

Anisakis pegreffii: A quantitative fluorescence PCR assay for detection in situ

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ABSTRACT

To facilitate improved diagnosis and detection of the third stage larva (L3) of *Anisakis pegreffii* from the Minnan-Taiwan bank fishing ground in Taiwan Strait, a real-time PCR method for the detection in situ and differentiation was developed to amplify a region of the second internal transcribed spacer (ITS-2) of this parasite. The real-time PCR assay was capable of detecting 1/3 of a single L3 in 30 mg of marine fish tissue, and also exhibited a high level of specificity for *A. pegreffii*, no fluorescence signals were observed in other five major larval anisakid species found in commercial marine fishes caught in this fishing ground.

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1. Introduction

The nematode *Anisakis simplex* sensu lato is a representative zoonotic parasite that invades marine animals and occasionally causes not only anisakiasis but also allergic reactions in sensitized subjects when raw or undercooked seafoods with third-stage larvae (L3) of this parasite are ingested (Sakanari and Mckerrow, 1989; Kobayashi et al., 2007). With the increased popularity of eating undercooked or raw fish dishes, the number of anisakiasis cases may be expected to increase (Chai et al., 2005; Umehara et al., 2008; Cristian, 2009). Anisakids, including *A. simplex* s. l., have a global distribution among a wide variety of marine fishes, and their high prevalence and heavy infections of economically important fishes may have adverse financial effects on fisheries (Farjallah et al., 2006) and on food hygiene and safety control.

Accurate detection of infection is paramount in the management and therapy of food-borne parasitic zoonosis, however traditional techniques, such as microscopy-based methods, detection for *A. simplex* s. l. infection have low sensitivity and are labor intensive. The PCR method has the advantage of being less influenced by observer variation/bias as compared to microscopy-based methods, and many successful PCR-based assays have been developed to date for the diagnosis of *A. simplex* (D'Amelio et al., 1999; Nadler et al., 2005; Zhu et al., 2007; Mattiucci and Nascetti, 2008;

Umehara et al., 2008; Fang et al., 2010). Unfortunately, these assays are time-consuming and do not provide quantitative data. Real-time PCR allows for both differentiation and quantification within a single assay (Harmon et al., 2007), besides, it proved to be more quickly to perform and require no manipulations post-amplification (Yin et al., 2001; MacMillan et al., 2006). So, the purpose of this study is to develop a Taqman probe real-time PCR assay for the detection in situ of *Anisakis pegreffii* based on nuclear ribosomal RNA gene (rDNA) region spanning the second internal transcribed spacer (ITS-2).

2. Materials and methods

2.1. Nematode materials and specificity control DNA

The six major larval anisakid species, *A. pegreffii* (one of the sibling species of *A. simplex*), *Anisakis physeteris*, *Raphidascaris trichiuri*, *Hysterothylacium aduncum*, *Contracaecum muraenesoxi*, and *Contracaecum* sp., found in commercial marine fishes caught in the Minnan-Taiwan bank fishing ground (23.3–24.0°N, 117.2–118.3°E) of Taiwan Strait were identified based on the morphological characteristics (Luo and Fang, 2000; Zhang et al., 2007) and on a PCR method (Fang et al., 2010). One *A. pegreffii* L3 weighs approximately 1 mg. All materials preserved in 75% ethanol. DNA from the parasitic nematodes *A. physeteris*, *R. trichiuri*, *H. aduncum*, *C. muraenesoxi*, and *Contracaecum* sp. were used as specificity controls. Additionally, DNA from the host tissue of big hairtail *Trichiurus haumela* was additionally tested.

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2.2. DNA extraction, ITS-2 sequences amplification and DNA sequencing

Genomic DNA from *A. pegreffii* was extracted by a method of SDS/proteinase K treatment and purified with a Wizard™ DNA Clean-Up columns (Promega) kit used according to the manufacturer's instructions. Purified DNA was stored at -20°C until use. The rDNA ITS-2 sequence was amplified by PCR using two primers IT2 and BD2 (Zheng et al., 2007). PCR assays were performed in a final reaction volume of 50 μL that consisted of 3 μL of genomic DNA, 0.25 μL Taq polymerase (5 U μL^{-1}) (TaKaRa, China), 10 $\times$ PCR buffer 5 μL (TaKaRa, China), 4 μL dNTPs mixture (2.5 mM each) (TaKaRa, China), 3 μL MgCl_2 (25 mM) (TaKaRa, China) and 1.25 μL forward and reverse primers (10 μM each) and 32.25 μL of dH_2O .

An initial denaturation step at 95°C for 5 min was followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 40 s with a final extension step at 72°C for 7 min. PCR products were checked on 1.5% (w/v) agarose gels and visualized using ethidium bromide and UV illumination to confirm product size. Target fragments (about 500 bp) were purified with EZ-10 Spin Column DNA Gel Extraction Kit (Sangon, China) and products were ligated into pMD18-T vector (TaKaRa, China), then transformed into competent *Escherichia coli* DH5 α . Product was bidirectionally sequenced (Invitrogen, Shanghai) and blasted the GenBank Database. Following extraction and identification, the positive recombinant plasmids were used as quantitative template to generate standard curve.

2.3. Primer and probe design

According to ITS-2 obtained above (497 bp), a dual-labeled probe ASsl and two primers FP and RP were designed for real-time PCR using the primer design software Primer Premier v5.0. The FP/RP primer pair amplified a 161 bp product. All primers and probe used in this study were shown in Table 1.

2.4. Plasmid DNA, standard curve generation and real-time PCR detection

Plasmid DNA that including an entire ITS-2 region was extracted using a TIANprep Mini Plasmid Kit (Tianzen, China) and was quantified using a spectrophotometer (NanoDrop ND-1000 USA). A stock solution of purified plasmid DNA was estimated to contain 7.40×10^{10} DNA copies μL^{-1} , a standard curve for *A. pegreffii* was prepared from the stock by 10-fold serial dilution in dH_2O to a lowest concentration of 1 copy μL^{-1} .

Real-time PCR assays were performed in a final reaction volume of 20 μL that consisted of 2 μL plasmid DNA, 2.5 $\times$ RealMasterMix 8 μL , 20 $\times$ Probe Enhancer solution 1 μL ; 0.5 μL of 10 μM forward and reverse primer, 0.25 μL of 10 μM dual-labeled probe and 7.75 μL of dH_2O . Cycling conditions involved an initial activation step of 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 62°C for 40 s using a Rotor Gene 3000 quantitative PCR system (Corbett Research, AUS). For all assays, positive (high-concentration *A. pegreffii* DNA) and negative (absence of DNA template)

controls were included and PCR products were separated on a 2% agarose gel and visualized using ethidium bromide and UV illumination to confirm product size. Threshold cycle (CT) values were determined with the Rotor Gene 4.6.94 software (Corbett Research) utilizing adaptive baseline. After reaching the threshold, the sample was considered positive. When amplification did not occur (no statistical increase in fluorescence), samples were assigned as negative.

2.5. Detection of *A. pegreffii* within the intermediate host tissue

As capacity constraints of kit, DNA were extracted from 1/5, 1/4, 1/3, 1/2, 1, 2, 5, 7, and 10 L3 of *A. pegreffii* mixing with 30 mg tissue of *T. haumela*, respectively, the PCR assay procedure as mentioned above.

2.6. Specificity, sensitivity, and reproducibility of the assay

The specificity was tested by including nucleic acid extracts of *A. physeteris*, *H. aduncum*, *R. trichiuri*, *C. muranesoxi* and *Contracaecum* sp., relevant anisakid nematodes. These samples consisted of the major larval anisakid species found in commercial marine fishes caught in the Taiwan Strait. The target specificity of the probes was tested by sequences BLAST in the GenBank Database. The sensitivity was assessed by testing 10-fold dilutions of the positive plasmids DNA whose amplification copy numbers were diluted from 1×10^8 to 1×10^0 copies μL^{-1} . Fluorescence quantitative PCR reaction was performed in gradient of each dilution as a template. Within the linear range of the minimum initial template concentration shall be the sensitivity of real-time PCR reaction. To assess the reproducibility of the TaqMan® assay results from an intra-assay reproducibility test and an inter-assay reproducibility test (Chen et al., 2009) were conducted. The intra-assay reproducibility of the assay was evaluated by testing six replicates of five different concentrations of reference sample for *A. pegreffii*. The inter-assay reproducibility was determined with five different titers of *A. pegreffii* plasmids for six times interval of 7 days separately. Based on the Ct value obtained from this experiment, coefficient of variation (CV) was calculated. $\text{CV} = s/\bar{x}$, s : standard deviation, $\bar{x}$: average Ct value.

2.7. Comparison of the real-time PCR to conventional PCR assays

The conventional real-time PCR and the TaqMan® RT-PCR were compared in terms of sensitivity by carrying out serial 10-fold dilutions of plasmids as tested samples using the primers FP/RP. Briefly, the PCR assay was carried out in the same 20 μL volume containing 2 μL plasmid, 0.5 U Taq polymerase, 10 $\times$ PCR buffer 2 μL , 0.08 mM dNTP mixture, 0.6 mM MgCl_2 and 0.25 μM forward and reverse primers. An initial denaturation step at 95°C for 3 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 40 s with a final extension step at 72°C for 7 min. The amplified products were analyzed on 2% agarose gel electrophoresis.

3. Results

3.1. Standard curve

Standard curves for *A. pegreffii* was constructed with 10-fold dilution series ranging from 1×10^8 to 1×10^2 (copies μL^{-1}) per reaction. Ct values were obtained after amplification and were plotted against DNA copies. A rise in Ct value was associated with a decrease in DNA content, giving a strong linear relationship between fluorescence and target copy number for each of the

Table 1
Oligonucleotide primers and probe used in this study.

Primer	Sequence (5–3')	locus
IT2	GGTGGATCACTGGGCTCGTG	5.8S rRNA gene
BD2	TATGCTTAATTCAGCGGGT	28S rRNA gene
FP	GACTGTGAAGCATTCGGCAAG	ITS-2
RP	ACCACCAAAGCACCTCACCC	ITS-2
ASsl probe	FAM – CTATCATGGACAATATGAC GAGCGGTTTC – TAMRA	ITS-2

plasmid DNA. The standard curve had a slope of -3.32 , a correlation coefficient R of 0.999 , and amplification efficiency (E) of 1.00 with the dynamic range of detection between 10^8 and 10^2 copies per reaction (Fig. 1).

3.2. Detection of *A. pegreffii* in the intermediate host tissue

One set of primer FP/RP and dual-labeled Probe ASsI specific to *A. pegreffii* were combined in an optimized real-time PCR system. The FAM fluorescent signal could be observed specifically when the templates DNA were extracted from 1, 2, 5, 7, and 10 L3 of *A. pegreffii* mixing with 30 mg tissue of *T. haumela*, respectively (Fig. 2). None positive fluorescent signal was not observed as the templates DNA were only from the fish tissue.

Results from the test to determine the maximum detection limit of this method showed that, 1/2 and 1/3 worm mixed in 30 mg tissue could be detected in less than 30 cycles, the rest couldn't be detected (Fig. 3). So, at this detection limit, 1/3 of a single L3 of *A. pegreffii* in 30 mg of marine fish tissue can be detected.

3.3. Specificity of the real-time PCR

In the specificity test, a single positive fluorescent signal could be detected by the amplification system for *A. pegreffii*. When nucleic acid extracts of *A. physeteris*, *H. aduncum*, *R. trichiuri*, *C. muraenesoxi* and *Contracaecum* sp. were added to the amplification system, none of the positive fluorescent signals could be collected or signals higher than the detection threshold (Fig. 4).

3.4. Sensitivity of the real-time PCR assay

By using 10-fold serial dilutions of *A. pegreffii* plasmids DNA, the sensitivities of the real-time PCR assay were determined to be 1×10^2 copies μL^{-1} , which are about 100 times higher than those conventional PCR assays (Fig. 5).

3.5. Reproducibility of the real-time PCR

The coefficients of variation (CVs) of Ct values for detection of *A. pegreffii* were carried out in six replicates (Fig. 6). The intra-assay CVs for Ct values ranged between 0.46% and 1.25%, and the CVs of the inter-assay ranged between 1.64% and 2.96%, indicating the reproducibility of the assay (Table 2).

4. Discussion

The present study is actually the extension of our previous work (Fang et al., 2010). The advent of real-time quantitative PCR technique has proven useful in various applications, including pathogen detection, gene expression and regulation, and allelic discrimination (Das et al., 2000; Fujii et al., 2000). The genetic marker commonly used in real-time PCR assay for detection of anisakid larvae were rDNA ITS-1 sequence (Mossali et al., 2010) and cytochrome c oxidase II gene (Lopez and Pardo, 2010). Comparing with ITS-1, ITS-2 was more variable and have more informative site (Zhu et al., 2000; Zhang et al., 2009). In the present study, a Taqman real-time quantitative PCR assay for the detection of *A. pegreffii*

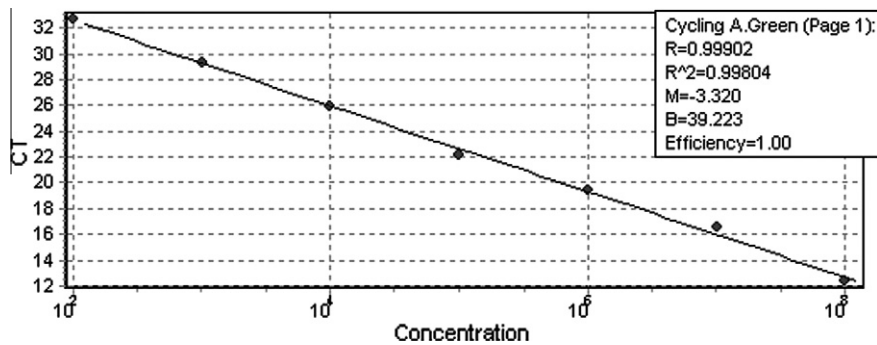


Fig. 1. Standard curve establishment for quantification of *A. pegreffii*. Serial dilutions of *A. pegreffii* plasmids DNA, ranging from 1×10^8 to 1×10^2 (copies μL^{-1}), were used as the template for real-time PCR analyses. Notes: vertical ordinate: Ct (cycle threshold) value; horizontal ordinate: the original copies; R: correlation coefficient; R^2 : coefficient of determination; M: slope; B: intercept; E: efficiency.

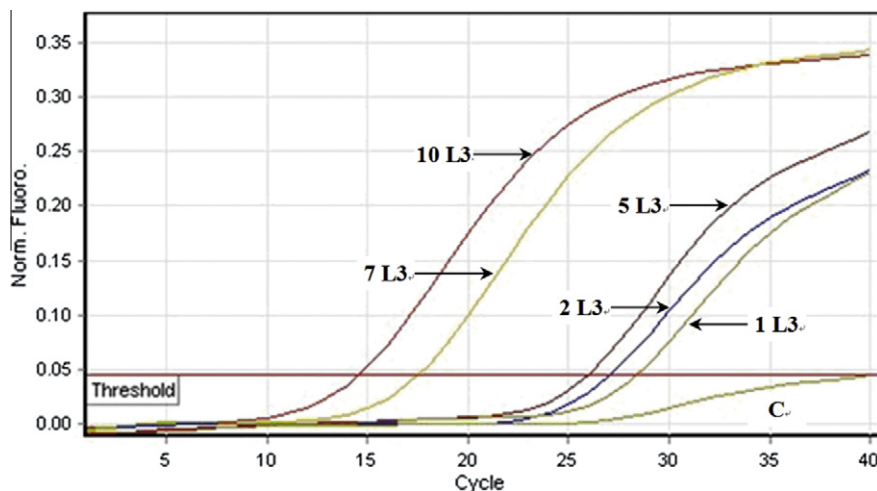


Fig. 2. Detection of *A. pegreffii* in the intermediate host tissue. FAM fluorescent signals were observed in the real-time PCR for *A. pegreffii* with DNA extracted from 1, 2, 5, 7, and 10 L3 of *A. pegreffii* mixing with 30 mg tissue of *T. haumela* as templates, respectively. C, DNA template from tissue of *T. haumela* as negative control.

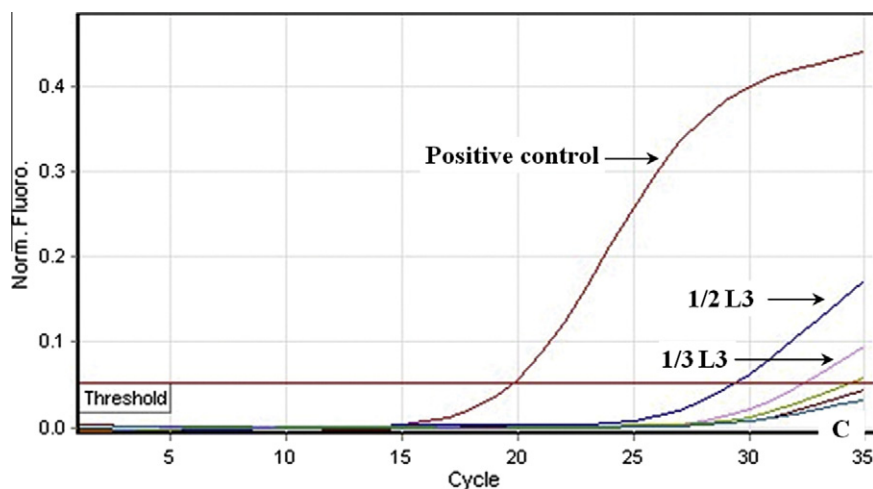


Fig. 3. Real-time PCR for *A. pegreffii* with DNA extracted from 1/2, 1/3, 1/4, and 1/5 L3 of the worm mixing with 30 mg tissue of *T. haumela*. FAM fluorescent peaks could be only detected from 1/2 and 1/3 L3 in less than 30 cycles, the rest couldn't be detected. Positive control, DNA template from *A. pegreffii* was used as positive control. C, DNA template from tissue of *T. haumela* as negative control.

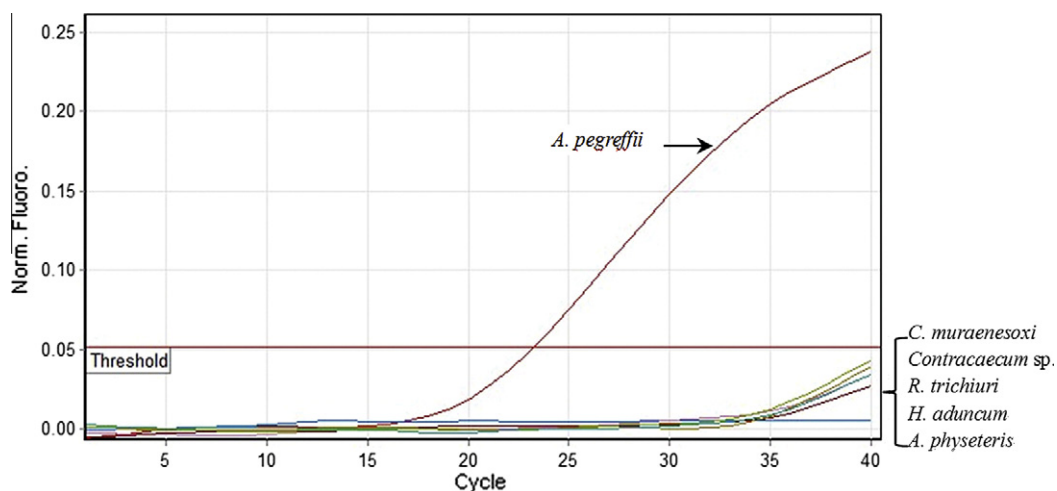


Fig. 4. Specific amplification curve for *A. pegreffii*. A positive FAM fluorescent signal special for *A. pegreffii* was collected in the real-time PCR. None of FAM signals could be observed or signals higher than the detection threshold when other five anisakid species as templates.

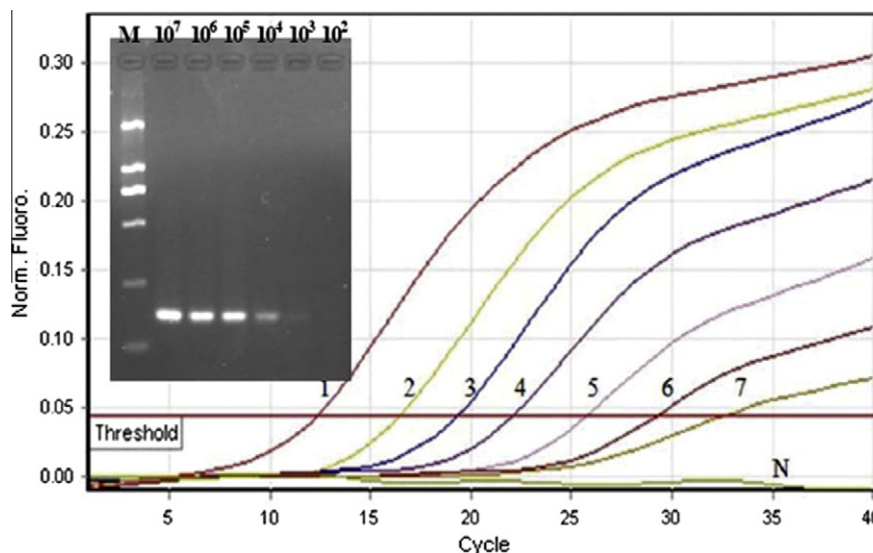


Fig. 5. Sensitivity comparison for *A. pegreffii* detection between the real-time PCR and the conventional PCR. Samples were diluted serially 10-fold DNA plasmids. *M* = 2 kb DNA ladder. 1: 1×10^8 , 2: 1×10^7 , 3: 1×10^6 , 4: 1×10^5 , 5: 1×10^4 , 6: 1×10^3 , 7: 1×10^2 , N: negative control. Unit: copies μL^{-1} .

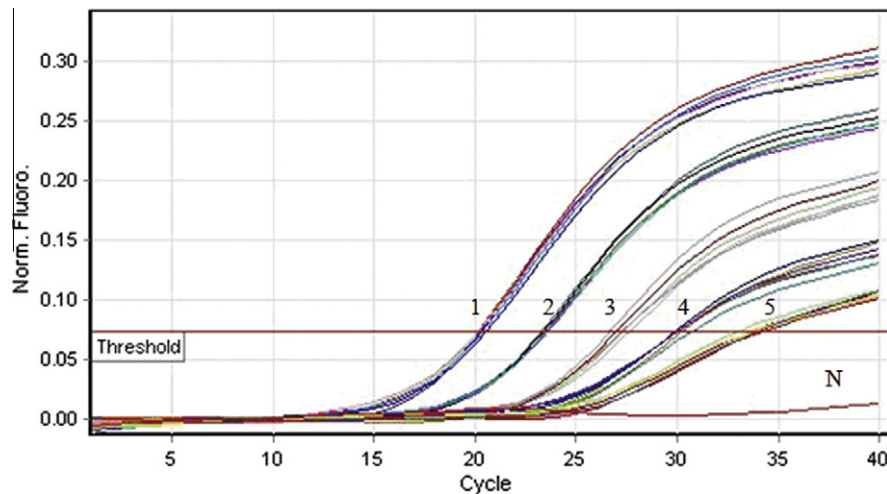


Fig. 6. The dynamic curve of the real-time PCR for reproducibility assay. 1–5: real-time PCR results of the recombinant plasmid concentrations of 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , and 1×10^2 copies μL^{-1} ; N: negative control.

Table 2
The reproducibility assay of the TaqMan real-time PCR.

	Concentration (copies μL^{-1})	$\bar{x} \pm s$	Coefficient of variation (%)
The intra-assay reproducibility	1×10^6	20.24 ± 0.16	0.77
	1×10^5	23.46 ± 0.11	0.46
	1×10^4	27.18 ± 0.32	1.17
	1×10^3	30.07 ± 0.30	1.00
	1×10^2	33.59 ± 0.42	1.25
The inter-assay reproducibility	1×10^6	20.75 ± 0.35	1.71
	1×10^5	23.88 ± 0.49	2.03
	1×10^4	27.32 ± 0.45	1.64
	1×10^3	30.83 ± 0.91	2.96
	1×10^2	34.35 ± 0.90	2.63

was established using rDNA ITS-2 region as molecular target. In test to determine the maximum detection limit of this method, only 1/2 worm mixed in 30 mg tissue is stable and a peak can be detected every time in less than 30 cycles. Although the peak appeared late for 1/3 of the worm mixed with 30 mg tissue can be detected within 30 cycles, its repetitive was a little bit worse.

Recently, it has been recognized that the morphospecies *A. simplex* s. l. 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. As we have not found *A. simplex* s. s. and *A. simplex* C in the economic fishes from Taiwan Strait, we adopted a strategy to blast our probe sequence to the GenBank database, the result revealed maximum identity to no other DNA sequences available other than ITS-2 sequences of *A. simplex* s. s., *A. pegreffii* and *A. simplex* C (Jefferies et al., 2009). Therefore the primer pair and probe provided here might be universal for *A. simplex* s. l. As *A. simplex* s. s. and *A. pegreffii* widely extend across geographic ranges (Mat-tiucci and Nascetti, 2006; Umehara et al., 2008), they might coexist in marine fishes. Under this circumstance, real-time PCR combined with conventional PCR-RFLP assay is recommended to determine what exactly the sibling species is at present.

As the quality of extracted DNA from mixed materials of nematodes and fish tissues is not easy to control, so that will affect the tissue detection curve of the linear relationship as compare with the extracted plasmid DNA, but the detection in this study is still has a relatively higher sensitivity. The ASsl probe specific for *A. pegreffii* in this study showed satisfying specificity, and no cross-reactivity was observed with *A. physeteris*, and other major larval

anisakid species distributed in commercial marine fishes caught in the Minnan-Taiwan bank fishing ground.

The rapid identification of the pathogenic zoonotic parasite is a crucial parameter, and is especially important for food-borne disease control of human. The real-time PCR is capable of rapid and accurate diagnosis of *A. pegreffii*. When comparing with the conventional PCR assays, the real-time PCR in this study is less labor intensive. Real-time PCR method also has the advantage of being less time-consuming (Bell and Ranford-Cartwright, 2002), since there is no need for post-PCR handling, such as agarose gel electrophoresis of the PCR product. In our hands, it takes a mere 2.5 h to complete the detection, as opposed to approximately 4 h for conventional PCR. Finally, the adaptability of real-time PCR to a high-throughput 100-well format should significantly reduce the overall time spent per sample.

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