

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.

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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 morphos-

pecies *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'-TAGGTGAACCTGCG-GAAGGATCATT-3') and NC2 (reverse; 5'-TTAGTTTCTTTCTCCGCT-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 MgCl₂), 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, *Hinf* 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
<i>Anisakis simplex</i> sensu stricto	<i>Pleurogrammus azonus</i>	10	Hokkaido
<i>A. pegreffii</i>	<i>Scomber japonicus</i>	10	Kyushu
<i>A. physeteris</i>	<i>Scomber japonicus</i>	1	Kyushu
<i>Contracaecum osculatium</i>	<i>Pleurogrammus azonus</i>	3	Hokkaido
<i>Hysterothylacium aduncum</i>	<i>Hypomesus pretiosus japonicus</i>	5	Hokkaido
<i>Pseudoterranova decipiens</i>	<i>Pleurogrammus azonus</i>	5	Hokkaido

^aParasites 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'-TTAGTTTCTTTCTCCGCT-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'-GGCTGGTTGATGAAGTGTG-3') specific for *Anisakis physeteris*; PD (5'-CGAGTACTTTTATGGTCGTGAAGT-3') specific for *Pseudoterranova decipiens*; AC (5'-GACATTGTTATTTTCATTGTATGTGTTGAAAATG-3') specific for *A. simplex* but, common to *A. simplex* sensu stricto and *A. pegreffii*; COS (5'-TGATATGCTTGAAAGGCAGG-3') specific for *Contracaecum osculatium* and HAD (5'-GCCTTCCA-TATGCGCGTATA-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'-GAGCAGCAGCTTAAGGCA-GAGGC-3' and APE2: 5'-GAGCAGCAGCTTAAGGCA-GATGC-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'-GCCGGATCC-GAATCCTGGTTAGTTTCTTTCTCC-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

A. simplex s. str. **GAGCAGTAGCTTAAGGCAGAGTT**
A. pegreffii **GAGCAGCAGCTTAAGGCAGAGTC**
 APE1 **GAGCAG**CAGCTTAAGGCAGAGG**C**
 APE2 **GAGCAG**CAGCTTAAGGCAGATT**GC**

Fig. 1. The sequences of the intentional mismatch primers (APE1 and APE2) compared with those of *A. simplex* s. str. and *A. pegreffii* in the corresponding region. The primers were constructed based on the sequence of *A. pegreffii* and mismatches were introduced at the second base for APE1 (T to G) and at the second and third bases for APE2 (GT to TG). There are two more base differences between *A. simplex* s. str. and the primers (boxed C).

template DNA of each anisakid species were prepared. Following optimization of the annealing temperatures of primers by gradient analysis and primer ratios by titration, a multiplex PCR protocol was established. All reactions were carried out in a final volume of 20 µl containing 0.5 units TaKaRa Ex *Taq* (Takara), 0.2 mM each dNTP, 0.07 µM HAD primer, 0.3 µM other five forward primers, 0.6 µM reverse primer, and various amounts of template DNA (1–40 ng) in 1× PCR buffer. PCR reactions were performed as follows: initial denaturation at 95 °C for 3 min, followed by 30

cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 7 min. All products were electrophoresed in 2.0% agarose gel, followed by staining with ethidium bromide and then visualized under ultraviolet light.

3. Results

3.1. PCR–RFLP analysis

PCR products were digested with restriction enzymes to identify the characteristic of RFLPs. The larvae were distinguished based on combinations of different RFLP patterns. *A. simplex* s. str., *A. pegreffii* and *A. physeteris* were identified based on *HinfI* and *HhaI* restriction profiles (Table 1). On the other hand, *C. osculatum*, *H. aduncum* and *P. decipiens* were distinguished based on *RsaI* and *HaeIII* restriction profiles (Fig. 2).

3.2. Sequence analysis

The nucleotide sequences determined in this study have been deposited in DDBJ/EMBL/GenBank databases under accession numbers AB277821 to AB277826.

A total of 1300 bp sequence of *A. physeteris* (AB277821), 1353 bp sequences of both *A. simplex* s. str. (AB277822) and *A. pegreffii* (AB277823), 1305 bp sequence of *P. decipiens* (AB277824), 1364 bp sequence of *C. osculatum* (AB277825) and 1433 bp sequence of *H. aduncum* (AB277826) were obtained. The aligned sequences of six anisakids including sibling species showed high sequence differences in the ITS region. Sequence differences were 58.4% and 91.3% in the ITS-1 and ITS-2 regions, respectively. However, *A. simplex* s. str.

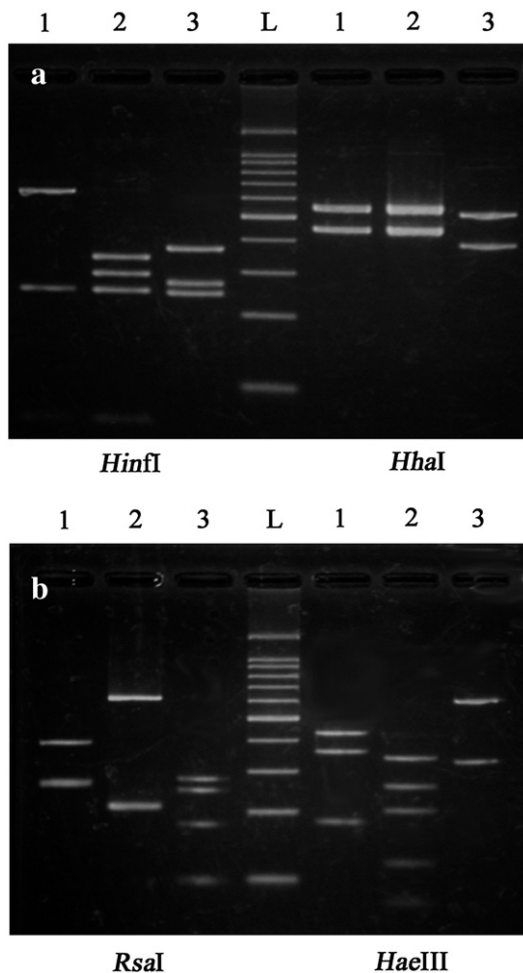


Fig. 2. (a) RFLP analysis using *HinfI* and *HhaI* enzymes. Lane 1, *A. simplex* s. str.; Lane 2, *A. pegreffii*; Lane 3, *A. physeteris*; L, 100 bp ladder. (b) RFLP analysis using *RsaI* and *HaeIII* enzymes. Lane 1, *C. osculatum*; Lane 2, *H. aduncum*; Lane 3, *P. decipiens*; L, 100 bp ladder.

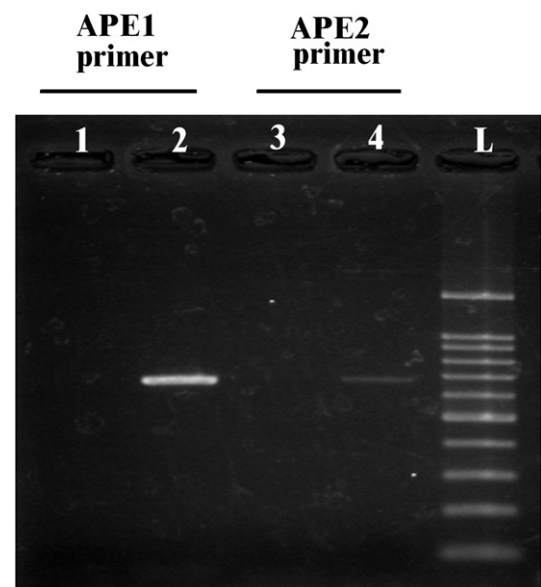


Fig. 3. Specificity test of intentional mismatch primers for *A. simplex* s. str. and *A. pegreffii*. PCR was performed using the intentional mismatch primers APE1 and APE2 (see Fig. 1) as the former, and universal primer B as the reverse primer. Lanes 1, 3, *A. simplex* s. str.; Lanes 2, 4, *A. pegreffii*; L: 100 bp ladder.

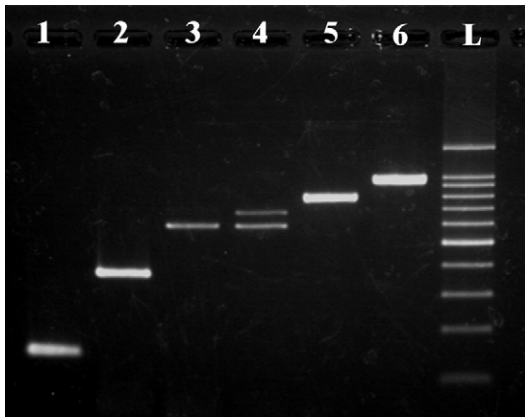


Fig. 4. Molecular identification of anisakid nematodes by multiplex PCR. Lane 1, *A. physeteris*; Lane 2, *P. decipiens*; Lane 3, *A. simplex* s. str.; Lane 4, *A. pegreffii*; Lane 5, *C. osculatum*; Lane 6, *H. aduncum*; L: 100 bp ladder.

exhibited a high level of similarity to *A. pegreffii*, these two sibling species differing only by two base pairs. We detected two C–T transitions in the ITS-1 region between two sibling species.

3.3. The specificity of intentional mismatch primers and multiplex PCR

Intentional mismatch primers APE1 and APE2 (Fig. 1) in PCR were tested experimentally using *A. simplex* s. str. and *A. pegreffii* genomic DNA as a template. Each primer was tested separately. The expected size of the PCR product (672 bp) was obtained only when the template DNA of *A. pegreffii* was present. However, comparison of the sensitivity of each intentional mismatch primer resulted in PCR reactions with the APE2 primer producing only a faint band (Fig. 3). Therefore, six specific forward primers, including APY, PD, APE1, AC, COS and HAD and one reverse primer (primer B) were used in the multiplex PCR reaction. The multiplex PCR products with molecular sizes of 143, 370, 799, and 991 bp were amplified in *A. physeteris* (Lane 1), *P. decipiens* (Lane 2), *C. osculatum* (Lane 5) and *H. aduncum* (Lane 6), respectively (Fig. 4). PCR products with a molecular size of 588 bp were amplified in both *A. simplex* s. str. (Lane 3) and *A. pegreffii* (Lane 4). PCR products of 672 bp were amplified only for *A. pegreffii* (Lane 4) (Fig. 4). The PCR product amplified by APE1 primer can distinguish between *A. simplex* s. str. and *A. pegreffii*. The most reliable SNP genotyping could be obtained using 1 ng samples of genomic DNA.

4. Discussion

Genetic markers provide specific identification of anisakid larvae that lack morphologically distinct characteristics. Molecular techniques using PCR amplification [7,9] and restriction enzyme digestion [4–10] to differentiate anisakid species have been described. In this study, we established a multiplex PCR assay for easy identification of six anisakid species, including two sibling species of *A. simplex*.

We confirmed the presence of specific nucleotide sequences of *A. simplex* s. str., *A. pegreffii*, *A. physeteris*, *C. osculatum*, *P. decipiens* and *H. aduncum* in the ITS region. Therefore, we designed forward primers according to the specific sequences in the ITS region. However, between *A. simplex* s. str. and *A. pegreffii*, 18S–28S rRNA gene sequences are highly conserved; sequence comparison by alignment showed only two C–T transitions in the ITS-1 region. This finding corresponds to those in previous reports [15,17]. For SNP genotyping, we designed intentional mismatch primers containing one or two artificial mismatched bases within the third position from the primer 3' end. The specificity of these primers to discriminate between two sibling species was increased due to the introduction of artificial mismatched bases. Based on this study, it was shown that a mismatch incorporated at the second base from the 3' end in the primer could optimize PCR specificity and reactivity. Greater duplex thermal stability was observed for a duplex containing one mismatch than that containing two mismatches. In addition, we found that the amount of template DNA strongly influenced the result of the multiplex PCR reaction. When the amount of template DNA was greater than 1 ng, nonspecific amplicons were observed.

The PCR product of predicted size (672 bp) was observed only in *A. pegreffii* as a result of introducing an artificial mismatched base. It is possible to differentiate between *A. simplex* s. str. and *A. pegreffii* in multiplex PCR. However, it is not determined yet whether some recombinant genotypes of *A. simplex* s. str. and *A. pegreffii* could be detectable by the present method. Nevertheless, the six multiplex PCR amplicons of different sizes could be visualized by agarose gel electrophoresis. The identity of individual multiplex PCR products was confirmed by specific PCR with each of the single primer pair. Our results indicated that this novel assay is rapid, accurate and cost-effective for the identification of anisakid species. Therefore, multiplex PCR is useful for diagnostic purposes and molecular epidemiological studies of anisakid species.

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