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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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), cytochrome C oxidase subunit (CO), and nicotinamide adenine dinucleotide hydrogen dehydrogenase (NAD) subunits. The molecular analyses claimed that C. sinensis is genetically distinct from other trematodes.

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. 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. 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. 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.

For aDNA extraction, we followed the method in our previous report. 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.

| 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.
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. We compared the consensus sequences of ancient *C. sinensis* to GenBank taxa by NCBI/BLAST tools. 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 (CO1), Kimura 2-parameter (ITS1), Hasegawa-Kishino-Yano model (NAD2 and NAD5) for Model/Method. We performed bootstrap test to estimate the reliability of the tree. The number of bootstrap replicates was 500.

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.

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 ACC TAT CAT AGT ACG CG | 54 | 207 |
| ITS1       | ITS1-2 | ITS1 F2 | CTG GCA CGT GTA CCC AAT A TCA CCC CCA ATA TGG ACT | 56 | 122 |
| ITS1       | ITS1-3 | ITS1 F3 | TGG GTA TGC TCG CTT CCG TTG CCG TTT GAA ATG ACG AAC AA | 62 | 151 |
| ITS1       | ITS1-4 | ITS1 F4 | GAG TGG GCA TGA TGT GTC TC GGC GGT ATC AGT CGT ACC CGG | 63 | 215 |
| NAD2       | NAD2-1 | NAD2 F1 | GCT ATG TTG TTG TTG CTG TTG ATG ACC ACC TCT TCA AAA TGG TT | 56 | 194 |
| NAD2       | NAD2-2 | NAD2 F2 | TGA AGT TTG GTC TTT TTT TCA TGA TGC ACT GGA ACT AAT CA | 54 | 260 |
| NAD2       | NAD2-3 | NAD2 F3 | TGG GGG TTT AAC GTT TAT TT CTC AGC AAT AT ACC ACC AT | 56 | 195 |
| NAD2       | NAD2-4 | NAD2 F4 | GAG CTT TCT CCT GAT TTG CT ATG GAT AAA GAC CCT GGA AA | 56 | 164 |
| NAD2       | NAD2-5 | NAD2 F5 | CCG CAG TTG GGA TAT ATT TAA GGA AAT CAT CTC CCA CCA AAT AT | 54 | 159 |
| NAD5       | NAD5-1 | NAD5 F1 | GAT GCC GTC CTT GAT ATT TT | 54 | 164 |
| NAD5       | NAD5-2 | NAD5 F2 | TGC TAA ACC TCG GAT GCA ACC AAT ACT | 58 | 191 |
| NAD5       | NAD5-3 | NAD5 F3 | CAG AAT TGG GGT GAT AG TGC TTG CTC ATA GCA GAA TAA CG | 56 | 200 |
| NAD5       | NAD5-4 | NAD5 F4 | CCC CAG TTA GGT TTG TTG TTG TGA ACA AAT TTG TCA CCA GCA GGA TAA CG | 56 | 211 |

CO = cytochrome C oxidase subunit, ITS = internal transcribed spacer, NAD = nicotinamide adenine dinucleotide hydrogen dehydrogenase subunits.
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 NAD5 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; AY188122.2; AY184619.2), Russia (MF406205.1; MF406204.1), and Vietnam (MF287785.1; KJ104609.1). *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; KF404425.1; 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 NAD5 sequences from Korea (NAD2, JF729304.1; NAD5, FJ729304.1), China (NAD2, KJ170192.1; 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. 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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Clonorchis sinensis DNA from Joseon Mummy Specimens

Fig. 1. BLAST analyses of the consensus DNA sequences from ancient C. sinensis and other sequences in GenBank. (A) COI, (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.
Table 3. BLAST searching results of ancient *C. sinensis* CO1, ITS1, NAD2, and NAD5 consensus sequences obtained from Andong mummy DNA region

| DNA region | Species            | Coverage, % | Percent identity, % | Accession No. | Geographical region |
|------------|--------------------|-------------|---------------------|---------------|---------------------|
| CO1        | *C. sinensis*      | 100         | 100                 | KY564177.1    | Korea               |
|            |                    | 100         | 100                 | MF387785.1    | Vietnam             |
|            |                    | 100         | 100                 | MF406205.1    | Russia              |
|            |                    | 100         | 100                 | FJ965391.1    | China               |
|            |                    | 100         | 99                  | EF688130.1    | Japan               |
|            |                    | 100         | 99                  | FJ654383.1    | China               |
|            |                    | 100         | 99                  | KJ024609.1    | Vietnam             |
|            |                    | 100         | 99                  | JX040566.1    | Russia              |
|            |                    | 100         | 99                  | JF729304.1    | Korea               |
|            |                    | 100         | 98                  | MF406206.1    | Russia              |
|            |                    | 100         | 97                  | AF188122.2    | China               |
|            |                    | 96          | 96                  | AF184619.2    | China               |

|            | *P. summa*         | 100         | 100                 | AF181184.3    | 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                 | MF316655.1    | Vietnam             |
|            |                    | 100         | 100                 | KJ137226.1    | China               |
|            |                    | 100         | 99                  | JQ46578.1     | Russia              |
|            |                    | 100         | 99                  | JN638320.1    | Korea               |
|            |                    | 100         | 99                  | KF740425.1    | China               |
|            |                    | 100         | 99                  | MF319650.1    | Vietnam             |
|            |                    | 100         | 98                  | MF319653.1    | Vietnam             |
|            |                    | 100         | 98                  | HJ166255.1    | China               |
|            | *M. bilis*         | 95          | 93                  | KY356536.1    | Russia              |
|            | *O. felineus*      | 95          | 93                  | DQ465683.1    | 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                  | MK033123.1    | Pakistan            |
|            | *M. orientalis*    | 100         | 76                  | KT239342.1    | China               |
|            | *O. felineus*      | 100         | 76                  | EU921260.2    | Russia              |

| NAD5       | *C. sinensis*      | 100         | 100                 | JK729304.1    | Korea               |
|            |                    | 100         | 99                  | KY564177.1    | China               |
|            |                    | 100         | 99                  | FJ381664.2    | Russia              |
|            | *O. sudarikovi*    | 99          | 81                  | MK033123.1    | 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.
**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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