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A study on *Kudoa septempunctata* infection from sashimi and sushi of olive flounder *Paralichthys olivaceus* in Busan, South Korea

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Abstract

Kudoa septempunctata has been reported as a new parasite in aquacultured olive flounder *Paralichthys olivaceus*, and also as a causative agent of food poisoning in humans. This paper investigated the infection of *K. septempunctata* in 216 sashimi and 20 sushi made of olive flounders in Busan, Korea. Among 236 samples, *K. septempunctata* was detected in eleven sashimi with 6–7 polar capsules by the microscopy. Among eleven sashimi, five sashimi were positive in Polymerase Chain Reaction (PCR) assay with the targets of 18S rDNA and 28S rDNA. The genotype of all the five PCR results is identified as the genotype ST3 which is common in Korea. *K. septempunctata* was found in olive flounders sashimi from Samcheonpo and Wando outside of Jeju Island. These findings would contribute to establish the standard of *K. septempunctata* for preventing food-borne outbreaks in advance and providing further preventive management for the seafood safety.

Keywords: *Kudoa septempunctata*, Food poisoning, Olive flounder, ST3

Introduction

Kudoa septempunctata has been known to be a parasite on fish muscles, digestive tract and kidneys, and has 6–7 polar cysts per spore, but does not affect fish physiology or survival (Matsukane et al., 2010). Some studies have reported that eating raw fish infected with *K. septempunctata* causes food poisoning in humans (Ahn et al., 2015; Jang et al., 2016). Kawai et al. (2012) analyzed 113 food poisoning patients in eight prefectures of Japan in 2010, and reported that the main cause was olive flounders infected with *K. septempunctata*. Since *K. septempunctata* was also detected in the feces of patients associated with consumption of sliced raw olive flounders, the Ministry of Health, Labor, and Welfare of Japan declared the *K. septempunctata* as a new causative agent of foodborne disease, and the associated foodborne illness was named Kudoa food poisoning (Harada et al., 2012; Jeon & Kim, 2016), which has a shorter incubation period of 2–20 hours compared to other food poisoning-causing pathogens (Matsukane et al., 2010). The genotypes of *K. septempunctata* are classified as three types: ST1, ST2, and ST3. Both ST1

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and ST2 were found mainly in Japan, but ST3 is mostly known to be detected in Korea (Takeuchi et al., 2016). Some animal tests using Asian house shrew *Suncus murinus* found that 10^7 spores of *K. septempunctata* per 1 g, caused food poisoning symptoms such as vomiting and diarrhea (Kawai et al., 2012; Ohnishi et al., 2013). In the analysis on the residues of the food poisonings, more than 10^6 spores were found out of 1 g of olive flounders, and this concentration was set as the standard for food poisoning in Japan (Kawai et al., 2012).

So far some studies have been conducted to investigate the infection of *K. septempunctata* of olive flounders in Korea. Song et al. (2013) collected 143 olive flounders obtained from 26 fish farms in Jeju, and 4 samples out of 143 detected as *K. septempunctata* positive. Moreover, Song et al. (2014) collected 1,107 olive flounders from 89 fish farms in Jeju, Jeollanam-do, Gyeongnam, Gyeongbuk, and Gangwon-do in Korea, and 0.9% of the olive flounders in Jeju were only found to be *K. septempunctata* positive, but *K. septempunctata* were not detected in olive flounders in other regions. *K. septempunctata* infection was detected in olive flounders exported from Korea to Japan, so the Japanese government tightened quarantine inspection on olive flounders from Jeju in Korea, using 10^6 spores as the judgement standard (Jeon & Kim, 2016; Kim et al., 2015). As a result, the Ministry of Oceans and Fisheries of Korea has implemented Kudoa management measures for fish farms at the same level as Japan since April 2014. However, the actual concern is the food poisoning caused from eating sliced raw seafood such as sashimi or sushi with *K. septempunctata* infected. Not only in Korea but also in all of the world, the *K. septempunctata* standard for sashimi or sushi has not been established, which requires the management strategy for preventing the Kudoa food poisoning.

Within the authors' knowledge, the previous studies have focused mainly on *K. septempunctata* infection of olive flounders from fish farms, and there has been no research on *K. septempunctata*-infected sashimi or sushi that people can eat right away. Therefore, this study is to investigate the contamination status of *K. septempunctata* in sashimi and sushi in Busan, Korea, where eating the raw seafood is traditional food culture, and provide basic data necessary for preventing and managing Kudoa food poisoning.

Materials and Methods

Test samples

From January to December in 2020, 236 samples including 216 sashimi and 20 sushi made of olive flounders were collected from seafood markets and sashimi restaurants in Busan. The samples used in the study were purchased from the market by government employees of the Environmental Sanitation Department of 16 district offices in Busan. The samples were refrigerated and transported to the laboratory, and the experiments were conducted immediately.

Sample preprocessing and screening tests

According to the Korean Food Code, the *K. septempunctata* infection test in this paper is carried out as follows; (1) scratch the surface of the sashimi and sushi at least five places and obtain 1 g, (2) apply saline solution to it, (3) mash the bottom lightly with a flat surface, (4) centrifuge the solution through the mesh at 1,500×g at 4℃ for 15 minutes, (5) dispose of the top part of the solution and add saline to the sediment and mix it to obtain a homogenized solution as a test solution. DNA was extracted according to the manual of the Fast DNA Spin Kit for Soil (Mpbio, Santa Ana, CA, USA) manufacturer. The extracted DNA was inserted into the PowerCheck™ Kudoa Real-time PCR Kit (Kogenbiotech, Seoul, Korea) and the Kudoa Screening Test was performed using the Real-time PCR (ABI 7500 Fast, Alameda, CA, USA), which was also followed by the manufacturer's manual.

Microscopic examination

From the Real-time PCR results, if an amplification appears when the Ct value is less than 35, and if the quantitative value is 1.0×10^5 or higher, then we carried out a microscopic examination to check the spores of *K. septempunctata* as follows; (1) add 10 uL trypan blue solution to 10 uL test solution and mix it, (2) inject the obtained 20 uL solution into the hemocytometer, (3) examine the presence of Kudoa spores containing 6 to 7 cysts under a microscope (Carl zeiss, Oberkochen, Germany) and count them.

Genetic identification and genotype analysis

If the Real-time PCR test results show an amplification curve, then the nested PCR was carried out for detecting *K. septempunctata* using primers to detect 18S rDNA and 28S rDNA, according to Grabner et al. (2012) (Table 1). The target size was verified using Automatic Electrophoresis (QIAxcel advanced, Cologne, Germany), and we analyze the sequence obtained from the gene amplification PCR, and confirm whether it is equivalent to the standard strains of NCBI Blast (https://www.ncbi.nlm.nih.gov/Blast).

According to Takeuchi et al. (2015), we performed nested

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Table 1. Primer sequences

Target	Primer	Sequence (5´- 3´)
18S rDNA	Ks 18S 1st-F	GGTGGGAGCATTTATTAGACT
	Ks 18S 1st-R	AATCGAGACCACTGTCAAC
	Ks 18S 2nd-F	AGAAATACCGGAGTGGACCGTAAAATG
	Ks 18S 2nd-R	GTTCCATGCTATAACATTCAAGCGTTCG
28S rDNA	Ks 28S 1st-F	TGCGTAGTGAAGCGGGAAAA
	Ks 28S 1st-R	GTGTTTCAAGACGGGTCGG
	Ks 28S 2nd-F	GTGTGTGATCAGACTTGATATG
	Ks 28S 2nd-R	AAGCCAAAACTGCTGGCCATTT
Cytochrome coxidase subunit 1	cox1 1st-F	TTTGTTCATCGGCACAATTC
	cox1 1st-R	ATAGCCTGGAACAAGGAATC
	cox1 2nd-F	TATGGCAAAGAAGGTCTGAT
	cox1 2nd-R	TCTAGGGATTCCACAAAGAC
Large subunit rRNA	rnl 1st-F	TGCCGTCAATTCTGTTGTATT
	rnl 1st-R	AATACCCATGCTGTGTTCAT
	rnl 2nd-F	GTTCCAACAAGTCCATGAA
	rnl 2nd-R	GACTTTATGGACAACTCAGC

PCR for the genotype analysis of *K. septempunctata* under the conditions of 95℃ for 3 minutes, 35 cycles of 95℃ for 30 seconds, 55℃ for 30 seconds, 68℃ for 60 seconds, and 68℃ for 5 minutes (Table 1). The gene sequence from PCR results is aligned by using the ClustalX BioEdit program, the genotype analysis was carried out by comparing the sequence with the standard strain and the sequence variation at a certain size (Table 2).

Statistics analysis

For the statistical significance tests, the Chi-square test is conducted using SPSS 22.0 software (IBM, Armonk, NY, USA), which is determined to be statistically significant when the *p*-value is less than 0.05.

Results and Discussion

The genotype analysis for detected *K. septempunctata*

We conducted a Real-time PCR screening test on 236 samples of sashimi and sushi of the olive flounders. In order to perform the quantitative analysis, we made the calibration curve from the five standard reference materials for 1.0×10^7, 1.0×10^5, 1.0×10^4, 1.0×10^3 Kudoa DNA copy. As shown in Table 3, *K. septempunctata* was detected in 29 samples (13.4%) out of 216 sashimi, but not detected in sushi.

K. septempunctata was detected mainly in the summer, more specifically with 2 samples (0.8%) in June, 25 samples (10.6%) in July, and 2 samples (0.8%) in August. Using the Chi-square analysis, we confirmed statistically that the *K. septempunctata* detection rates vary significantly from month to month ($p = 0.000$). Kim et al. (2018) also found that Kudoa-related food poisoning cases in 2015 and 2016 were the most common in April, followed by October and November in Gyeonggi Province, and were the highest in May and followed by August in Korea as a whole. Conversely, Song et al. (2013) reported that *K. septempunctata* is detected throughout the year from January to December. Therefore, further research is needed on the monthly infection trend of *K. septempunctata*.

By analyzing the results of the Real-time PCR screening, we found that the number of copies of Kudoa rDNA in 29 samples varies from 5×10^3 to 2.6×10^8 per 1 g. By using the microscopy, we found *K. septempunctata* in 11 sashimi samples (Table 3). From the average value of the spores measured by four times in the hemocytometer, the number of the spores detected by *K. septempunctata* ranges from 1.0×10^4 to 9.0×10^5 spores per 1 g, all of which were less than 10^6 spores per 1 g of the standard for managing *K. septempunctata* in Japan.

Kudoa nested PCR results

We conducted the PCR test on eleven samples with *K. septempunctata* spores observed by comparing with the 18S rDNA and 28S rDNA of *K. septempunctata*, and found that the 18S rDNA target size is 333 bp and the 28S rDNA target size is 356 bp, which is a specific part of the *K. septempunctata* (Fig. 1). Among the eleven samples, five (2.1%) samples were determined to be positive with *K. septempunctata* genes for nested PCR with both 18S rDNA and 28S rDNA. The distribution of olive flounders by original region is three from Jeju, one from Samcheonpo,

Table 2. The genotypes of *Kudoa septempunctata*

Genotype[1)]		Target gene	Accession no.	Target gene	Accession no.
ST1	Combined type of	cox1-1	AB915831	rnl-1	AB915833
ST2	Combined type of	cox1-2	AB915830	rnl-2	AB915832
ST3	Combined type of	cox1-3	LC014799	rnl-2	AB915832

[1)] The Genotype of *K. septempuntata* is determined by the combination of the cox1 gene and rnl gene.

Table 3. Quantification of *Kudoa septempunctata* by real-time PCR screening test and microscopic examination

Month	No. of samples (no. of fish)	No. of *K. septempunctata* detected samples by real-time PCR (no. of fish)	Amounts by real-time PCR (Kudoa rDNA copy/g)		No. of *K. septempunctata* detected samples by microscopic examination (no. of fish)	Amounts by microscopic examination (spore/g)	
			Min	Max		Min	Max
Jan	2 (1)	-	-	-	-	-	-
Feb	3 (1)	-	-	-	-	-	-
Mar	6 (2)	-	-	-	-	-	-
Apr	12 (5)	-	-	-	-	-	-
May	14 (8)	-	-	-	-	-	-
Jun	34 (13)	2 (2)	5.0×10^3	1.5×10^5	-	-	-
Jul	41 (22)	25 (13)	6.3×10^3	2.6×10^8	11 (6)	1.0×10^4/g	9.0×10^5/g
Aug	35 (14)	2 (2)	2.9×10^5	2.2×10^8	-	-	-
Sep	38 (10)	-	-	-	-	-	-
Oct	27 (11)	-	-	-	-	-	-
Nov	20 (15)	-	-	-	-	-	-
Dec	4 (1)	-	-	-	-	-	-
Total	236 (103)	29 (17)	5.0×10^3	2.6×10^8	11 (6)	1.0×10^4/g	9.0×10^5/g

PCR, polymerase chain reaction.

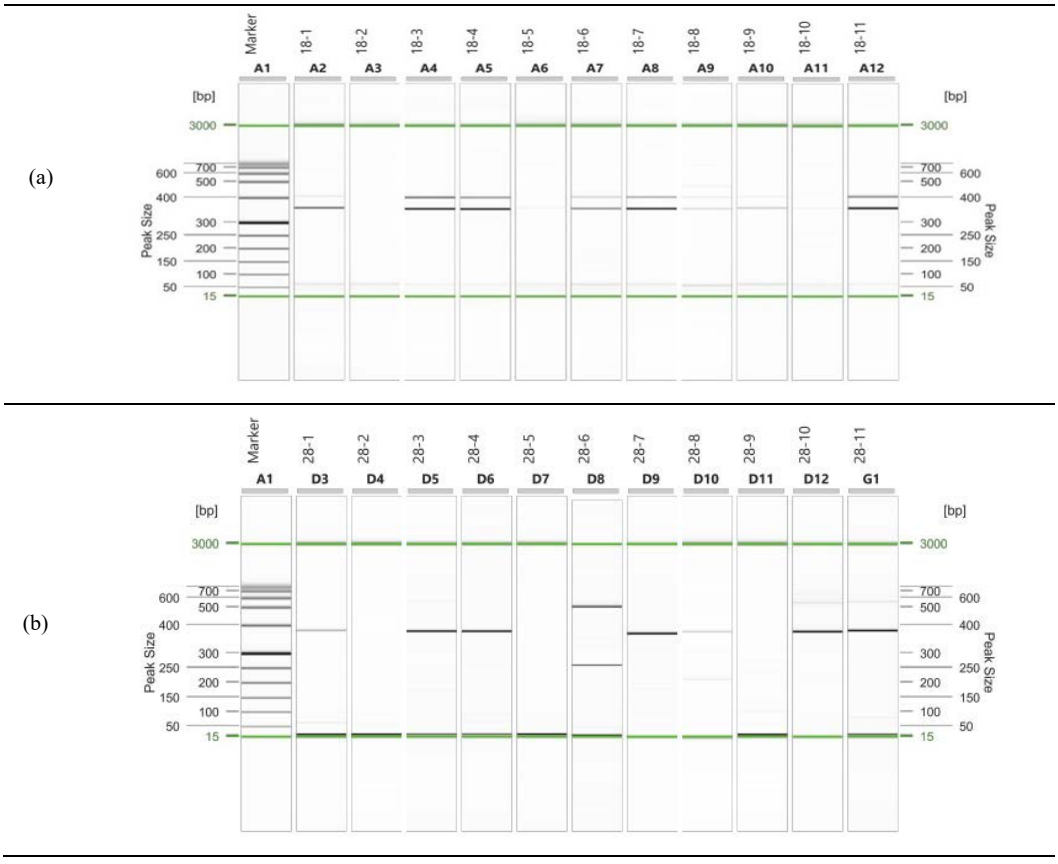

Fig. 1. 18S rDNA (a) and 28S rDNA (b) PCR results. The target size of 18S rDNA is 333bp (a) and the target size of 28S rDNA is 356 bp (b). The five samples (1, 3, 4, 7, 11) are positive for both 18S rDNA and 28S rDNA target of *Kudoa septempunctata*. PCR, polymerase chain reaction.

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and one from Wando (Table 4).

Song et al. (2013) surveyed 270 adult flounders and flounder fries from 26 fish farms in Jeju in 2012, and reported that 4 samples (2.8%) out of 143 adult flounders were detected with *K. septempunctata* genes. In addition, Song et al. (2014) collected 1,107 olive flounders from 89 fish farms in Jeju, Jeollanam-do, Gyeongnam, Gyeongbuk, and Gangwon-do, and reported that *K. septempunctata* genes were detected in 10 samples (3.1%) out of the 318 olive flounders in Jeju, but were not detected in other regions. Kim et al. (2015) surveyed 660 olive flounders in 11 fish seeding farms on the southwest coast in Korea between 2014 and 2015, and *K. septempunctata* genes were not detected. In contrast to previous papers, *K. septempunctata* were detected in the sashimi which made of olive flounders from Samcheonpo and Wando in addition to olive flounders from Jeju. In this study, a completely new result was found with the previous papers that *K. septempunctata* were detected only in olive flounders on Jeju. Therefore, further monitoring is needed for sashimi and sushi with olive flounders from other domestic regions.

Kudoa gene homogeneity and genotype analysis

In order to evaluate the gene consistency of *K. septempunctata* of the detected samples with the standard strains, we compared the gene sequence with 18S rDNA standard strains AB731754.1 and 28S rDNA standard strains AB731755.1. The five samples with *K. septempunctata* positive are identical from 98.4% to 100% consistent with the standard strains via NCBI Blast (https://www.ncbi.nlm.nih.gov/Blast) (Table 5).

The genotypes of *K. septempunctata* are ST1, ST2, and ST3, which are determined by combination of gene cox1 (cytochrome coxidase subunit 1) and gene rnl (large subunit rRNA). Gene cox1 is divided into cox1-1, cox1-2, and cox1-3 due to the differences of six nucleotides, while gene rnl is divided into rnl-1 and rnl-2 due to the differences of two nucleotides. By using Clustal W of the Bioedit program, we found that the gene sequences of *K. septempunctata* in the five samples are identical to ST3 with cox1-3 and rnl-2 (Fig. 2).

The genotypes of *K. septempunctata* vary depending on the country; ST1 and ST2 are mainly detected in Japan, while only ST3 is known to be detected in Korea (Takeuchi et al., 2015). In this study, the detected genotype of *K. septempunctata* is also

Table 4. The number of *Kudoa septempunctata* isolated from sashimi and sushi

Food type	No. of samples	No. of *K. septempunctata* detected by microscopic examination	No. of *K. septempunctata* detected by PCR test with 18S rDNA and 28S rDNA	Region (no. of positive samples)
Sashimi	216	11	5	Jeju (3), Samcheonpo (1), Wando (1)
Sushi	20	0	0	-
Total	236	11 (4.7%)	5 (2.1%)	

PCR, polymerase chain reaction.

Table 5. Results by microscopic examination, PCR assays with *Kudoa septempunctata* in positive samples

Food type	Region	*K. septempunctata* spore	*K. septempunctata* detected by PCR		Identity of *K. septempunctata*	
			18S rDNA	28S rDNA	AB731754.1 (%)	AB731755.1 (%)
Sashimi-1	Wando	8.4×10^5 /g	P	P	99.74	100
Sashimi-2	Samcheonpo	8.0×10^4 /g	ND	ND		
Sashimi-3	Samcheonpo	9.0×10^5 /g	P	P	99.48	99.72
Sashimi-4	Jeju	6.0×10^5 /g	P	P	98.45	99.45
Sashimi-5	Jeju	2.0×10^4 /g	ND	ND		
Sashimi-6	Jeju	1.8×10^5 /g	ND	ND		
Sashimi-7	Jeju	5.0×10^5 /g	P	P	98.43	100
Sashimi-8	Jeju	2.0×10^5 /g	ND	ND		
Sashimi-9	Jeju	2.0×10^5 /g	ND	ND		
Sashimi-10	Jeju	1.0×10^4 /g	ND	ND		
Sashimi-11	Jeju	3.4×10^5 /g	P	P	99.74	99.72

PCR, polymerase chain reaction; P, positive; ND, not detected.

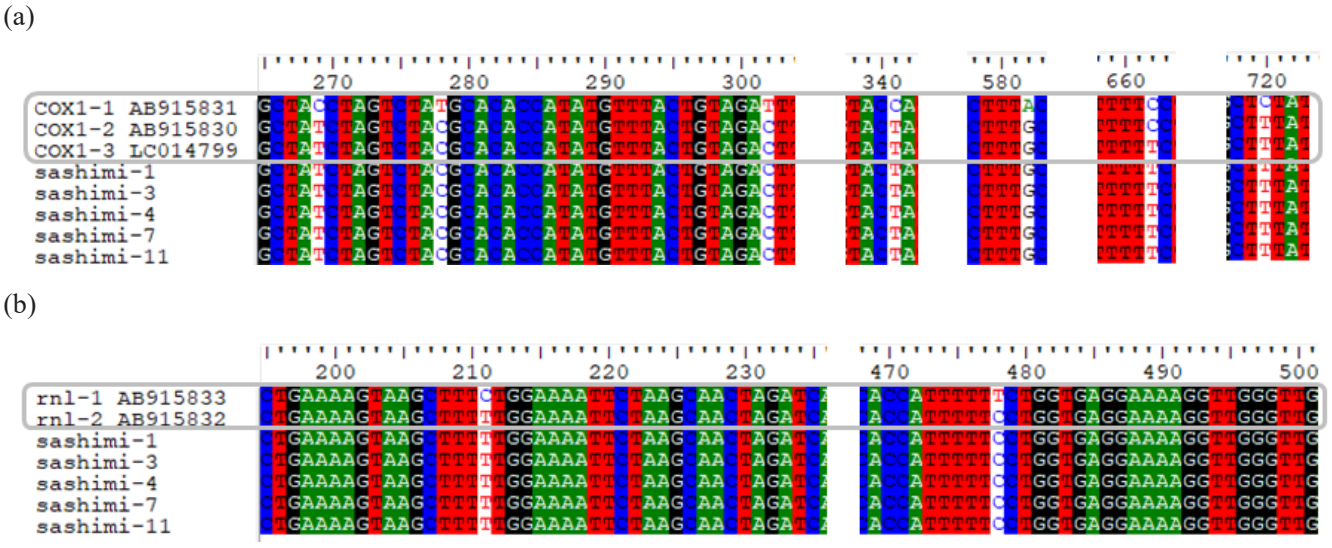

Fig. 2. The gene results of sequence analysis. The five samples are consistent with cox1-3 (a) and rnl-2 (b) on *Kudoa septempunctata*.

ST3, which is consistent with the results of the existing studies; Kim et al. (2018) reported all the genotypes of *K. septempunctata* detected in food poisoning patients in Gyeonggi-do Province were ST3. However, Jang et al. (2016) could not find any connection with the foodborne diseases in animal tests by eating olive flounders with *K. septempunctata* of ST3. Since the infection path of *K. septempunctata* and its connection with the intermediate host has not been identified, the additional studies are needed to identify it. Eventually, further studies are needed on the connection between sliced raw olive flounders and Kudoa food poisoning (Ahn et al., 2015).

In the experiments, we first performed the Real-time PCR to screen the olive flounder samples, and then check the Kudoa spores with the microscopy, and finally determine whether they are positive by the PCR test. This study detected 29 samples to be *K. septempunctata* positive in the Real-time PCR, and the microscopy confirmed 11 samples among 29 samples, and the PCR tests finally confirmed 5 samples as positive which are detected from both 18S rDNA and 28S rDNA target of *K. septempunctata*. The number of detected samples decreased as they went through the steps, because the Real-time PCR test had unusual reactions or very little DNA in some samples (Grabner et al., 2012). In the future, the Loop-mediated isothermal amplification method (LAMP) or more advances in the Real-time PCR method is needed on preventing Kudoa food poisoning or tracking the infection path (Jeon & Kim, 2016; Kim et al., 2015; Song et al., 2014).

The previous researches only focused on the spread of *K. septempunctata* infections in olive flounders in fish farms, but no researches have been performed so far on the infection of *K. septempunctata* on sashimi and sushi in markets. In fact, the serious concern is that people have foodborne outbreaks from eating sashimi or sushi with *K. septempunctata* infected, so this study can be seen as the first step on preventing food poisoning. Now not only in Korea but also in the world, the detection criteria of *K. septempunctata* in sashimi and sushi have not been established yet, therefore we believe that the further research will be prioritized on the detection criteria for *K. septempunctata* in sashimi and sushi.

All the previous studies have detected *K. septempunctata* only in Jeju. However, this study found *K. septempunctata* in olive flounders sashimi from Samcheonpo and Wando other than Jeju. Within authors' knowledge, this is the first study to detect *K. septempunctata* outside of Jeju. It is necessary to continuously monitor whether the detection region of *K. septempunctata* widen depending on climate change and environmental factors.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

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