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▣ BRIEF COMMUNICATION

Monitoring of *Fasciola* Species Contamination in Water Dropwort by *COX1* Mitochondrial and ITS-2 rDNA Sequencing Analysis

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Abstract: Fascioliasis, a food-borne trematode zoonosis, is a disease primarily in cattle and sheep and occasionally in humans. Water dropwort (*Oenanthe javanica*), an aquatic perennial herb, is a common second intermediate host of *Fasciola*, and the fresh stems and leaves are widely used as a seasoning in the Korean diet. However, no information regarding *Fasciola* species contamination in water dropwort is available. Here, we collected 500 samples of water dropwort in 3 areas in Korea during February and March 2015, and the water dropwort contamination of *Fasciola* species was monitored by DNA sequencing analysis of the *Fasciola hepatica* and *Fasciola gigantica* specific mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear ribosomal internal transcribed spacer 2 (ITS-2). Among the 500 samples assessed, the presence of *F. hepatica cox1* and 1TS-2 markers were detected in 2 samples, and *F. hepatica* contamination was confirmed by sequencing analysis. The nucleotide sequences of *cox1* PCR products from the 2 *F. hepatica*-contaminated samples were 96.5% identical to the *F. hepatica cox1* sequences in GenBank, whereas *F. gigantica cox1* sequences were 46.8% similar with the sequence detected from the *cox1* positive samples. However, *F. gigantica cox1* and ITS-2 markers were not detected by PCR in the 500 samples of water dropwort. Collectively, in this survey of the water dropwort contamination with *Fasciola* species, very low prevalence of *F. hepatica* contamination was detected in the samples.

Key words: *Fasciola* species, water dropwort, *cox1*, ITS-2, DNA sequencing analysis

Fascioliasis is a zoonosis caused by *Fasciola hepatica* and *Fasciola gigantica*, 2 trematode species of the genus *Fasciola*, prevalent in cattle and emerging as a cause of disease in humans. Humans are infected mainly by ingesting raw water plants that are contaminated with the metacercariae [1]. Several reports have indicated that water plants such as watercress, rice, dandelion, *Nasturtium*, and *Mentha* spp. harbor *Fasciola* metacercariae [2].

Water dropwort (*Oenanthe javanica*) is a perennial herb with a distinctive aroma and is cultivated in marshy areas of Asia and Australia. The fresh stems and leaves are used as a salad or as a seasoning in soups and stews in Korea [3]. Water dropwort has also been used in Korea as a folk medicine for the treatment of jaundice, hypertension, fever, abdominal pain, leucorrhea, mumps, and urinary difficulty [4]. In a biological hazard analysis of the water dropwort, it was reported that *Escherichia coli* was detected in samples of the herb collected from water dropwort fields [5]. However, there has been no information on *Fasciola* species contamination in water dropwort. Here, to obtain basic information regarding *Fasciola* species contamination in water dropwort in Korea, we collected a total of 500 samples from 3 areas, and evaluated *Fasciola* species contamination by mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear ribosomal internal transcribed spacer 2 (ITS-2) DNA sequencing analysis.

Water dropwort samples were obtained between February and March 2015. A total of 500 samples were collected, and the lower parts of water dropwort was initially examined using a stereomicroscope (×10 magnification, Zeiss, Oberkochen, Germany). Next, we determined the presence of *cox1* and ITS-2 genes of *Fasciola* species in each sample using PCR amplification. Briefly, the surface of the lower 20 cm of the water dropwort stem was peeled using a sterile scalpel, and genomic DNA was isolated using a G-DEX™ genomic DNA extraction

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kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Genomic DNA isolated from an adult *F. hepatica* worm (Prof. Sung-Jong Hong, Chung-Ang University, kindly provided) and adult *F. gigantica* worm (Prof. Keeseon S. Eom and Hyeong-Kyu Jeon, Chungbuk National University, kindly provided) were used as a positive control. The primers used for PCR amplification are listed in Table 1. The PCR mixture for the PCR amplification contained 5 µl genomic DNA, 3 µl each of forward and reverse primers, 4 µl dNTP, 5 µl 10× Ex Taq buffer, 0.25 µl Ex Taq polymerase, and 29.75 µl DDW. PCR assays were performed with an initial denaturation step of 94˚C for 30 sec, followed by 30 cycles of denaturation at 98˚C for 10 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec, followed by 1 cycle at 72˚C for 10 min and a final hold at 4˚C. Amplifications were generated using a TaKaRa PCR Thermal Cycler (Takara Bio Inc., Otus, Japan). Agarose gel electrophoresis (1.5%) with ethidium bromide staining was used to visualize the ITS-2 PCR products.

Furthermore, to identify the sequence of the PCR products from *Fasciola*-contaminated water dropwort, we purified the PCR products. Briefly, after electrophoretic separation, the *cox1* and ITS-2 PCR products were clearly delineated and sequenced directly by SolGent (Daejeon, Korea). The sequence of PCR products were compared with the complete *cox1* and *ITS-2* sequences of *F. hepatica* obtained from GenBank (accession no. GU112476.1 and AJ272053.1, respectively) using Clone Manager software (Sci-Ed Software, Cary, North Carolina, USA). Also, the sequence of PCR products were compared with the

Table 2. Results for the detection of the *cox1* and ITS-2 genes of *F. hepatica* or *F. gigantica* from water dropwort by PCR

Areas	No. of samples	No. of PCR positive samples (%)	
		F. hepatica	*F. gigantica*
A	150	0 (0.0)	0 (0.0)
B	200	1 (0.5)	0 (0.0)
C	150	1 (0.67)	0 (0.0)
Total	500	2 (0.4)	0 (0.0)

Table 1. Primers used for detection of *Fasciola hepatica* and *F. gigantica* from water dropwort in Korea

Target name	Oligonucleotide sequence (5'-3')	Product size (bp)	GenBank accession No.
Fasciola hepatica COX1	F: TTTGCCTGGGTTTGGAGTTA R: CCACACAACAGGATCCCATA	283	GU112476.1
Fasciola hepatica ITS-2	F: GTTATAAACTATCACGACGCCCAAA R: GAAGACAGACCACGAAGGGTA	364	AJ272053.1
Fasciola gigantica COX1	F: GGTCTTTGGGGTGGATTTTT R: GTCCAACCAACACCCATACC	308	AB983838.1
Fasciola gigantica ITS-2	F: TATCACGACGCCCAAAAAGT R: CCAAGTTCAGCATCAAACCA	300	EU260059.1

COX1, mitochondrial cytochrome *c* oxidase subunit 1; ITS-2, nuclear ribosomal internal transcribed spacer 2.

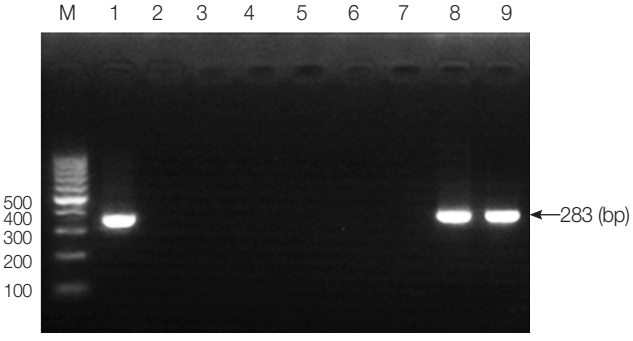

Fig. 1. Agarose gel electrophoresis of PCR products containing the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) marker of *Fasciola hepatica*. M, 100 bp marker; lane 1, positive control (adult *F. hepatica* worm); lanes 2-7, *F. hepatica* negative samples of water dropwort; lane 8; No. 11 *F. hepatica* positive sample of water dropwort, lane 9; No. 18 *F. hepatica* positive sample of water dropwort.

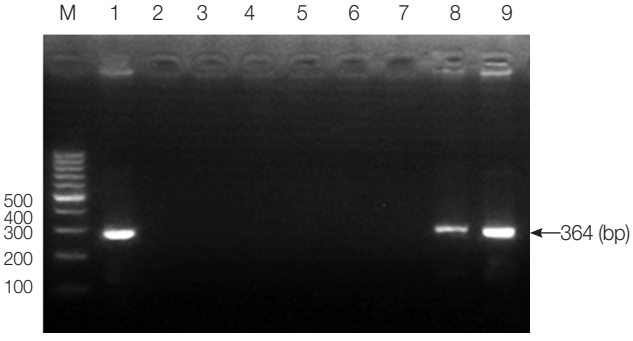

Fig. 2. Agarose gel electrophoresis of PCR products containing the nuclear ribosomal internal transcribed spacer 2 (ITS-2) marker of *F. hepatica*. M, 100 bp marker; lane 1, positive control (adult *F. hepatica* worm); lanes 2-7, *F. hepatica* negative samples of water dropwort; lane 8; No. 11 *F. hepatica* positive sample of water dropwort, lane 9; No. 18 *F. hepatica* positive sample of water dropwort.

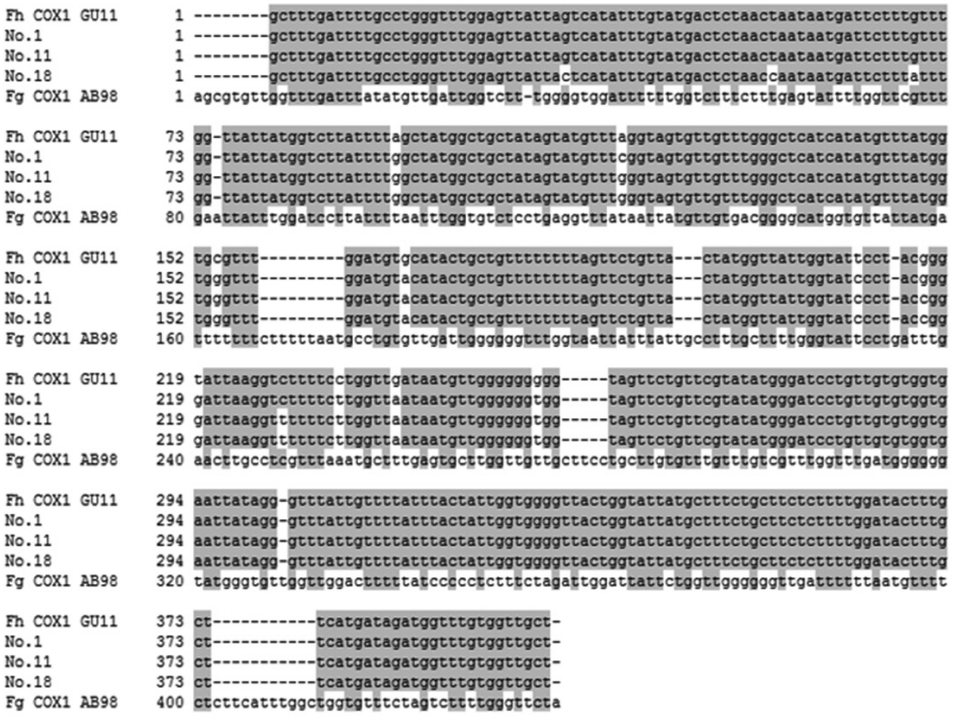

Fig. 3. *F. hepatica cox1* nucleotide sequences of 2 positive samples obtained from PCR products compared with a GenBank sequence (accession no. GU112476.1). Base homologies are indicated by a dot (·); base changes are shown in orange. Fh *COX1* GU11, *F. hepatica cox1* GenBank sequence (accession no. GU112476.1); No. 1, positive control (adult *F. hepatica* worm); No. 11, No. 11 *F. hepatica* positive sample of water dropwort; No. 18, No. 18 *F. hepatica* positive sample of water dropwort; Fg *COX1* AB98, *F. gigantica cox1* GenBank sequence (accession no. AB983838.1).

complete *cox1* and *ITS-2* sequences of *F. gigantica* obtained from GenBank (accession no. ab983838.1 and EU260059.1, respectively).

We tried to detect the metacercariae of *Fasciola* species from the surface of water dropwort using a stereomicroscope. However, metacercariae were not observed at the stack of water dropwort. Next, we performed PCR analysis on the 500 specimens of water dropwort to monitor *Fasciola* species contamination. Among the 500 specimens collected from 3 areas, *cox1* and ITS-2 bands of *F. hepatica* were detected in 2 specimens (Table 2; Figs. 1, 2), which were exactly consistent with the PCR amplification of the positive control, adult *F. hepatica* DNA (283 bp for *cox1* and 346 bp for ITS-2). We also performed PCR analysis to evaluate *F. gigantica* contamination of water dropwort using *F. gignatica cox1* and ITS-2 gene primers. However, the *F. gigantica cox1* and ITS-2 markers were not detected by PCR using 500 water dropwort (data not shown). To confirm whether the positive PCR products were real *F. hepatica*, the complete DNA sequence of *cox1* and ITS-2 PCR products were compared with those of GenBank. The nucleotide sequences of the *cox1* PCR products from the 2 *F. hepatica*-contaminated samples were 96.5% identical to the *F. hepatica cox1* sequences of GenBank (accession no. GU112476.1; Fig. 3), whereas *F. gigantica cox1* sequences were 46.8% similar with *cox1* positive samples. The ITS-2 sequences of 2 PCR positive samples were 100% identical to those of GenBank (accession no. aj272053.1) and positive control sample (adult *F. hepatica* worm); however, *F. giganica* ITS-2 sequences were 97.5% identical to those of ITS-2 positive PCR samples (Fig. 4). Thus, *Fasciola* species PCR positive samples were confirmed to be *F. hepatica*, and the overall prevalence of *F. hepatica* infection in water dropwort was 0.4%, ranging from 0.0% to 0.67% depending on the collection area.

Fascioliasis in animals and humans is caused by *F. hepatica* and *F. gigantica*. It is difficult to accurately discriminate between 2 species because their size varies depending on the age of the fluke and species of the host [6-8]. PCR technology and DNA sequencing techniques facilitate species identification, clarification of strains, and genetic populations. Genes in the mitochondrial and nuclear DNA (the genes encoding ribo-

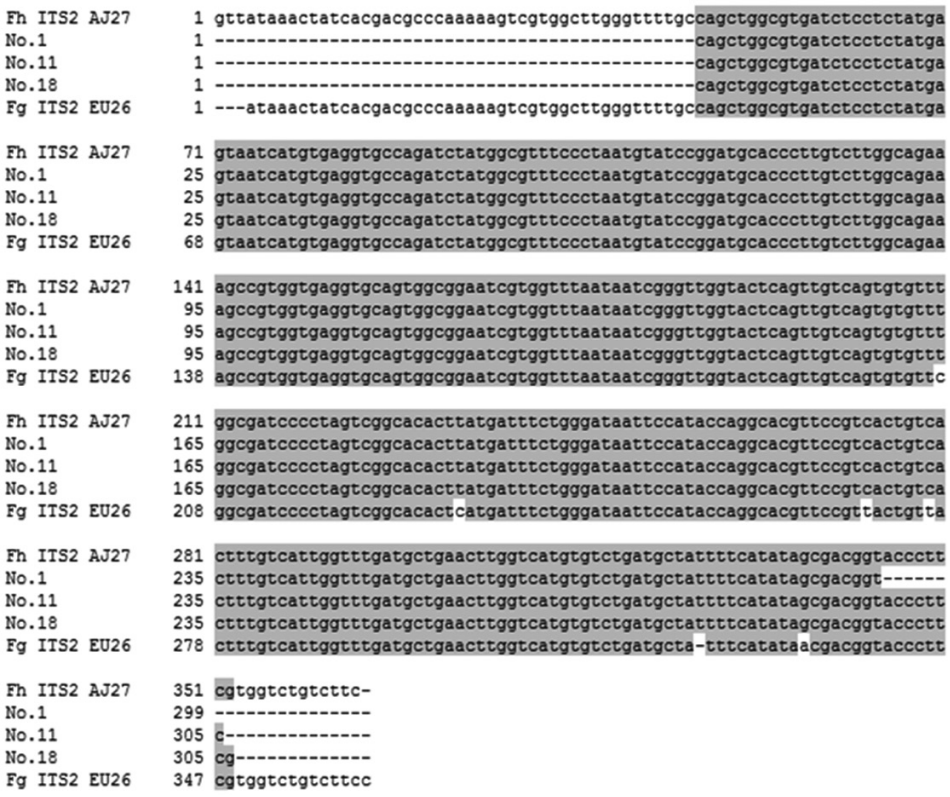

Fig. 4. *F. hepatica* ITS-2 nucleotide sequences of 2 positive samples obtained from PCR products compared with a GenBank sequence (accession no. AJ272053.1). Base homologies are indicated by a dot (·); base changes are shown in orange. Fh ITS-2 AJ27, *F. hepatica* ITS-2 GenBank sequence (accession no. AJ272053.1); No. 1, positive control (adult *F. hepatica* worm); No. 11, No. 11 *F. hepatica* positive sample of water dropwort; No. 18, No. 18 *F. hepatica* positive sample of water dropwort; Fg ITS-2 EU26, *F. gigantica* ITS-2 GenBank sequence (accession no. EU260059.1).

somal RNAs) have been used as marker(s) in population genetics and phylogeny for fasciolid classification [6-8]. The prevalence of fascioliasis was greatly reduced in the 2000s in Korea. However, human cases of *F. hepatica* infection have been continuously reported [9,10]. Humans and cattle are most commonly infected by ingestion of water plants contaminated with encysted metacercariae. Water dropwort is one of the major sources of *F. hepatica* infection in Korea [2,9]. In this study, the overall prevalence of *F. hepatica* infection in water dropwort was 0.4%, which was much lower than that of snails in water dropwort fields in Korea [11]. Moreover, the prevalence in this study was lower than that in watercress in France (1.2-2.4% annually) [12]. Sources of *F. hepatica* contamination in agricultural products include soil, feces, irrigation water, inadequately composted manure, wild and domestic animals, dirty equipment, and human handling [13]. Differences in prevalence may be induced by various factors such as host distribution, locality, and environmental conditions. In this study, we used the repetitive DNA sequences of *cox1* and ITS-2 regions specific for *F. hepatica* or *F. gigantica* to identify the species of genus *Fasciola* because these genes were used efficiently to identify liver fluke species collected from various hosts and geographic regions [6-8]. From this study, *F. hepatica cox1* and ITS-2 DNA were detected at 2 samples among 500 samples, but not *F. gigantica* contamination. These results were further confirmed by sequence analysis of positive PCR products in comparison to *cox1* and ITS-2 gene sequences of *F. hepatica* and *F. gigantica*.

Taken together, of 500 water dropwort samples, 2 water dropwort samples displayed the DNA bands of *F. hepatica* via PCR, and these findings were confirmed by sequencing analysis. This is the first study regarding parasitological examination of *Fasciola* species in water plants in Korea, suggesting that we need to improve the biosafety of aquatic plants during the pre- and postharvest periods.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

REFERENCES

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 2005; 35: 1255-1278.
2. Slifko TR, Smith HV, Rose JB. Emerging parasite zoonoses associated with water and food. Int J Parasitol 2000; 30: 1379-1393.
3. Seo WH, Baek HH. Identification of characteristic aroma active compounds from water dropwort (*Oenanthe javanica* DC). J Agri Food Chem 2005; 53: 6766-6700.
4. Yang SA, Jung YS, Lee SJ, Park SC, Kim MJ, Lee EJ, Byun HJ, Jhee KH, Lee SP. Hepatoprotective effects of fermented field water-dropwort (*Oenanthe javanica*) extract and its major constituents. Food Chem Toxicol 2014; 67: 154-160.
5. Kim YR, Lee KA, Choi IW, Lee YH, Kim SR, Kim WI, Ryu SH, Lee HS, Ryu JG, Kim HY. Investigation of microbial contamination in *Oenanthe javanica* at postharvest environments. J Food Hyg Safety 2014; 29: 268-277.
6. Itagaki T, Kikawa M, Terasaki K, Shibahara T, Fukuda K. Molecular characterization of parthenogenic *Fasciola* sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene. J Vet Med Sci 2005; 67: 1115-1118.
7. Choe SE, Nguyen TT, Kang TG, Kweon CH, Kang SW. Genetic analysis of *Fasciola* isolates from cattle in Korea based on second internal transcribed spacer (ITS-2) sequence of nuclear ribosomal DNA. Parasitol Res 2011; 10: 833-839.
8. Wannasan A, Khositharattanakool P, Chaiwong P, Piangjai S, Uparanukraw P, Morakote N. Identification of *Fasciola* species based on mitochondrial and nuclear DNA reveals the co-existence of intermediate *Fasciola* and *Fasciola gigantica* in Thailand. Exp Parasitol 2014; 146: 64-70.
9. Kim YH, Kang KJ, Kwon JH. Four cases of hepatic fascioliasis mimicking cholangiocarcinoma. Korean J Hepatol 2005; 11: 169-175.
10. Kang BK, Jung BK, Lee YS, Hwang IK, Lim H, Cho J, Hwang JH, Chai JY. A case of *Fasciola hepatica* infection mimicking cholangiocarcinoma and ITS-1 sequencing of the worm. Korean J Parasitol 2014; 52: 193-196.
11. Kim HY, Choi IW, Kim YR, Quan JH, Ismail HA, Cha GH, Hong SJ, Lee YH. *Fasciola hepatica* in snails collected from water-dropwort fields using PCR. Korean J Parasitol 2014; 52: 645-652.
12. Dreyfuss G, Vignoles P, Rondelaud D. *Fasciola hepatica*: epidemiological surveillance of natural watercress beds in central France. Parasitol Res 2005; 95: 278-282.
13. Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P, Frankel G. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ Microbiol 2010; 12: 2385-2397.