Jpn. J. Infect. Dis., 68, 235–238, 2015

Short Communication

Molecular Epidemiological Analysis of *Kudoa septempunctata* by Random Amplified Polymorphic DNA Analysis

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SUMMARY: Molecular epidemiological analysis of *Kudoa septempunctata* isolates from 34 olive flounders associated with foodborne disease outbreaks and from 6 reference samples was performed using random amplified polymorphic DNA (RAPD) analysis. The *K. septempunctata* isolates analyzed in this study were divided into 8 groups. Eight isolates obtained from the large Ehime Prefecture outbreak in Japan that had occurred on October 8, 2010, were further divided into 4 groups. Eight isolates obtained from Korean samples were divided into 3 groups. These groups included isolates that had been identified from the large Ehime Prefecture outbreak. These results indicated that the Korean isolates had similar genetic backgrounds to those involved in the Ehime Prefecture outbreak. Isolates associated with outbreaks with similar dates of onset tended to be classified in the same group, suggesting that the strains involved in these incidents were genetically related. These results demonstrated that RAPD analysis is a useful molecular epidemiological analysis method for *K. septempunctata*.

In Japan, outbreaks of unidentified foodborne diseases associated with the consumption of raw olive flounder (*Paralichthys olivaceus*) have increased (1). The symptoms include transient but severe diarrhea and emesis; patients generally recover within 24 h after the onset of symptoms (1). Illness occurs 2–20 h after ingestion (1). We previously reported that *Kudoa septempunctata*, a newly described myxosporean species that lives in olive flounder, is the causative agent of this foodborne disease (1,2). Sporoplasms released from *K. septempunctata* spores in the human intestine invade the mucosal monolayer (3). The invasion of intestinal epithelial cells by *K. septempunctata* sporoplasms is believed to be a contributing factor in causing the diarrhea associated with this pathogen (3).

Olive flounders, which are consumed in Japan, are obtained from natural waters, cultured in Japanese fish farms, or imported from foreign countries. Therefore, a wide diversity in *K. septempunctata* strains potentially exists within Japan. However, the origin and diversity in *K. septempunctata* has not been well studied. *K. septempunctata* is not currently cultured in laboratories. When surveying and analyzing outbreaks, only a few grams of olive flounder can usually be obtained from the meal remnants of patients. Both these factors make it difficult to obtain a large amount of *K. septempunctata* genomic DNA, making genetic analyses very difficult. In addition, the details of the *K. septempunctata* genome are not currently published. Random amplified polymorphic DNA (RAPD) analysis (4,5) enabled us to perform whole-genome analysis without requiring a large amount of DNA or detailed genomic information (6,7). The aim of this study was to characterize *K. septempunctata* in olive flounder remnants collected from the meals of patients by performing RAPD analysis to clarify the genetic relationship between *K. septempunctata* strains.

A total of 34 olive flounder samples associated with the outbreaks were provided by local Japanese governments. Olive flounders cultured at fish farm A in Oita Prefecture were provided by the National Research Institute of Aquaculture. Several olive flounder samples were purchased from a Japanese market. Olive flounders obtained from fish farm A and those purchased from the Japanese market were used as reference samples in this study. *K. septempunctata* spores were purified from olive flounder flesh as described previously (1). DNA was extracted from 5×10^6 spores using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Nineteen RAPD primers contained in a RAPD 10-mer kit (Operon Biotechnology, Tokyo, Japan) that produced reproducible results were used in this study. Polymerase chain reaction (PCR) was performed using 50 ng of template DNA, 0.5 U of Ex Taq (Takara Bio, Inc., Shiga, Japan), 1 × PCR buffer, 0.2 mM dNTPs, and 5 μM RAPD primer in a final volume of 20 μl. Amplification was performed using a T100 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: initial denaturation at 95°C for 1 min, followed by 45 cycles of 95°C for 30 s, 36°C for 30 s, and 72°C for 1 min, and final primer extension at 72°C for 10 min. PCR products were subjected to gel electrophoresis using 1.5% agarose gel, and DNA was detected using SYBR Safe DNA gel stain (Thermo Fisher Scientific Inc., Waltham, MA, USA). The pattern of DNA bands was analyzed using TotalQuant gel analysis software (TotalLab Ltd., Newcastle, UK). The genetic

Received May 2, 2014. Accepted August 26, 2014.
J-STAGE Advance Publication December 24, 2014.
DOI: 10.7883/yoken.JJID.2014.190

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distances among *K. septempunctata* strains were represented as dissimilarity (8,9), which was calculated as follows: dissimilarity = $1 - 2N_{12}/(N_1 + N_2)$. N_{12} represents the number of bands shared between samples 1 and 2; N_1 represents the total number of bands amplified using 19 RAPD primers; and N_2 represents the total number of bands amplified using 19 RAPD primers. The genetic distance matrix between samples was constructed and then utilized to construct a neighbor-joining dendrogram (10). Cluster analysis and dendrogram construction were performed using MEGA6 Freeware (11).

In this study, we analyzed 34 olive flounder samples that were associated with foodborne disease outbreaks (Table 1, ID 1–34). Olive flounders ID 2 and 3 were collected from the same outbreak that occurred in Oita Prefecture, Japan. Olive flounders ID 23–30 were different individuals related to an outbreak in Ehime Prefecture. Three olive flounders that were raised in fish farm A in Oita Prefecture and 3 others that were purchased from Japanese markets were used as reference samples in this study. Fish farm A was heavily contaminated with *K. septempunctata*. The sources of olive flounders used in this study were as follows (Table 1): 8 fishes from Oita Prefecture (ID 2, 3, 8, 15, 21, and 36–38), 8 fishes from Ehime Prefecture (ID 23–30), 1 fish each from Mie Prefecture (ID 5) and Fukui Prefecture (ID 13), 8 fishes from Korea (ID 22, 31–35, 39, and 40), and 14 fishes of unknown origin (ID 1, 4, 6, 7, 9–12, 14, and 16–20). A single wild olive flounder was analyzed (ID 18). Spores were purified from these olive flounders and subjected to RAPD analysis. The genetic distance matrix between samples was constructed (Table 2) and then utilized to construct a dendrogram (Fig. 1). A total of 40 *K. septempunctata* isolates in this study were divided into 8 groups (Fig. 1, groups A–H). The isolates obtained from Korean olive flounders were divided into 3 groups (Fig. 1, groups B, G, and H) and those isolated from olive flounders raised at fish farm A (ID 36–38) were divided into 2 groups (Fig. 1, groups G and H). On October 8, 2010, a large *K. septempunctata* outbreak occurred in Ehime Prefecture, representing the largest outbreak of this pathogen at that time. During this outbreak, 113 of 534 patients developed symptoms. The olive flounders causing this outbreak were provided from a single fish farm in Ehime Prefecture and approximately 195 olive flounders were probably associated with this outbreak (a personal report from Ehime Prefecture). *K. septempunctata* spores were purified from 8 olive flounders (ID 23–30) associated with this outbreak and then subjected to RAPD analysis. The isolates from this outbreak were divided into 4 groups (Fig. 1, groups B, D, G, and H). Groups B, G, and H included isolates from olive flounders originating from Korea, fish farm A, and Ehime Prefecture outbreak. This result suggested that the large outbreak in Ehime Prefecture was caused by multiple strains that included close relatives of isolates obtained from Korean olive flounders. Isolates obtained from olive flounders raised in Oita Prefecture (ID 2, 3, 15, 21, and 36–38) were divided into groups A (ID 2 and 3), F (ID 15), G (ID 36 and 37), and H (ID 21 and 38), indicating that the isolates from the same prefecture, and even from the same fish farm (fish farm A), showed high genetic diversity.

Table 1. Details of the origin of *K. septempunctata* isolates analyzed in this study

ID	Onset Date (m/d/y)	Prefecture of incident	Source of olive flounder	Note
1	7/14/2009	Fukuoka	Unknown	
2	9/4/2009	Oita	Oita	Same outbreak
3	9/4/2009	Oita	Oita	
4	9/8/2009	Saitama	Unknown	
5	11/28/2008	Fukui	Mie	
6	4/11/2009	Fukui	Unknown	
7	12/7/2009	Shizuoka	Unknown	
8	10/11/2009	Hiroshima	Oita	
9	3/31/2010	Hiroshima	Unknown	
10	5/4/2010	Ishikawa	Unknown	
11	3/18/2010	Hiroshima	Unknown	
12	8/1/2010	Hiroshima	Unknown	
13	5/4/2010	Fukui	Fukui	
14	8/22/2011	Aichi	Unknown	
15	9/5/2011	Kagawa	Oita	
16	9/23/2011	Chiba	Unknown	
17	4/6/2013	Fukuoka	Unknown	
18	4/24/2013	Hiroshima	Unknown · Wild	
19	9/26/2013	Yamaguchi	Unknown	
20	10/14/2013	Hyogo	Unknown	
21	12/9/2011	Fukuoka	Oita	
22	2/26/2011	Chiba	Korea	
23	10/8/2010	Ehime	Ehime	Large outbreak related to Iyo Bank
24	10/8/2010	Ehime	Ehime	
25	10/8/2010	Ehime	Ehime	
26	10/8/2010	Ehime	Ehime	
27	10/8/2010	Ehime	Ehime	
28	10/8/2010	Ehime	Ehime	
29	10/8/2010	Ehime	Ehime	
30	10/8/2010	Ehime	Ehime	
31	8/12/2009	Tokyo	Korea	
32	10/26/2009	Fukui	Korea	
33	9/6/2009	Yamaguchi	Korea	
34	10/11/2010	Hiroshima	Korea	
35	7/2/2013[1)]	Reference	Korea	
36	2/16/2011[1)]	Reference	Fish farm A (Oita)	
37	6/8/2011[1)]	Reference	Fish farm A (Oita)	
38	11/15/2011[1)]	Reference	Fish farm A (Oita)	
39	8/31/2012[1)]	Reference	Korea	
40	6/5/2013[1)]	Reference	Korea	

[1)]: Date that sample arrived at National Institute of Health Sciences.

One possibility is that multiple lots of olive flounder are introduced to fish farm; alternatively, this diversity may merely represent the natural variety of *K. septempunctata* strains. Additional studies are needed to elucidate the noticeable genetic diversity of *K. septempunctata* strains.

Groups E and F included 10 isolates; however, the origins of these olive flounders were unknown, except for 2 isolates (ID 8 and 15). Group E included 6 strains, and 4 olive flounders of group E were derived from incidents that occurred at Hiroshima Prefecture. The 3 incidents in Hiroshima Prefecture (ID 9, 11, and 12) occurred at around the same date. Similarly, isolates from outbreaks with close dates of onset frequently clustered

Table 2. Matrix of genetic distance of 40 isolates of *K. septempunctata*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
1-Fukuoka_(Unknown)																																							
2-Oita_(Oita)	0.395																																						
3-Oita_(Oita)	0.419	0.460																																					
4-Saitama_(Unknown)	0.260	0.461	0.387																																				
5-Fkui_(Mie)	0.319	0.429	0.396	0.221																																			
6-Fukui_(Unknown)	0.251	0.443	0.361	0.144	0.234																																		
7-Shizuoka_(Unknown)	0.405	0.564	0.437	0.306	0.362	0.269																																	
8-Hiroshima_(Oita)	0.304	0.513	0.429	0.217	0.243	0.210	0.086																																
9-Hiroshima_(Unknown)	0.331	0.487	0.405	0.191	0.218	0.203	0.103	0.123																															
10-Ishikawa_(Unknown)	0.296	0.455	0.390	0.163	0.267	0.173	0.172	0.222	0.183																														
11-Hiroshima_(Unknown)	0.271	0.463	0.388	0.175	0.220	0.179	0.184	0.197	0.170	0.184																													
12-Hiroshima_(Unknown)	0.319	0.500	0.453	0.208	0.262	0.253	0.157	0.190	0.182	0.247	0.132																												
13-Fukui_(Fukui)	0.296	0.376	0.458	0.271	0.295	0.274	0.284	0.339	0.320	0.295	0.257	0.292																											
14-Aichi_(Unknown)	0.270	0.402	0.422	0.267	0.247	0.260	0.268	0.320	0.350	0.257	0.210	0.333	0.223																										
15-Kagawa_(Oita)	0.296	0.436	0.437	0.244	0.243	0.224	0.205	0.236	0.223	0.160	0.233	0.265	0.254	0.242																									
16-Chiba_(Unknown)	0.382	0.369	0.325	0.376	0.377	0.369	0.312	0.436	0.436	0.371	0.378	0.452	0.345	0.351	0.355																								
17-Fukuoka_(Unknown)	0.364	0.464	0.397	0.227	0.288	0.235	0.147	0.227	0.216	0.263	0.259	0.241	0.282	0.365	0.232	0.370																							
18-Hiroshima_(Unknown-Natural)	0.286	0.348	0.410	0.266	0.306	0.232	0.253	0.290	0.305	0.250	0.262	0.328	0.210	0.205	0.236	0.360	0.237																						
19-Yamaguchi_(Unknown)	0.353	0.422	0.441	0.308	0.313	0.314	0.324	0.369	0.344	0.344	0.317	0.354	0.254	0.330	0.303	0.362	0.316	0.226																					
20-Hyogo_(Unknown)	0.384	0.512	0.507	0.313	0.299	0.330	0.235	0.267	0.316	0.386	0.286	0.296	0.312	0.365	0.368	0.480	0.321	0.322	0.284																				
21-Fukuoka_(Oita)	0.330	0.567	0.434	0.275	0.300	0.269	0.225	0.288	0.274	0.290	0.242	0.278	0.367	0.308	0.333	0.441	0.321	0.331	0.358	0.346																			
22-Chiba_(Korea)	0.288	0.403	0.406	0.272	0.313	0.286	0.348	0.379	0.377	0.303	0.283	0.380	0.238	0.207	0.312	0.353	0.350	0.236	0.299	0.385	0.312																		
23-Ehime_(Ehime)	0.345	0.525	0.436	0.215	0.293	0.208	0.200	0.221	0.205	0.228	0.190	0.221	0.339	0.301	0.261	0.443	0.222	0.290	0.365	0.318	0.229	0.314																	
24-Ehime_(Ehime)	0.361	0.476	0.394	0.284	0.314	0.270	0.190	0.308	0.297	0.320	0.294	0.295	0.333	0.331	0.336	0.377	0.277	0.318	0.300	0.339	0.313	0.358	0.298																
25-Ehime_(Ehime)	0.278	0.382	0.410	0.240	0.281	0.243	0.273	0.315	0.313	0.289	0.254	0.295	0.218	0.230	0.274	0.328	0.301	0.209	0.292	0.348	0.305	0.163	0.307	0.341															
26-Ehime_(Ehime)	0.357	0.546	0.433	0.244	0.286	0.228	0.186	0.200	0.192	0.238	0.242	0.232	0.372	0.328	0.263	0.490	0.257	0.307	0.364	0.327	0.223	0.364	0.139	0.315	0.307														
27-Ehime_(Ehime)	0.314	0.480	0.477	0.224	0.285	0.212	0.205	0.277	0.229	0.211	0.182	0.273	0.279	0.266	0.237	0.401	0.230	0.274	0.331	0.327	0.292	0.263	0.185	0.323	0.298	0.205													
28-Ehime_(Ehime)	0.352	0.535	0.447	0.232	0.291	0.236	0.188	0.205	0.189	0.211	0.239	0.246	0.361	0.325	0.228	0.455	0.189	0.312	0.370	0.342	0.254	0.353	0.152	0.312	0.312	0.157	0.185												
29-Ehime_(Ehime)	0.376	0.537	0.466	0.244	0.311	0.239	0.191	0.216	0.200	0.214	0.258	0.257	0.381	0.345	0.216	0.449	0.208	0.362	0.406	0.372	0.258	0.347	0.172	0.347	0.331	0.176	0.197	0.117											
30-Ehime_(Ehime)	0.336	0.406	0.460	0.330	0.342	0.331	0.373	0.377	0.396	0.372	0.303	0.366	0.249	0.271	0.314	0.382	0.370	0.271	0.336	0.389	0.370	0.172	0.368	0.393	0.172	0.392	0.325	0.381	0.358										
31-Tokyo_(Korea)	0.276	0.413	0.432	0.276	0.299	0.284	0.304	0.328	0.323	0.315	0.254	0.331	0.277	0.221	0.259	0.364	0.361	0.256	0.319	0.360	0.324	0.177	0.342	0.376	0.152	0.317	0.283	0.331	0.333	0.136									
32-Fukui_(Korea)	0.325	0.369	0.424	0.348	0.359	0.317	0.354	0.408	0.402	0.363	0.312	0.391	0.285	0.290	0.339	0.364	0.419	0.304	0.336	0.414	0.404	0.235	0.383	0.408	0.192	0.431	0.325	0.412	0.419	0.170	0.198								
33-Yamaguchi_(Korea)	0.243	0.431	0.400	0.158	0.237	0.192	0.207	0.269	0.225	0.216	0.187	0.258	0.248	0.219	0.194	0.350	0.242	0.222	0.298	0.319	0.241	0.257	0.190	0.307	0.222	0.225	0.182	0.206	0.205	0.276	0.236	0.269							
34-Hiroshima_(Korea)	0.247	0.387	0.391	0.188	0.255	0.209	0.227	0.245	0.227	0.172	0.198	0.260	0.258	0.213	0.174	0.341	0.228	0.202	0.258	0.362	0.269	0.243	0.210	0.308	0.194	0.212	0.194	0.209	0.224	0.269	0.194	0.286	0.130						
35-Reference_(Korea)	0.320	0.458	0.465	0.297	0.285	0.250	0.284	0.291	0.293	0.285	0.313	0.282	0.328	0.358	0.293	0.425	0.289	0.268	0.336	0.359	0.300	0.320	0.263	0.351	0.283	0.257	0.286	0.269	0.259	0.331	0.302	0.362	0.250	0.230					
36-Referenc_(Fish_farm_Oita)	0.286	0.447	0.438	0.183	0.239	0.224	0.217	0.295	0.243	0.242	0.212	0.280	0.286	0.229	0.193	0.377	0.262	0.265	0.313	0.346	0.300	0.244	0.224	0.355	0.231	0.244	0.181	0.257	0.213	0.263	0.214	0.299	0.162	0.182	0.277				
37-Reference_(Fish_farm_Oita)	0.306	0.435	0.427	0.204	0.247	0.228	0.241	0.255	0.219	0.249	0.229	0.269	0.267	0.254	0.172	0.361	0.211	0.216	0.292	0.304	0.278	0.276	0.184	0.310	0.231	0.195	0.159	0.184	0.177	0.286	0.252	0.336	0.142	0.185	0.206	0.138			
38-Reference_(Fish_farm_Oita)	0.386	0.568	0.467	0.275	0.333	0.312	0.171	0.251	0.234	0.274	0.295	0.278	0.405	0.377	0.258	0.466	0.252	0.336	0.422	0.379	0.297	0.387	0.224	0.352	0.336	0.193	0.241	0.190	0.183	0.416	0.344	0.439	0.236	0.278	0.307	0.281	0.213		
39-Reference_(Korea)	0.354	0.525	0.449	0.224	0.314	0.233	0.111	0.220	0.199	0.236	0.231	0.254	0.382	0.364	0.244	0.424	0.214	0.310	0.366	0.322	0.280	0.341	0.187	0.318	0.318	0.183	0.203	0.141	0.161	0.393	0.312	0.399	0.208	0.256	0.276	0.249	0.194	0.144	
40-Reference_(Korea)	0.233	0.427	0.420	0.204	0.240	0.215	0.204	0.255	0.244	0.268	0.205	0.272	0.270	0.230	0.244	0.385	0.254	0.257	0.322	0.318	0.291	0.253	0.251	0.339	0.216	0.245	0.217	0.267	0.261	0.264	0.222	0.291	0.172	0.176	0.270	0.153	0.190	0.282	0.258

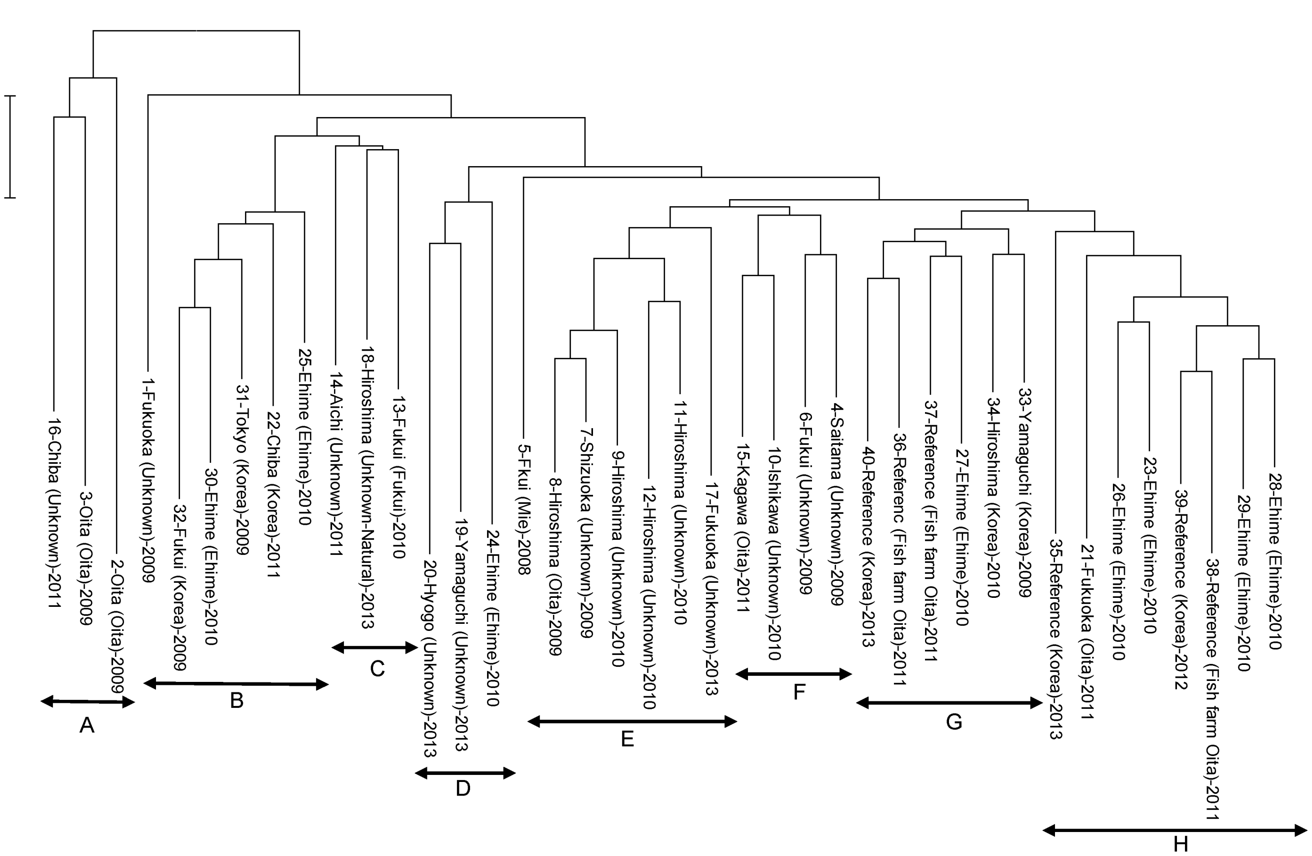

Fig. 1. Dendrogram of 40 *K. septempunctata* isolates obtained from the cluster analysis based on RAPD data. ID-Prefecture-(Source of olive flounder)-Onset year.

into the same groups: ID 7 (December 7, 2009) and ID 8 (October 11, 2009); ID 19 (September 26, 2013) and ID 20 (October 14, 2013). These results suggested that the incidents with close dates of onset were caused by genetically related strains. Thus, the olive flounders associated with these incidents potentially originated from the same fish farm. Further investigation will be needed to identify the origin of these olive flounders.

In this study, the genetic relationships among *K. septempunctata* isolates associated with foodborne disease outbreaks were examined using RAPD analysis. RAPD analysis is useful for genetic analysis of organisms that cannot be cultured in laboratories or those with limited genetic information available. RAPD analysis in this study suggested that the large Ehime Prefecture outbreak was caused by multiple strains that were genetically related to those isolated from Korean olive flounders. Therefore, RAPD analysis enabled us to obtain information that complements conventional epidemiological surveys on *K. septempunctata*-associated outbreaks. Moreover, RAPD analysis was shown to be effective for the analysis of *K. septempunctata*-associated outbreaks.

Acknowledgments We thank the local governments in Japan and the National Research Institute of Aquaculture Fisheries Research Agency for kindly providing olive flounder samples. This study was supported by the Ministry of Education, Culture, Sports, Science, and Technology (Grant-in-Aid for Scientific Research (C) 24501026).

Conflict of interest None to declare.

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