.
PUBMED | CROSSREF
26. Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* 1985;22(2):160-74.
PUBMED | CROSSREF
27. Hall BG. Building phylogenetic trees from molecular data with MEGA. *Mol Biol Evol* 2013;30(5):1229-35.
PUBMED | CROSSREF