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Multiplex PCR for the identification of *Anisakis simplex* sensu stricto, *Anisakis pegreffii* and the other anisakid nematodes

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Abstract

A multiplex PCR method was established for the rapid identification of *Anisakis simplex* sensu stricto, *A. pegreffii*, *A. physeteris*, *Pseudoterranova decipiens*, *Contracaecum osculatum* and *Hysterothylacium aduncum*. The sequence alignment of the internal transcribed spacer 1 region (ITS-1) between *A. simplex* s. str. and *A. pegreffii* showed a high degree of similarity, but only two C–T transitions were observed. To differentiate *A. simplex* s. str. from *A. pegreffii*, an intentional mismatch primer with an artificial mismatched base at the second base from the primer 3′ end was constructed. This intentional mismatch primer, which produced a PCR band only from *A. pegreffii* DNA, was able to differentiate the two morphologically indistinguishable sibling species of *A. simplex*. Specific forward primers for other anisakid species were also designed based on the nucleotide sequences of the ITS region. The multiplex PCR using these primers yielded distinct PCR products for each of the anisakid nematodes. The multiplex PCR established in this study would be a useful tool for identifying anisakid nematodes rapidly and accurately.

Keywords: *Anisakis*; *Pseudoterranova*; *Contracaecum*; *Hysterothylacium*; Multiplex; Polymerase chain reaction

1. Introduction

Anisakiasis is caused by the ingestion of larval nematodes belonging to the genera *Anisakis, Pseudoterranova, Contracaecum* and *Hysterothylacium* in raw or undercooked seafood. Regional eating habits are recognized as a factor in the high prevalence of anisakiasis [1]. With the increased popularity of eating undercooked or raw fish dishes, the number of anisakiasis cases may be expected to increase. The species of Anisakidae most often associated with anisakiasis is *Anisakis simplex.* Recently, it has been recognized that the morphospecies *A. simplex* does not consist of only a single species but a complex of three sibling species, namely, *A. simplex* sensu stricto, *A. pegreffii* and *A. simplex* C. Especially, *A. simplex* s. str. and *A. pegreffii* widely extend across geographic ranges and the numbers of hosts [2,3].

To date, anisakid larvae recovered from patients have been identified based on their morphology. However, it is very difficult to identify worms that have been partly destroyed or lack key morphological features. Thus, DNA differential diagnosis is considered very useful for the definitive identification of clinically obtained worms. Several methods for identification of anisakid species such as PCR–RFLP [4–10] and sequencing of rRNA gene [4,7–9,11] or mitochondrial DNA [12] have been developed. Furthermore, RFLP of the ITS and the 5.8 subunit rRNA gene have been successfully employed for identification of sibling species of *A. simplex* [5,13–20]. We report the establishment of a multiplex PCR for the differential diagnosis of anisakid nematodes as an alternative to conventional methods.

Abbreviations: rRNA, ribosomal RNA; ITS, internal transcribed spacer; SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism.

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2. Materials and methods

2.1. Parasite materials

A total of thirty-four anisakid nematodes were collected from fish caught in waters off the Japanese coast. The parasites were identified to the genus level based on the morphological characteristics described by Koyama et al. [21] and Kagei [22]. Species identification was carried out by sequencing 18S–28S rRNA genes (see Section 2.4). The anisakids used in this study and the locality of their hosts are summarized in Table 1.

2.2. DNA extraction

DNA from individual worms was extracted using QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instruction. DNA was eluted in elution buffer and kept at − 20 °C until use.

2.3. PCR–RFLP analysis

Larvae were distinguished based on the genetic markers described by Zhu et al. [4], Amelio et al. [5] and Abe and Yagi [9]. The ITS region including 5.8S rRNA gene was amplified using two universal primers NC5 (forward; 5′-TAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′). All PCR reactions were carried out in a final volume of 30 μl containing 1 μl of genomic DNA, 0.75 units TaKaRa Ex *Taq* (Takara Bio Inc., Japan), 1× PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP mixture and 0.3 μM forward and reverse primers. An initial denaturation step at 94 °C for 5 min was followed by 26 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s with a final extension step at 72 °C for 7 min. The amplification was performed in a RoboCycler Gradient 96 with Hot Top (Stratagene Japan K.K., Japan). The PCR products were digested with restriction enzymes, *Hin*f I, *Hha*I, *Rsa*I and *Hae*III (Toyobo, Japan). The digested products were analyzed by electrophoresis on a 3.0% Seakem GTG agarose gel (Cambrex Bio Science, USA) and visualized by illumination with shortwave ultraviolet light after ethidium bromide staining.

Table 1
Anisakid nematodes used in this study and locality of their hosts

Anisakid species[a]	Host species	No. of worms	Locality
Anisakis simplex sensu stricto	*Pleurogrammus azonus*	10	Hokkaido
A. pegreffii	*Scomber japonicus*	10	Kyushu
A. physeteris	*Scomber japonicus*	1	Kyushu
Contracaecum osculatum	*Pleurogrammus azonus*	3	Hokkaido
Hysterothylacium aduncum	*Hypomesus pretiosus japonicus*	5	Hokkaido
Pseudoterranova decipiens	*Pleurogrammus azonus*	5	Hokkaido

[a]Parasites were first identified to the genus level, then species were identified by sequencing of 18S–28S rRNA genes.

2.4. DNA sequencing

We analyzed 18S–28S rRNA gene sequences of each parasite. The following pair of primers for PCR reactions was used: nemspec 18SF (forward; 5′-TCTAGCCTACTAAATAGTCATC-3′), complementary to the region coding for the 18S rRNA, and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′), complementary to the region coding for the 28S rRNA. All PCR reactions were carried out in a final volume of 30 μl containing 3 μl of genomic DNA, 0.75 units TaKaRa Ex *Taq* (Takara), 1× PCR buffer, 0.2 mM dNTP mixture, and 0.3 μM forward and reverse primers. An initial denaturation step at 93 °C for 1 min was followed by 30 cycles of denaturation at 93 °C for 30 s, annealing at 52 °C for 1 min and extension at 72 °C for 75 s with a final extension step at 72 °C for 7 min. The PCR products were purified, ligated into the pGEM-T easy vector (Promega, USA), and transformed into competent *Escherichia coli* DH5α cells. Plasmids were extracted using a Wizard Plus SV Minipreps DNA Purification System (Promega). The DNA sequences were defined using Big Dye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Nucleotide sequences obtained from each species were aligned using the multiple sequence alignment program Clustal W (http://clustalw.ddbj.nig.ac.jp/top-j.html).

2.5. Primer design

Based on the aligned 18S–28S rRNA gene sequences, the following forward primers were designed to amplify different size products; APY (5′-GGCTGGTTGATGAACTGTTG-3′) specific for *Anisakis physeteris*; PD (5′-CGAGTACTTTTTATGGTCGTGAAGT-3′) specific for *Pseudoterranova decipiens*; AC (5′-GACATTGTTATTTCATTGTATGTGTTGAAAATG-3′) specific for *A. simplex* but, common to *A. simplex* sensu stricto and *A. pegreffii*; COS (5′-TGATATGCTTGAAAGGCAGG-3′) specific for *Contracaecum osculatum* and HAD (5′-GCCTTCCATATGCGCGTATA-3′) specific for *Hysterothylacium aduncum*. Moreover, we also designed two intentional mismatch primers to obtain PCR product only from *A. pegreffii* DNA. The sequences of these primers were APE1: 5′-GAGCAGCAGCTTAAGGCAGAGGC-3′ and APE2: 5′-GAGCAGCAGCTTAAGGCAGATGC-3′. The underlined nucleic acid bases indicate artificial mismatched sites (Fig. 1). Artificial mismatched bases were introduced into the terminal region using the methods previously described [23,24]. Universal primer B (5′-GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT-3′) [5] was used as reverse primer.

2.6. PCR and multiplex PCR

To select the optimal position for the artificially mismatched bases in the intentional mismatch primers, PCR reactions were carried out with forward primers having artificial mismatched bases in different positions in the terminal region, APE1 or APE2, and universal reverse primer.

In multiplex PCR, six specific forward primers and one universal reverse primer were used. Six separate tubes containing