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Detection of Japanese flounder-specific DNA from gut contents of potential predators in the field

KENJI SAITOH,[1]* MAMORU TAKAGAKI[2] AND YOH YAMASHITA[1†]

[1]*Tohoku National Fisheries Research Institute, Shiogama, Miyagi 985-0001 and* [2]*Fukui Prefectural Sea Farming Center, Obama, Fukui 917-0116, Japan*

ABSTRACT: Predation by invertebrates may be one of the major factors influencing the mortality of released flounder juveniles in the field. We developed a polymerase chain reaction (PCR)-based technique for the detection of Japanese flounder material from the gut contents of potential predators in the field. The PCR primers used here are flounder specific and encompass a 153 bp region from the right domain of the mitochondrial control region. With these oligonucleotide primers, PCR reactions yielded specific amplification products from the gut contents of some putative predators, including *Matuta lunaris*, *Portunus gladiator*, *Sepioteuthis lessoniana*, *Suggrundus meerdervoorti*, *Platycephalus* sp. and *Rhyncopelates oxyrhynchus*. Many animals with gut contents of unidentified fish material showed indication of predation on the flounder after release of hatchery reared flounder juveniles. The technique developed in the present study is useful not only for the survey of predation on the flounder by known species, but also for the search for novel potential predatory species in the field.

KEY WORDS: **aquaculture, mitochondrial DNA, mortality, *Paralichthys olivaceus*, predation, sea ranching, survival.**

INTRODUCTION

Low survival of released stock is a difficulty for the promotion of stock-enhancement programs.[1,2] Starvation and predation are the major factors influencing fish mortality in the early stages.[3] In many fish species for stocking, hatchery raised fish are released at sizes at which they have tolerance to starvation and predation may be responsible for their low survival.[4,5]

Japanese flounder *Paralichthys olivaceus* is commercially important and one of the most extensively stocked fishery resources. Recent advances in rearing methods and releasing protocols improved the survival and growth of released flounder juveniles in the field.[5,6] However, a rapid decrease in the numbers of released flounder in the field still occurred and intensive predation by crustacean animals, as well as by piscivorous fish, was suspected.[7] Many invertebrate predators mince their preys with maxilae, whereas predatory fish swallow their prey. Therefore, it has been difficult to identify prey fish species on the basis of invertebrate gut contents to assess predation on flounder.[7]

Molecular techniques have enabled sensitive and specific detection for the rapid identification of fish species with small and various sources of DNA.[8–11] The DNA were successfully polymerase chain reaction (PCR) amplified, even from heavily degraded DNA.[12] Here, we present a species-specific PCR-based DNA detection method from the gut contents of putative predators on released flounder juveniles in the field. The existence of flounder genetic material in the gut contents of a wide range of animals indicates that predation is the important factor influencing the survival of released flounder juveniles, especially within a few days after the release date.

MATERIALS AND METHODS

Fish and invertebrates were collected with a gill net (mesh size 70 mm) on the coast of Takahama Cove (35.29 N, 135.32°E; 2–20 m in depth) where 94 000 hatchery flounder (approximately 65–95 mm in total length (*TL*)) were released during the daytime of 2 July 2001. The gill net was set at

*Corresponding author: Tel: 81-22-365-9932. Fax: 81-22-367-1250. Email: ksaitoh@affrc.go.jp

†Present address: Kyoto University, Fisheries Research Station, Maizuru, Kyoto 625-0086, Japan.

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17.00 h on 2, 5 and 17 July and 18 September. Animals entrapped onto the net were collected at 06.00 h the next morning. We checked the stomach contents of animals visually and preserved them individually in ethanol (> 95%) until DNA extraction when the stomachs contained unidentified or possible fish material. Forceps, scissors and dishes used in this procedure were washed well in running water from sample to sample to avoid contamination.

Gut contents were, in many cases, of mucus material with very small pieces of food items. Some contained sizable pelleted debris. Approximately 50–100 mg mucus material or of five small pieces collectively picked from different portions of the pellet were used as sources for DNA extraction. The method of DNA extraction was a modification of the procedure with a buffer containing 8 mol/L urea.[13] After lysis of mucus or pelleted material, the aqueous phase was extracted with Tris-saturated phenol with vigorous vortexing. When separation of aqueous and organic phases was incomplete, chloroform (0.1–0.2 volumes) was added to facilitate phase separation. The subsequent aqueous phase was extracted twice further with phenol–chloroform (1 : 1) with vortexing and then treated with RNaseA (2.5 ng/μL final concentration) for 5 min at room temperature. The aqueous phase was again extracted with phenol–chloroform, ethanol precipitated and redissolved in water. Along with DNA extracted from animal gut contents, we used genomic DNA from 12 flatfish species and plasmid DNA containing the flounder target sequence (pJFMT17)[14,15] to confirm the specificity and sensitivity of our method (Table 1). We determined the DNA concentration optically (GeneQuant; Pharmacia, Cambridge, UK).

Table 1 Related flatfish species used in the present study to test the specificity of polymerase chain reaction amplification with the primer pair

Paralichthyidae
Pseudorhombus pentophthalmus
Tarphops oligolepis
Pleuronectidae
Atheresthes evermanni
Clidoderma asperrimum
Dexistes rikuzenius
Eopsetta grigorjewi
Glyptocephalus stelleri
Kareius bicoloratus
Microstomus achne
Pleuronectes herzensteini
P. yakohamae
Cynoglossidae
Cynoglossus joyneri

All the polymerase chain reactions with genomic DNAs (10 ng/reaction) from the species listed above failed to amplify DNA fragments around the length expected. Plasmid pJFMT17, derived from *Paralichthys olivaceus* containing the target sequence,[14,15] was used as a positive control (0.5 pg/reaction).

We designed the PCR reaction to amplify flounder-specific mitochondrial (mt) DNA fragments encompassing nucleotides from 16 909 to 17 061 at the right domain of the control region.[14,15] The oligonucleotide primers used here were 5′-CATGAACCCCTTATTGAACA-3′ and 5′-GTCATGCAGTCCTTGTAA-3′. A preliminary survey of mitochondrial control region sequences from three pleuronectids[16] failed to find the corresponding sequences of the position where we constructed primers. The PCR reaction mix of 20 μL in the total volume contained various amounts of template DNA (0.5 pg to 200 ng according to the source of DNA), dNTPs (0.2 mmol/L each), primers (1 μmol/L each), bovine serum albumin (BSA; 20 μg; Boehringer Mannheim, Mannheim, Germany), 1× buffer and *Taq* DNA polymerase (0.5 U; Takara, Otsu, Japan). The PCR reaction consisted of an initial denaturation step at 95°C for 180 s, 40 cycles of incubation at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension step at 72°C for 300 s. Agarose gel electrophoresis (2% Agarose-21; Nippon Gene, Toyama, Japan) in 1× TAE (40 mmol/L Tris-acetate, 1 mmol/L EDTA, pH 8) offered separation of PCR products, 3 μL of which was loaded onto the gel unless specified otherwise. Subsequent ethidium bromide staining of the gel visualized the separated fragments.

We picked up some PCR products and sequenced them directly for confirmation. Sequencing primers were the same as primers used for the PCR reaction.

RESULTS

In total, 496 predatory animals were collected by gill nets. Most stomachs of the crustaceans and cephalopods were empty but for small pieces of molluscan shell. Stomaches of predatory fish were, in many cases, empty or with identifiable material. Predation on flounder juveniles was detected by morphological observation of gut contents of a seabass (*Lateotabrax japonicus*) and a flounder of 1+-year-old individual. From morphological inspection of gut contents, we decided to perform PCR-based examination of gut contents on 22 animals, the stomachs of which held unidentified fish material.

The DNA yield using the present method was 500–6900 ng (mean 3400 ng) from approximately 50–100 mg gut contents. Extracted DNA were

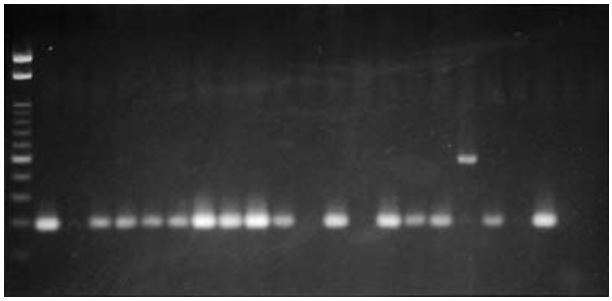

Fig. 1 Polymerase chain reaction (PCR) amplification of Japanese flounder mitochondrial (mt) DNA from the gut contents of potential predators. Each PCR reaction contained 20 ng extracted DNA. Lanes from left to right contain the following: lane 1, 100 bp ladder (Toyobo, Osaka, Japan); lanes 2–11, animals collected the day immediately after release of juvenile flounder; lanes 12–17, animals collected 4 days after release; lanes 18–21, animals collected 16 days after release; lanes 22–23, animals collected 78 days after release. Lanes, except for the molecular weight marker, contain PCR products from each animal in the same order as listed in Table 1.

heavily degraded and showed a powdery appearance upon ethanol precipitation.

The PCR reactions indicated the presence of Japanese flounder-specific mtDNA fragments in gut contents from various animal species collected in the field (Fig. 1; Table 2). In cases where DNA were separately extracted from mucus material and pelletted debris, results were consistent with both DNA except for one. Sequence analysis confirmed the amplified DNA fragment was the target region of flounder mtDNA (data not shown). In addition to crustaceans, which have been suspected as predators, a cephalopod species (*Sepioteuthis lessoniana*) harbored Japanese flounder mtDNA in its gut contents. Three fish species had well-digested unidentified matter in their stomachs and the flounder mtDNA fragment was amplified from this matter.

The same reaction conditions for PCR amplification did not yield specific amplification products from genomic DNA of 12 related flatfish species (Table 1). Different concentrations of template DNA from gut contents varying in the range of 0.2–200 ng template DNA/reaction gave consistent results (Fig. 2).

Nine of 10 animals collected the day immediately after the release date of flounder juveniles harbored flounder mtDNA, as did four of six animals collected 4 days after release. Some animals collected 16 days after release indicated the presence of flounder mtDNA. Animals collected 79 days after release did not show amplification of the target DNA sequence.

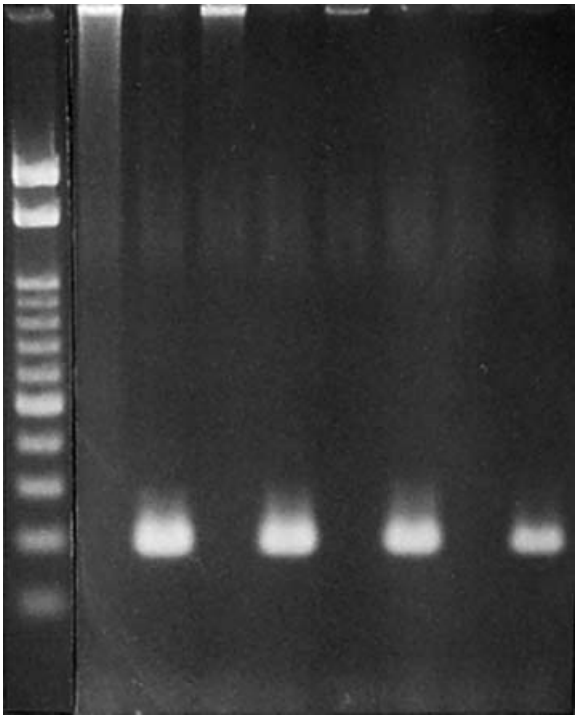

Fig. 2 Polymerase chain reaction (PCR) experiment with different amounts of template DNA. Lanes from left to right contain the 100 bp ladder and DNA negative or positive to flounder mitochondrial (mt) DNA in turn (see Table 1); lanes 2, 3, 200 ng template DNA; lanes 4, 5, 20 ng template DNA; lanes 6, 7, 2 ng template DNA; lanes 8, 9, 0.2 ng template DNA. Samples were overloaded (5 μL/lane).

DISCUSSION

We developed a PCR-based technique for the detection of Japanese flounder genetic material from the gut contents of potential predators in the field. It is easily predictable from phylogenetic information[16] that false-positive amplification due to sequence similarity is unlikely from template DNA containing other fish species or invertebrates. Failure of specific amplification from related flatfish DNA confirmed the specificity of our method. Although there were some non-specific amplification products, they are distinguishable from the flounder target sequence, which differs in length.

Consistent results with a wide range of template concentrations indicates sensitivity and usefulness for rapid screening without adjustment of template concentration. Amplification of the target DNA regardless of the source of the DNA also enables easy handling of materials. Furthermore, sequence analysis of the flounder mtDNA from 30 individuals showed no variation in primer annealing sites (K Saitoh, unpubl. obs., 2001), indicating

Table 2 Polymerase chain reaction amplification of Japanese flounder mitochondrial DNA from the gut contents of potential predators

Date/animals	Size*	Source of DNA†	Result
3 July 2001			
Crustacea			
Portunus gladiator	122	M	+§
Matuta lunaris	51	M	–
	45	M	+§
	53	M	+
	51	M, P	+, +§
	48	M	+
	50	M, P	+,§ +
Mollusca			
Sepioteuthis lessoniana	210	M, P	+,§ +
	150	P	+
Pisces			
Suggrundus meerdervoorti	485	M, P‡	+, +§
6 July 2001			
Crustacea			
Matuta lunaris	56	M‡	–
	42	M	+§
Portunus gladiator	52	M	–
Mollusca			
Sepioteuthis lessoniana	198	M, P	+, +§
	250	M, P	+, +
Pisces			
Rhyncopelates oxyrhynchus	210	M, P	–, +
18 July 2001			
Crustacea			
Portunus gladiator	55	P	–
	74	P	+
Mollusca			
Octopus vulgaris	275	P	–
Pisces			
Platycephalus sp.	388	P	+
19 September 2001			
Mollusca			
Sepioteuthis lessoniana	157	P	–
Sepia lycidas	131	P	–

* Carapace width (mm) for crustaceans, mantle length (mm) for moluscs except for the octopus (wet weight in g) and standard length (mm) for fish.
† DNA were extracted from mucus material (M) or pelleted debris (P).
‡ Used for the polymerase chain reaction experiment with different amounts of template DNAs
§ Sequenced for confirmation.
+ , positive for the specific amplification product; –, negative for the specific amplification product.

little possibility of a false-negative result due to sequence variation among flounder individuals. An experimental study showed that mtDNA fragment is amplifiable for at least 5 h after ingestion of stone flounder *Kareius bicoloratus* by a crustacean species (*Crangon affinis*) in captivity.[12] In the present study, the gill net was set for 13 h at night, when predatory crustaceans are active.[7] Although experimental studies are also necessary for predators observed in the present study to assess the sensitivity of our method over time, flounder-specific DNA fragments detected in the present study are of flounder body materials ingested possibly during the previous night. Therefore, our method is useful for field studies of flounder juvenile mortality by predation.

The presence of the flounder mtDNA in gut contents clearly indicates predation on the flounder. There were no dead flounder juveniles at release. Hatchery raised flounder juveniles show no instanteneous death just after release into fields (Y Yamashita, unpubl. obs., 2001). Therefore, ingestion of dead flounder juveniles by animals is unlikely. The present study successfully indicated

that some crustacean species are predators on the flounder, as assumed previously.[7] The number of released flounder juveniles overdominated in the field on the release date (approximately 150-fold; M Takagaki and Y Yamashita, unpubl. data, 2001). Hatchery raised flounder are more vulnerable to predation than wild fish.[17] Thus, we assume that most of the flounder mtDNA fragments detected in animal gut contents were from released stock. Thus, flounder body materials detected in the gut contents of predatory animals are an indication of mortality of released flounder by predation. However, observation of predatory behaviors in the field or captivity is necessary to assess how many flounder juveniles are ingested by each individual predator, because detection of flounder DNA indicates only whether the predator ate the flounder.

In the present study, a cephalopod species (*S. lessoniana*) turned out to be a predator on the flounder. Despite a small number of animals examined, we found a previously unknown predator of flounder. If a larger number of animals was to be examined, we would expect to encounter further novel predators. A search for potential predators over a wide spectrum of animal species is helpful for understanding the mortality of flounder juveniles in the field. Our method is especially useful for this purpose.

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