



Parasitism between *Anisakis simplex* (Nematoda: Anisakidae) third-stage larvae and the spotted mackerel *Scomber australasicus* with regard to the application of stock identification

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ABSTRACT

The nematode fauna of 369 spotted mackerel of the species *Scomber australasicus*, collected off the northeastern Taiwanese coast of the northwestern Pacific, was investigated monthly from April 2004 to March 2005. The following nematode species were recorded: *Anisakis simplex* complex, *Hysterothylacium aduncum*, *Porrocaecum decipiens* and *Raphidascaris trichiuri*. The seasonal variation in the infection with *A. simplex* third stage larva (L3) was studied throughout the 12 months. The prevalence of *A. simplex* L3 recorded for total fish samples was 93.6%, varying between 86.7 and 100%. There was an increase in the abundance of this nematode in spring, with the peak occurring in April. To reveal whether intrinsic factors of the spotted mackerel host contributed to infection with this nematode, fish were grouped according to their body weight, age and gonad development (reported as gonadosomatic index, GSI), respectively, and infection parameters (i.e., prevalence, abundance and intensity) were analyzed. Results showed that abundance was significantly higher in both larger (>450 g) and older (>3 years old) fish. The gonad development of the host fish was not correlated with the intensity of the larval infection in both female and male fish. Two distinct *Anisakis* species were identified by PCR-RFLP, namely *A. pegreffii* and a recombinant genotype of *A. pegreffii* and *A. simplex* sensu stricto. These species occurred with frequencies of 97% and 3%, respectively. The usefulness of using parasites as biomarkers for spotted mackerel stock identification around Taiwanese waters was confirmed herein. A second group of 58 spotted mackerel were obtained from the coastal waters off southwestern Taiwan. In addition to the two species, *A. pegreffii* and the recombinant one, which were found with frequencies of 63% and 9%, respectively, an additional *Anisakis* species *A. typica* was identified with a frequency of 28% from these fish. Two spotted mackerel stocks could thus be identified based on their infrastructure of *Anisakis* species community and their frequency. To the best of our knowledge, this is the first report of stock identification of spotted mackerel using endoparasite biomarkers.

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1. Introduction

Anisakis simplex is one of the most significant food-borne parasites in human infections (Dorny et al., 2009). More specifically, this zoonotic worm is a fish-borne nematode that utilizes a very large number of fish species as intermediate or paratenic hosts. Humans can become accidental hosts by consuming raw or undercooked fish or seafood that contains the third-stage larvae (L3) of *A. simplex*. Humans might subsequently suffer by two distinct clinical entities, namely gastrointestinal anisakiasis and allergic anisakiasis (Daschner et al., 2000; Audicana et al., 2002; Shih et al., 2010). The fact that high prevalences of this nematode have been found in multiple economically important fish species, including herring, hake, black plaice, cod and cobia (Dorny et al., 2009; Shih et al., 2010), indicates that damage to the fishing industry could occur by considerably reducing the quality of fish, leading to a loss in marketing values. Furthermore, the presence of larvae in the flesh of fish may significantly lower the aesthetic quality of the product, especially with regard to the possible allergenic nature of the larvae or even molecular traces of larvae (Levsen and Lunestad, 2010).

The spotted mackerel *Scomber australasicus* is a pelagic fish that is restricted to the Pacific Ocean, the southeastern Indian Ocean and the Red Sea; and it is one of the most important commercial fishing species in Taiwan (Tzeng, 1988). According to official information, the Taiwanese annual yield and output value of this fish species during the last two decades was approximately 30,000–80,000 metric tons and 3.0–4.5 million US dollars, respectively (<http://www.fg.gov.tw/>). In addition to the wide circulation and consumption of this fish in Taiwan, over 80% of catches were industrially produced as frozen, salted or canned fish and were exported worldwide. Careful monitoring of zoonotic infections to ensure the safety of this seafood is an important issue in fish processing. Unfortunately, the problem concerning both aesthetic quality and public health (as anisakid nematode larvae have appeared within tomato sauces and canned mackerel) has occurred in Taiwan (Shih, 2004a).

The aim of this study was to analyze the parasitism between spotted mackerel collected from the coastal waters of northeastern Taiwan and *A. simplex* L3 by investigating infection data, including prevalence, mean intensity and abundance. The seasonal dynamics of this larva and the influences of the three intrinsic factors of fish, i.e., body length, age and sex, on parasitism were examined. Furthermore, the usefulness of *Anisakis* species as biomarkers for spotted mackerel stock identification around Taiwanese waters was tested in this study.

2. Materials and methods

2.1. Parasite materials

Spotted mackerel used in this study were caught from the coastal waters of Tashi, in northeastern Taiwan (25°N 122°E) between April of 2004 and March of 2005. Generally, about 30 specimens were collected by a Danish seiner

on a monthly basis. A total of 369 spotted mackerel were examined. Fresh specimens were frozen and transported to the laboratory.

After thawing, the fish were numbered, measured (fork length) and weighed. Then they were dissected, sexed and their gonads were also weighed. The fish were filleted and deskinning, and the musculature was examined by candling and using a pair of tweezers. The body cavity, gastrointestinal tract and the viscera were examined to determine the presence of nematode larvae or adults either with the naked eye or under a dissecting microscope. Nematode samples were fixed, stored in 70% alcohol and cleared in glycerin before examination. Anisakid nematodes were identified to species and developmental stage based on morphological characters described previously (Smith, 1983; Anderson, 2000; Shih and Jeng, 2002; Shih, 2004b). The population descriptors (prevalence, mean intensity and abundance) were calculated according to definitions given by Bush et al. (1997).

2.2. Spotted mackerel age estimation and grouping

Age and growth of spotted mackerel from the waters near the sampling location of this study had been previously studied by means of scale reading (Ku and Tzeng, 1985). The von Bertalanffy growth equation obtained for this area is as follows:

$$L_t = 420.8 \text{ mm} \times [1 - e^{-0.319(t+1.143)}]$$

where L_t is the body length and t is the fish age.

Using this equation, fish age was calculated from body length measurements.

Based on their ages, fish were divided into five groups: group 0+ included fish younger than one year old, group 1+ included fish older than one but younger than two years old, group 2+ included fish older than two but younger than three years old, group 3+ included fish older than three but younger than four years old and group 4+ included fish older than four but younger than five years old. Each group consisted of 28, 113, 171, 44 and 13 fish, respectively.

Based on their body weights, fish were divided into four groups: <150, 150–300, 301–450 and >450 g in 150 g increments. Each group consisted of 95, 103, 129 and 42 fish, respectively.

2.3. Gonadosomatic index calculation

The gonadosomatic index (GSI) of each sample was calculated according to the equation provided by Wang and Chen (1989): $GSI = GW/SW \times 100$, where GW, refers to gonad weight and SW refers to the somatic weight of spotted mackerel. In total, 95 fish, including 49 female and 46 male, were sampled to calculate their GSI. These fish were mainly collected during the months when their gonads developed and spawned, i.e., April and December of 2004 and January through March of 2005.

2.4. Molecular delineation of *Anisakis* sp. third-stage larvae (L3)

The species of *Anisakis* sp. L3 were identified by a PCR-RFLP analysis as previously described (D'Amelio et al., 2000; Abollo et al., 2003). Larvae were randomly picked from the pooled monthly sample. To compare the infrastucture of the *Anisakis* spp. community inside the spotted mackerel, a second group of 58 fish were obtained by a Danish seiner from coastal waters off of Kaohsiung (22°N 120°E) in southwestern Taiwan, between March and April 2005. One hundred individual L3 from each group were randomly picked and assayed as described.

The same protocols and conditions for the PCR-RFLP analysis of ribosomal DNA (rDNA) internal transcribed spacers (ITS-1 and ITS-2) were followed in this study. Two endonucleases *Hinf*I and *Hha*I were used to delineate between the three sibling species of the *A. simplex* complex, namely *A. simplex* sensu stricto, *A. pegreffii*, and *A. simplex* C, and *A. typica*, which is not member of the *A. simplex* complex.

2.5. Statistical analysis

The standard parameters, prevalence (proportion of the population infected), abundance (mean number of parasites of both infected and uninfected fish), and mean intensity (mean number of parasites of infected fish) from monthly, fish body weight and fish age groups, were determined for the *Anisakis* infection on spotted mackerel (after Bush et al., 1997). In addition, extra parameter intensity (number of parasites of a single infected fish) was determined from 95 fish which were sampled to calculate their GSI. Chi-square was employed to test for statistically significant differences in prevalence between groups. Analysis of variance (ANOVA) and Duncan's new multiple range tests were used to test the significant differences in abundance between groups. Significance level was established at ($P < 0.05$). The relationships between parasite intensity and fish size, age and GSI value were tested by regression analysis. Analyses were performed using the SAS software for Windows Version 9.0.

3. Results

Four anisakid nematodes were identified as the most abundant parasites in *S. australasicus* collected from Taiwanese waters: *A. simplex* complex, *Hysterothylacium aduncum*, *Porrocaecum decipiens* and *Raphidascaris trichiuri*. In addition, a few adult digeneans belonging to the Hemuridae family were also observed (data not shown), with a prevalence of 0–4% throughout the year. Among these four anisakids from three developmental stages including L3, fourth-stage larvae (L4) and adults of *H. aduncum* and *R. trichiuri* were harbored in the gastrointestinal tracts of spotted mackerel. For *A. simplex* complex and *P. decipiens*, L3 were present only in the stomach or abdominal cavity, but never in the musculature.

The prevalence recorded for the total sample of spotted mackerel was 93.6% for *A. simplex* complex L3, 54.5% for all three developmental stages of *H. aduncum*, 35.2%

for all three developmental stages of *R. trichiuri* and 8.1% for *P. decipiens* L3. The abundance recorded from total fish samples was 16.2 for *A. simplex* complex L3, 1.6 for all developmental stages of *H. aduncum*, 0.8 for all developmental stages of *R. trichiuri* and 0.1 for *P. decipiens* L3. Among these anisakids, *A. simplex* complex L3 was found to be the dominant species as shown by Simpson's dominance index ($P_i = 85.9\%$) and one-way analysis of variance ($P < 0.05$), and thus, *A. simplex* complex L3 was selected to be the specific object in this study.

A. simplex complex L3 was distributed mainly in the body cavity of spotted mackerel examined in this study. Most of them were free and moved around actively. L3 were also found inside the stomach in lower quantities compared to those appearing in the body cavity. During the spotted mackerel breeding season (January to May) great amounts of L3 occasionally accumulated on the lateral, posterior portion of the fishes' gonad (Fig. 1a). L3 were mainly encapsulated in the peritoneal mesentery (Fig. 1b) and occasionally on the liver surface (Fig. 1c) of the fish during the summer time (May to July).

The abundance of *A. simplex* complex L3 recorded for total fish samples was 16.2, varying between 3.4 and 83.4 throughout the sampling period. The equivalent values for prevalence were found to be 93.6% and 86.7–100%, respectively. No significant difference in prevalence was observed between the monthly groups ($P = 0.10$). The equivalent mean intensity values were 17.1 and 3.9–89.4, respectively (Table 1). The progression of the abundance was relatively even from month to month with the exception of one peak in April. April and May, which had the highest and the second highest abundance levels, differed significantly from each other and from all other months ($P < 0.0001$). A distinct annual maximum occurred in April (Fig. 2).

The fish samples were then divided into four groups according to their body weight, and the prevalence of *A. simplex* complex L3 was noted for each group. Prevalence in fish with body weights of <150, 150–300, 301–450 and >450 g varied from 90.5%, 93.2%, 93.6% to 95.2%, respectively, with the abundance values of 4.7, 8.9, 14.9 and 34.1, respectively (Fig. 3). None of the prevalence values differed significantly from each other ($P = 0.99$). Fish with a body weight of >450 g had the highest abundance which differed significantly from the others ($P < 0.01$). Fish with body weight of 301–450 g had the second highest abundance which differed significantly from two groups (body weight <150 g and 150–300 g) ($P < 0.01$). However, the correlation analysis between the intensity of these larvae and the fish size showed no significant correlation was found ($R^2 = 0.1002$, $F = 39.3039$, $P > 0.05$), meaning that there was not a significant increase in total L3 burden with increasing body weight.

The total fish samples were divided into five groups according to their age. From group 0+ to group 4+, the prevalence of *A. simplex* complex L3 was 92.9%, 90.3%, 94.2%, 84.1% and 100%, respectively, and the abundance was 7.4, 6.2, 14.5, 42.9 and 27.2, respectively (Fig. 4). None of these prevalence values differed significantly from each other ($P = 0.84$). There was a significant increase in abundance between the three younger fish groups and the other two older ones ($P < 0.005$). However, no significant relation-

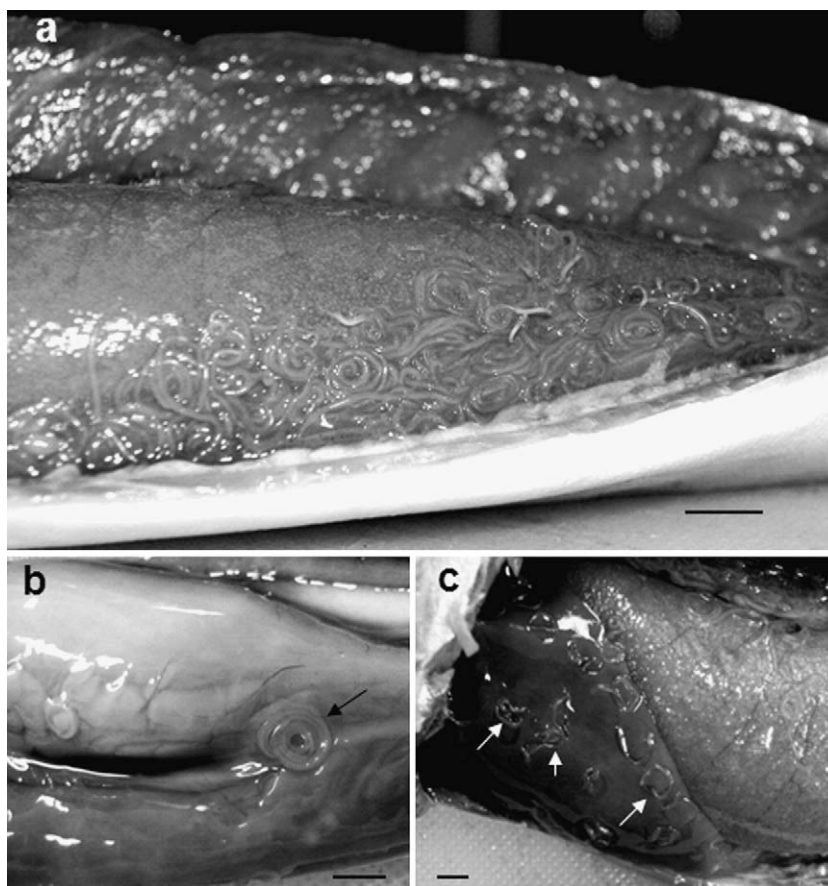


Fig. 1. *Anisakis simplex* third-stage larvae (L3) infection of the spotted mackerel, *Scomber australasicus*. (a) L3 accumulated on the lateral, posterior portion of fish gonad. Scale bar = 10 mm. (b) Encysted L3 in a typically spiral shape (arrow) was located mainly in mesentery tissue. Scale bar = 5 mm. (c) L3 encapsulated on the liver surface (arrows). Scale bar = 5 mm.

ship between the fish age and the intensity of these larvae was observed ($R^2 = 0.0333$, $F = 12.6479$, $P > 0.05$).

The correlation between the gonad development of spotted mackerel according to the gonadosomatic index (GSI) and the intensity of *A. simplex* complex larval infection was analyzed (Fig. 5). And no significant correlations

were found in both female ($R^2 = 0.0426$, $F = 1.9587$, $P > 0.05$) and male ($R^2 = 0.0503$, $F = 2.2785$, $P > 0.05$) fish.

PCR-RFLP patterns of all specimens corresponded to either that of *A. pegreffii*, *A. typica* or a recombinant genotype of *A. pegreffii* and *A. simplex* sensu stricto. Amplification of the rDNA region, spanning the ITS-1, ITS-2 and the 5.8S

Table 1

Infection parameters of *Anisakis simplex* complex L3 recovered from every monthly sample in the spotted mackerel, *Scomber australasicus*. Summed data for all months are given in *italics*.

Date	<i>n</i>	Σ No. of L3	Prevalence (%)	Mean intensity (range)	Abundance
2004					
Apr	30	2502	93.3	89.4 (2–550)	83.4
May	26	745	96.2	29.8 (1–89)	28.7
Jun	45	215	93.3	5.1 (1–23)	4.8
Jul	54	253	88.9	5.3 (1–19)	4.7
Aug	44	341	90.9	8.5 (1–47)	7.8
Sep	14	88	92.9	6.8 (1–15)	6.3
Oct	15	186	100.0	12.4 (1–60)	12.4
Nov	30	102	86.7	3.9 (1–12)	3.4
Dec	22	273	95.5	13.0 (1–126)	12.4
2005					
Jan	27	143	88.9	6.0 (1–31)	5.3
Feb	28	264	96.4	9.8 (1–54)	9.4
Mar	34	518	100.0	15.2 (1–270)	15.2
Total	369	5630	93.6	17.1	16.2

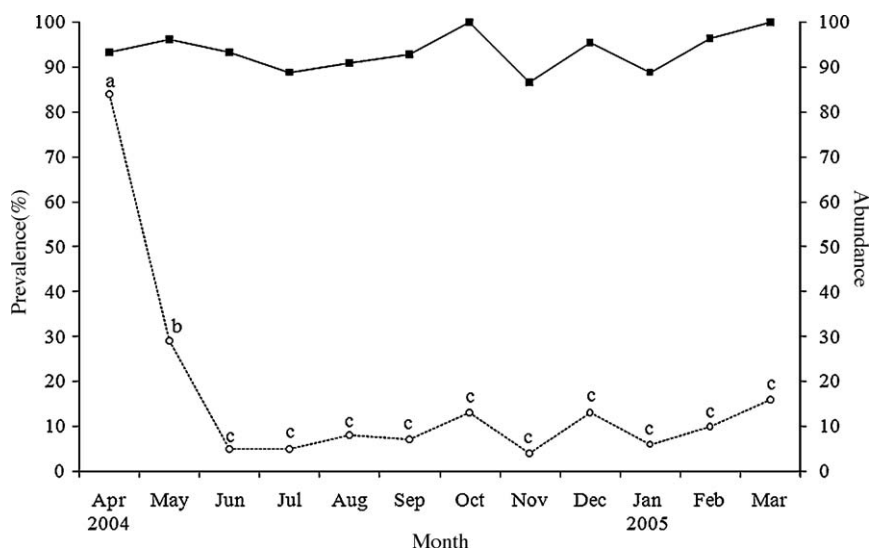


Fig. 2. Prevalence (black squares) and abundance (white circles) of *Anisakis simplex* L3 throughout the sampling period. Significant differences are indicated by different case letters ($P < 0.0001$).

subunit, produced a fragment of ~960 base pairs (bp). Digestion with *Hinf*I produced three bands of approximately 370, 300, and 250 bp in *A. pegreffii* (Fig. 6a, lane 1) and four bands of 620, 370, 300 and 250 bp in the recombinant genotype (Fig. 6b, lane 1). On the other hand, *A. typica* showed two bands of 620 and 350 bp (Fig. 6c, lane 1). Digestion with *Hha*I produced two major bands of 550 and 430 bp in both *A. pegreffii* (Fig. 6a, lane 2) and the recombinant genotype (Fig. 6b, lane 2), while *A. typica* showed four bands of 320, 240, 180 and 160 bp (Fig. 6c, lane 2). However, *A. simplex sensu stricto* was not found in this investigation.

The distribution of *Anisakis* sp. L3 in Taiwanese waters was revealed by PCR-RFLP analysis. Two species, i.e., *A. pegreffii* and the recombinant of *A. pegreffii* and *A. simplex* s.s., were identified in specimens pooled from both northeastern and southwestern Taiwan. One additional *Anisakis*

species *A. typica*, which is not a member of the *A. simplex* complex, was only identified in samples from southwestern Taiwan. *Anisakis pegreffii* was the most frequently found species in both the northeastern and southwestern samples (97% and 63%, respectively). The recombinant occurred with a frequency of 3% in samples from northeastern Taiwan and 9% in those from southwestern Taiwan (Fig. 7). *Anisakis typica* occurred only in the southwestern samples with a frequency of 28% (Fig. 7).

4. Discussion

The most important result of this study is related to the relationship between *A. simplex* complex L3 and the spotted mackerel collected from the Northwestern Pacific throughout the year of sampling. Although *A. simplex* s.s.

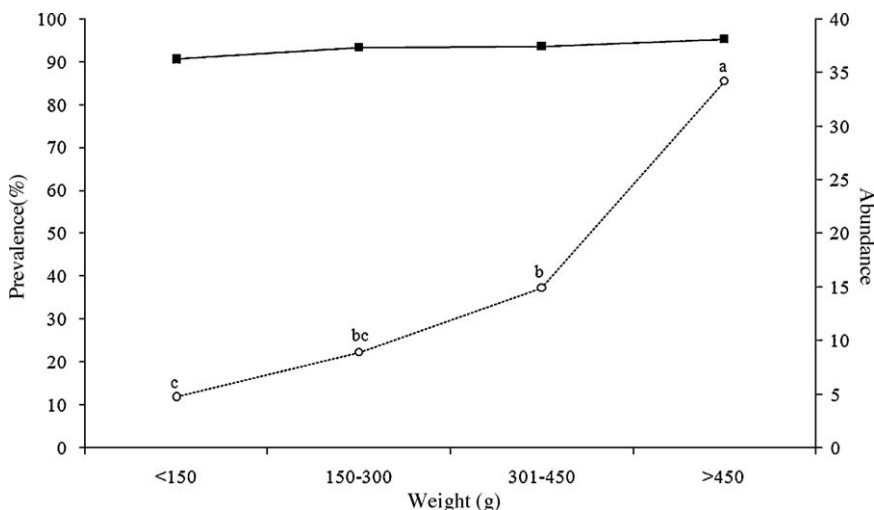


Fig. 3. Prevalence (black squares) and abundance (white circles) of *Anisakis simplex* L3 with the body weight of spotted mackerel hosts. Significant differences are indicated by different case letters ($P < 0.01$).

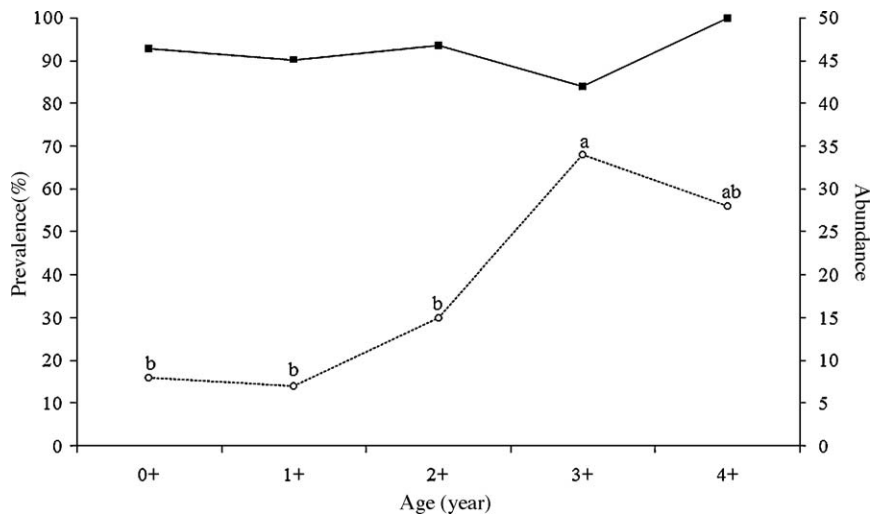


Fig. 4. Prevalence (black squares) and abundance (white circles) of *Anisakis simplex* L3 with the age of spotted mackerel hosts. Significant differences are indicated by different case letters ($P < 0.005$).

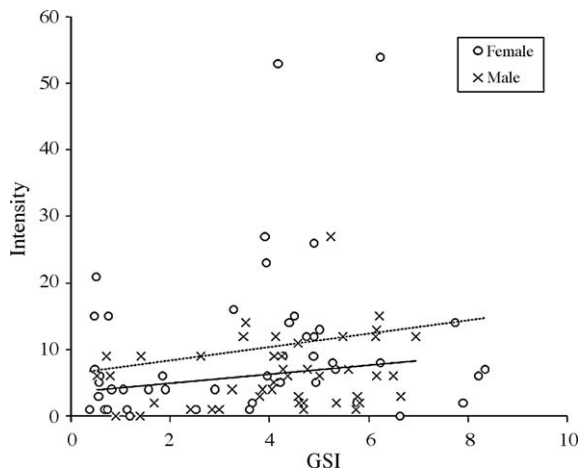


Fig. 5. Correlations of intensities of *Anisakis simplex* L3 with the gonadosomatic index (GSI) of spotted mackerel hosts.

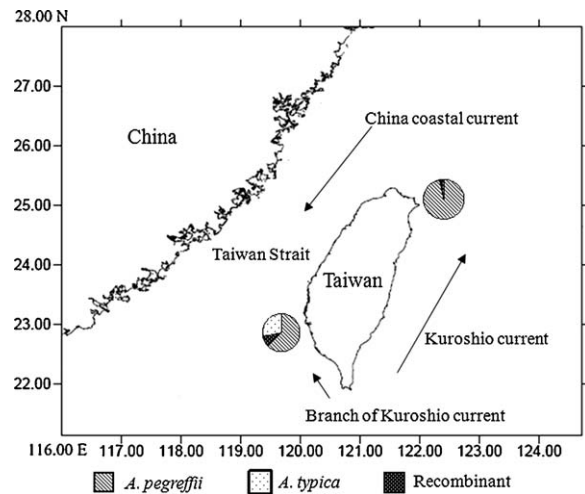


Fig. 7. Distribution of *A. simplex* L3 in Taiwanese waters.

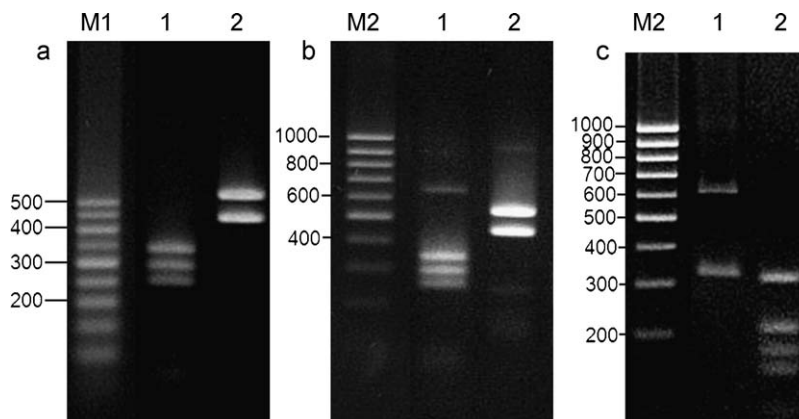


Fig. 6. PCR-RFLP analysis for the identification of *Anisakis simplex* L3. (a) *A. pegreffii*. (b) Recombinant genotype of *A. pegreffii* and *A. simplex* s.s. (c) *A. typica*. RFLP patterns digested by the endonucleases *HinI* (lane 1) or *HhaI* (lane 2). M1: DNA marker 1 (50-bp ladder), M2: DNA marker 2 (100-bp ladder).

had previously been recovered from this species of fish while studying the morphology of two *Anisakis* species (Quiazon et al., 2008), this is the first report concerning the endoparasitic, especially nematode, fauna of this fish. On the other hand, the assemblages of their ectoparasites, including monogeneans, trematodes, copepods and isopods, have been investigated previously (Perera, 1993; Hayward et al., 1998).

Seasonal studies of parasitic infections of marine fishes are important because low intensities of infection that are otherwise harmless may increase and become pathogenic only at certain times of the year (Perera, 1993). Although seasonality was not found in the ectoparasitic fauna of *S. australasicus* (Perera, 1993), it did occur in the endoparasitic nematode *A. simplex* L3 based on the present results. The distinct seasonal variation with a clear abundance peak in April was observed as well. A similar phenomenon was previously reported in three fish species of saithe (*Pol-lachius virens*), cod (*Gadus morhua*) and redfish (*Sebastes marinus*) from Norwegian waters. It is evident that the term "spring rise" has been used to describe the distinct increase in the abundance of *A. simplex* L3 during the springtime of March and April (Strømnes and Andersen, 2000). Two major factors were suggested to contribute to this phenomenon: the increased supply of parasite eggs from the northward-migration of whales and the general spring bloom of plankton (Strømnes and Andersen, 2000). Based on the observations that the major stomach contents of spotted mackerel studied herein were euphausiids (data not shown), which is an important paratenic host of *A. simplex* and that the northeastern Taiwanese waters where the fish were sampled include the whale southward-migration route during the spring and summer (Dei, 2003), the significant abundance peak of *A. simplex* L3 in April presented in this study might be possibly influenced by the same factors reported in the Norwegian waters. In addition, the fact that extraordinarily high prevalence values, from 86.7% to 100% with a mean of 93.6%, appeared through these successive sampling months indicated that *A. simplex* was beyond a doubt an endemic parasite around Taiwanese waters.

To investigate the influences of the intrinsic factors of the spotted mackerel host on infection with *A. simplex* L3, fish were grouped according to their body weight, age and gonad development (shown as GSI) and infection parameters were analyzed. The results showed that abundance was significantly higher in both larger and older fish groups. The cumulative effect was possibly a result of continuous re-infestation by feeding. Evidence that the nematodes remained in fish hosts long enough to accumulate as they grew (Sardella and Timi, 1996) was clear, and the main factor determining the infection was fish size and age within this parasitism. No significant correlation between larvae intensities and host GSIs of each sex showed that the infection status did not interfere with host fertility from the aspect of the gonad development; although, great amounts of L3 were occasionally found to have accumulated on fish gonads. Most importantly, it also suggested that host reproductive investment may not be a major factor contributing to parasite infection. The hypothesis that females invest more in reproduction by producing larger gametes than males and thus females become more susceptible to par-

asite infection was not supported in the present study, although this hypothesis was proposed in female stone loach (*Barbatula barbatula*) in which positive correlations between the abundance of *Gyrodactylus* spp. and *Raphidascaris acus*, and both a gonad mass and GSI were found (Šimková et al., 2005).

Parasites have been used as biological markers to provide information on various aspects of host biology for almost a century (MacKenzie, 1987). Furthermore, these markers were applied in population studies of marine fish, such as stock separation, migration, diet and phylogenetics (Williams et al., 1992). The usefulness of parasites as adequate tools to clarify the taxonomic status of their hosts belonging to the genus *Scomber* has also been demonstrated. The analyses of *Kuhnina* monogeneans and didymozoid digeneans of four populations of the chub mackerel *S. japonicus*, which came from two localities in the Atlantic Ocean and two localities in the Pacific Ocean, strongly suggest the need for a revision of the taxonomic status of *S. japonicus* from the Atlantic and Pacific coasts of America (Oliva et al., 2008).

With regard to spotted mackerels, monogeneans (*Kuhnina scomberi* and *Pseudokuhnina minor*) had been successfully used as tags to demonstrate different populations in New Zealand and southeastern Australia, based merely on the size of their sclerites (Rohde, 1987). However, studies focusing either on the endoparasites of this fish or on fish sampled from the Northwestern Pacific have not been reported. As revealed in the present study, two *Anisakis* species and a recombinant genotype were noted within spotted mackerels off Taiwanese waters but with differences in population composition and in the frequency of each species between the two sampling groups. Two species, *A. pegreffii* and the recombinant, were identified in the northeastern fish with extremely different frequencies of 97% and 3%, respectively. In addition to these two species, *A. typica* was also recovered from the southwestern samples and these three species appeared with frequencies of 63%, 9% and 28%, respectively. Based on the assemblages of these *Anisakis* nematodes, two stocks, namely the northeastern and the southwestern Taiwanese spotted mackerel, could be identified. This result is consistent with a previous research that stated that there are three morphologically distinguishable stocks of spotted mackerel off Taiwan (Tzeng, 2004), and the two sample groups studied herein were specifically captured from two of these three stocks. In conclusion, the present results suggest for the first time that endoparasites could be used as potential biomarkers of stock identification of spotted mackerel. Extensive sampling from all these three stocks to obtain more about infracommunity information of *Anisakis* nematodes within this fish population needs further investigations.

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