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Surface ultrastructure of larval Anisakidae (Nematoda: Ascaridoidea) and its identification by mensuration.

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Abstract

The surface ultrastructure of larval Anisakis type I, Anisakis type II, Raphidascaris, Contracaecum type A, Thynnascaris type A and Thynnascaris type B was examined by scanning electron microscopy. These species were identified clearly by the presence of a boring tooth, a mucron, and other morphological features. The means of the distances between transverse striations (DBTS) of larval Anisakis type I (5.45 +/- 0.125 micron), larval Raphidascaris (2.92 +/- 0.051 micron), and larval Contracaecum type A (1.68 +/- 0.056 micron) are significantly different (p less than 0.05). There was a correlation between the diameter of worm trunk (DOWT) and DBTS among these three larval types. In most cases a larva could be identified from the mean value of DBTS and DOWT even if obtained as a fragment from a patient.

KEYWORDS: Anisakidae, ultrastructure, surface striation, scanning ekectron microscopy

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Surface Ultrastructure of Larval Anisakidae (Nematoda: Ascaridoidea) and Its Identification by Mensuration

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The surface ultrastructure of larval *Anisakis* type I, *Anisakis* type II, *Raphidascaris*, *Contracaecum* type A, *Thynnascaris* type A and *Thynnascaris* type B was examined by scanning electron microscopy. These species were identified clearly by the presence of a boring tooth, a mucron, and other morphological features. The means of the distances between transverse striations (DBTS) of larval *Anisakis* type I ($5.45 \pm 0.125\,\mu m$), larval *Raphidascaris* ($2.92 \pm 0.051\,\mu m$), and larval *Contracaecum* type A ($1.68 \pm 0.056\,\mu m$) are significantly different ($p < 0.05$). There was a correlation between the diameter of worm trunk (DOWT) and DBTS among these three larval types. In most cases a larva could be identified from the mean value of DBTS and DOWT even if obtained as a fragment from a patient.

Key words : Anisakidae, ultrastructure, surface striation, scanning electron microscopy

Morphological features of anisakid larvae have been studied light microscopically by Koyama *et al.* (1), Smith & Wootten (2), and Sakaguchi *et al.* (3), and the larvae have been classified into 4 genera: *Anisakis* (type I and type II), *Contracaecum*, *Raphidascaris* and *Thynnascaris*. Early descriptions of the surface ultrastructure of these larvae have been given by Soleim (4), Aji *et al.* (5), Valter *et al.* (6), Smith (7), Fujino *et al.* (8), and Weerasooriya *et al.* (9).

Human cases of gastric or intestinal anisakiasis have increased recently in Japan (10, 11). Therefore, it has become important to be able to identify an anisakid larva by that part of the worm which has been surgically removed from an anisakiasis patient. As one approach, Oshima *et al.* (12) tried to classify parts of worms found in pathological sections using a light microscope. However, it proved difficult to identify the worm in every section. Weerasooriya *et al.* (9) reported the fine structure of anterior and posterior extremities, and differences in cuticular surface structures between anisakid larvae. Using a scanning electron microscope (SEM), they were able to classify even a small fragment of a worm. Fredericksen *et al.* (13) reported the use of the cuticular fine structure as revealed in sectioned material by transmission electron microscopy to identify anisakid larvae. In the present study, we observed the sur-

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face ultrastructure of anisakid larvae by SEM and prepared a guide for classification. Furthermore, we attempted to classify the worms by measuring the distance between transverse striations and the diameter of the worm trunk on scanning electron micrographs.

Materials and Methods

Third stage larvae (L3) of *Anisakis* type I, *Anisakis* type II, *Raphidascaris*, *Thynnascaris* type A, *Thynnascaris* type B and *Contracaecum* type A were collected from mackerel (*Scomber japonicus*) and jack mackerel (*Trachurus japonicus*) caught in the open sea. In the genus *Thynnascaris*, fourth stage larvae (L4) were also contained. Species of the genus *Thynnascaris* were classified as *Hysterothylacium* by Deardorff & Overstreet (14). Here, however, we have retained the genus *Thynnascaris*, following the classification of Fukuda *et al.* (15) and Koyama *et al.* (16). The specimens were fixed with 2% phosphate buffered glutaraldehyde at pH 7.4 for over 12 h, followed by post-fixing for 12 h in 1% osmium tetroxide solution in phosphate buffer, pH 7.4. The fixed materials were dehydrated through a graded ethanol series by routine methods. Then they were transferred into isoamyl acetate and dried in a critical point dryer using liquid carbon dioxide. The dried specimens were coated with carbon and gold. A JSM-25SII (JEOL Ltd.) SEM was used for observations.

The distances between transverse striations (DBTS) of larval *Anisakis* type I, *Raphidascaris* and *Contracaecum* type A were measured at the head, middle and tail portions of fixed materials. The average distance was calculated from measurements of 50 striations or more. The diameter of the worm trunk (DOWT) was also measured individually.

Results

Anisakis. The head of *Anisakis* type I was provided with a mouth and a boring tooth at the tip of the ventral lip (Fig. 1A). The mouth had a tri-radiate lumen. The orifice of an excretory pore was observed on the ventral side near the boring tooth (Fig. 1A, 2B). The tail end of *Anisakis* type I was rather globular and had a small mucron on its tip (Fig. 1B, 2A). The mucron showed transverse striation-like stripes (Fig. 2A). The anus, with a crescentic opening, was located ventrally near the tail end. The head of *Anisakis* type II had a tri-radiate mouth lumen and a boring tooth and did not differ much from the head of *Anisakis* type I (Fig. 3A). The tail of this species dose not bear a mucron, and tapered gradually from base to tip (Fig. 3B). The anus of *Anisakis* type II was almost the same as that of *Anisakis* type I.

Raphidascaris. The head of *Raphidascaris* had a boring tooth (Fig. 4A). The mouth showed a tri-radiate lumen, and did not differ externally from that of *Anisakis* type I or type II.

The tail of *Raphidascaris* tapered off to the tip, and resembled a mucron (Fig. 4B). The anus had a semilunar opening, and an anterior lip appears to serve as a cover for the anal opening (Fig. 4B).

Contracaecum. Only *Contracaecum* type A was studied. A tri-radiate mouth lumen and a boring tooth (Fig. 5A) were seen on the head. The head of *Contracaecum* type A did not differ much from that of *Anisakis* type I or type II, or *Raphidascaris*. A pit located ventrally at the base of the boring tooth appears to be the orifice of the excretory pore (Fig. 5A).

Contracaecum type A had a clavate tail. A mucron was not observed (Fig. 5B). The anus was similar to that of *Raphidascaris* (Fig. 5B) in having an anterior lip.

Thynnascaris. Both *Thynnascaris* type A and type B were studied. The head of *Thynnascaris* type A (L4) differed considerably from those of *Anisakis*, *Raphidascaris*, and *Contracaecum*. The mouth had a tri-

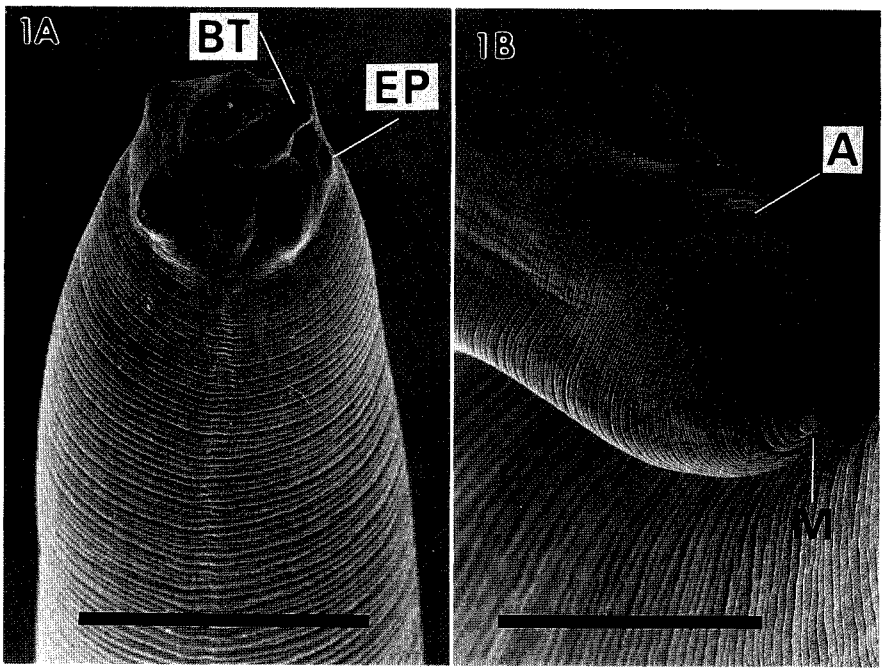

Fig. 1 Anterior (A) and tail end (B) of *Anisakis* type I. BT, boring tooth; EP, excretory pore; A, anus; M, mucron. Bar shows 100 μm.

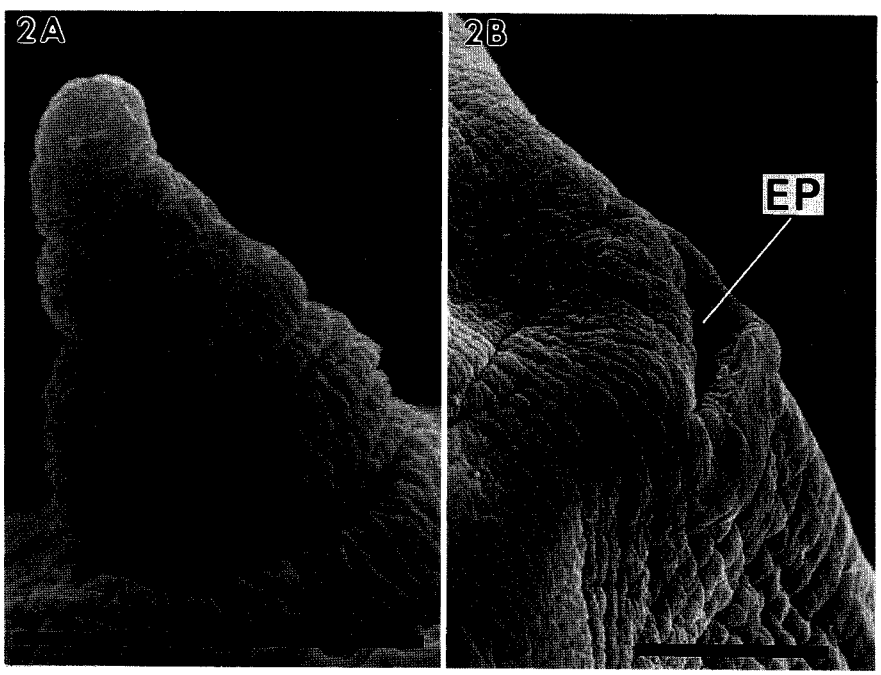

Fig. 2 High magnification of mucron (A) and excretory pore (B) of *Anisakis* type I. EP, excretory pore. Bar shows 10 μm.

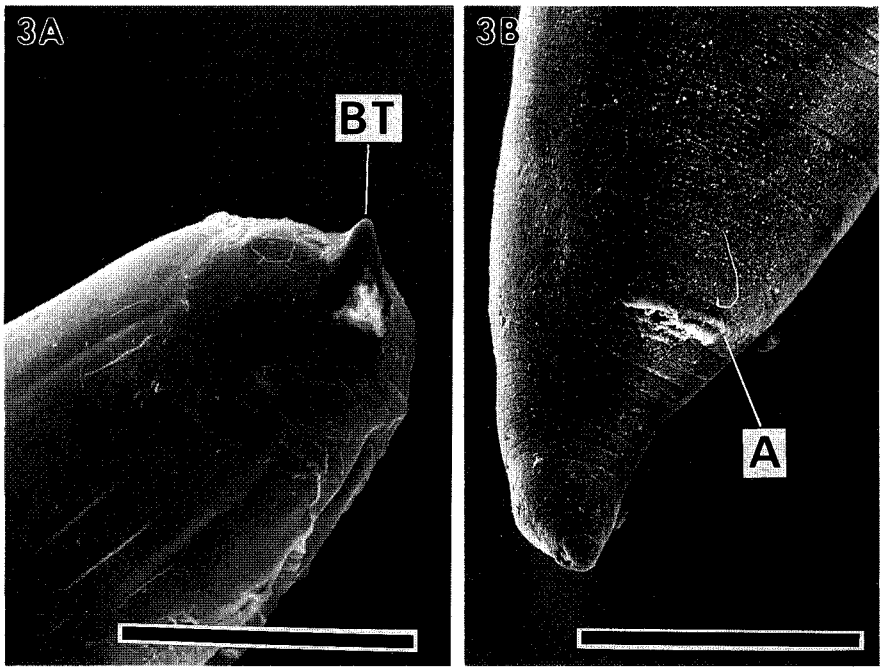

Fig. 3 Anterior (A) and tail end (B) of *Anisakis* type II. BT, boring tooth; A, anus. Bar shows 100 μm.

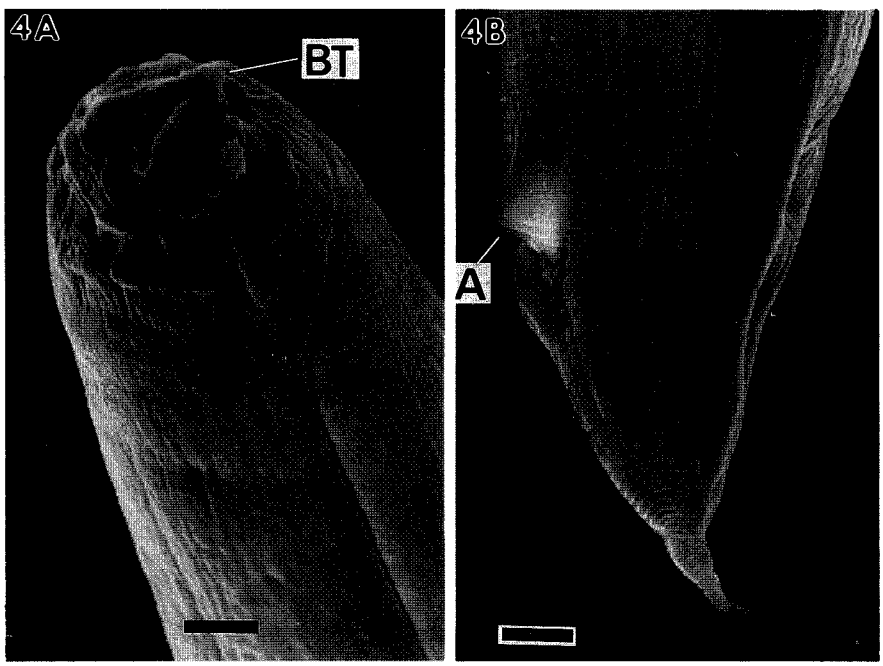

Fig. 4 Anterior (A) and tail end (B) of *Raphidascaris*. BT, boring tooth; A, anus. Bar shows 10 μm.

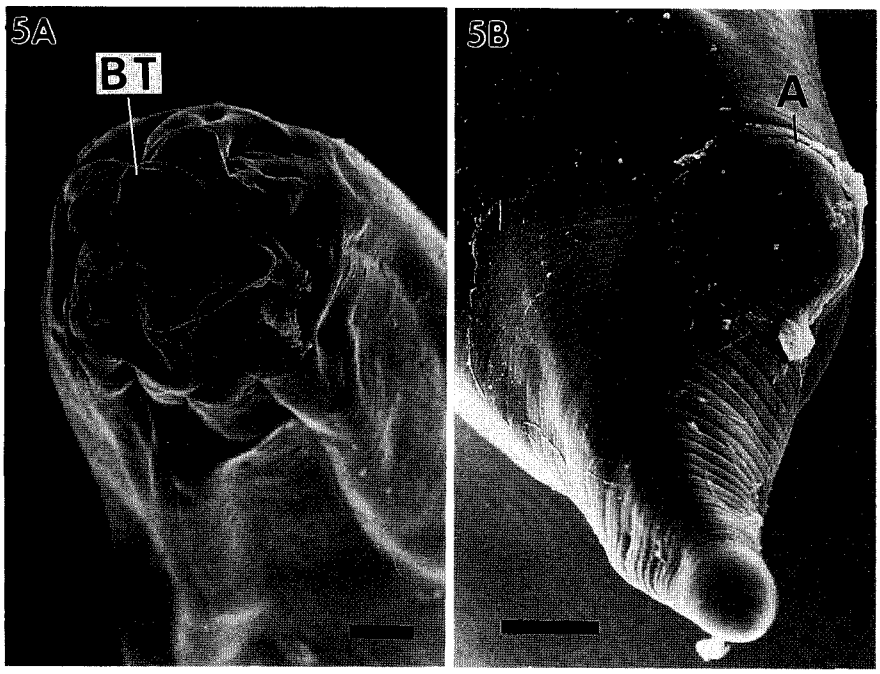

Fig. 5 Anterior (A) and tail end (B) of *Contracaecum* type A. BT, boring tooth; A, anus. Bar shows 10 μm.

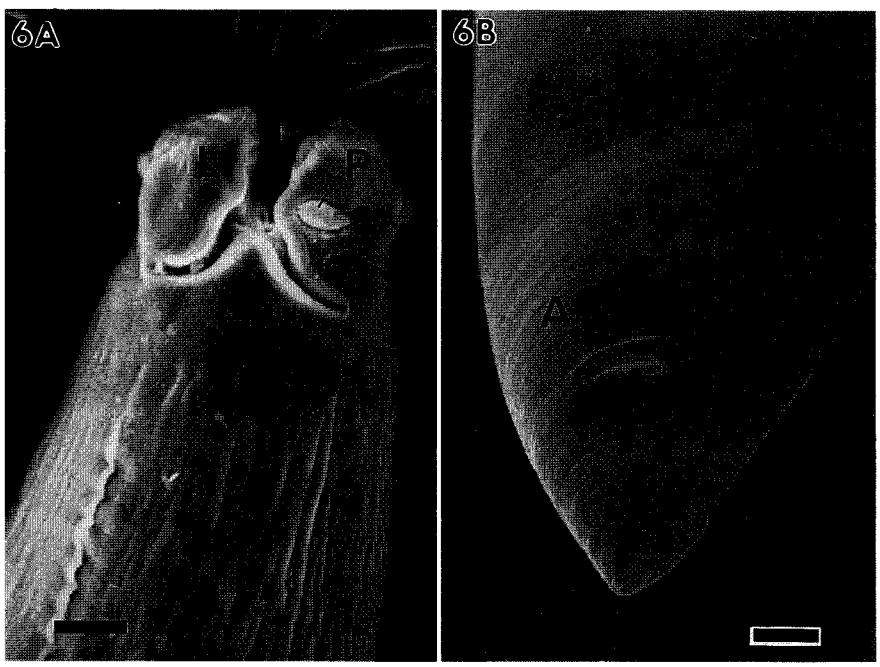

Fig. 6 Anterior end (A) of *Thynnascaris* type A (L4) and tail end (B) of *Thynnascaris* type A (L3). L, lip; IL, interlabium; P, papilla; A, anus. Bar shows 10 μm.

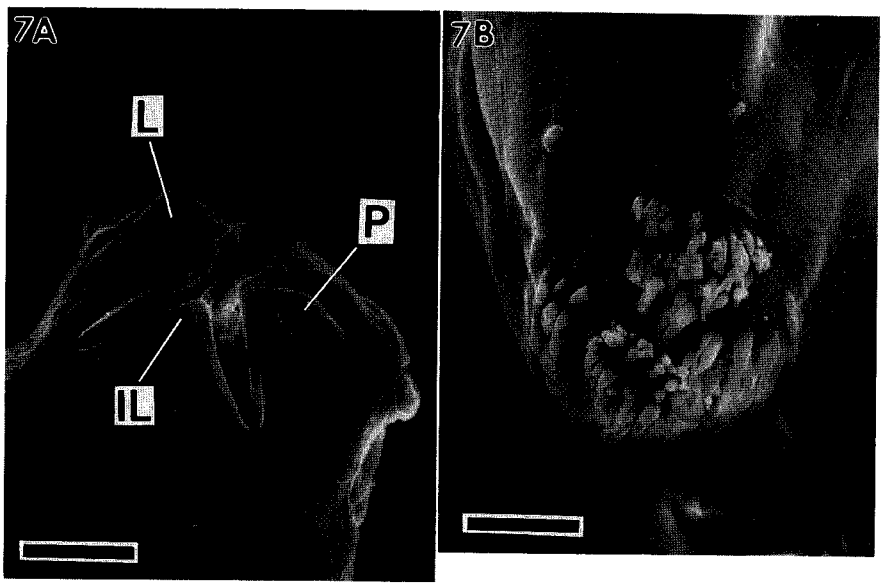

Fig. 7 Anterior (A) and tail end (B) of *Thynnascaris* type B (L4). L, lip; IL, interlabium; P, papilla. Bar shows 10 μm.

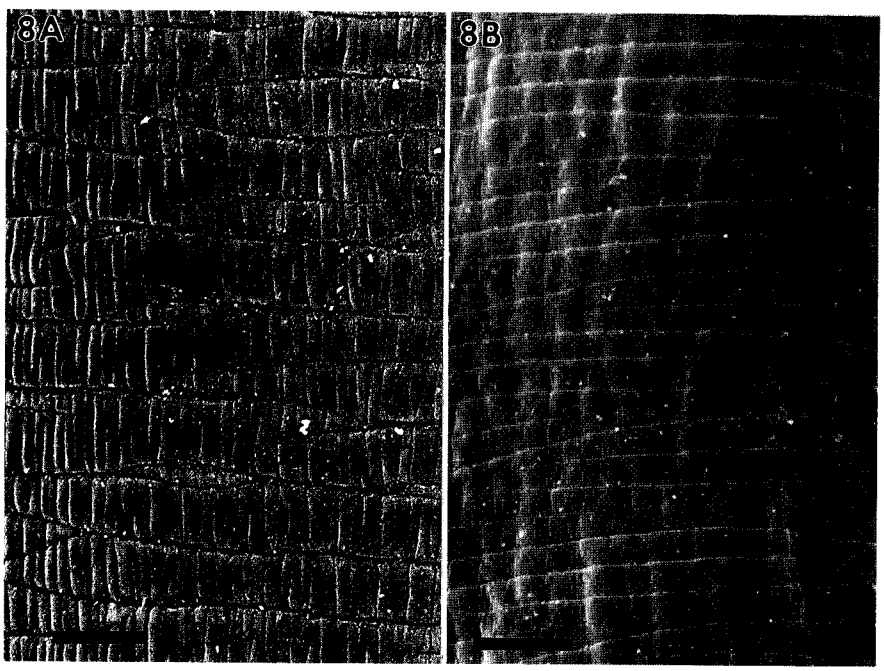

Fig. 8 Transverse striations of *Anisakis* type I (A) and *Raphidascaris* (B). Bar shows 10 μm.

radiate lip and interlabia (Fig. 6A). There was no boring tooth, but small papillae were observed on the lips (Fig. 6A). The tail (L3) was conical. The anus, with a curved opening, was located ventrally. A small mucron was observed (Fig. 6B).

The mouth of *Thynnascaris* type B (L4) showed a tri-radiate lip, interlabia, and papillae as in type A (Fig. 7A). However, the tail of *Thynnascaris* type B (L4) differed considerably from that of type A in bearing about 100 spines (Fig. 7B).

The distances between transverse striations of representatives of the three genera. DBTS of *Anisakis* type I (Fig. 8A), *Raphidascaris* (Fig. 8B) and *Contracaecum* type A are shown in Table 1. The mean and confidence interval ($p < 0.05$) were 5.45 ± 0.125 μm in *Anisakis* type I, 2.92 ± 0.051 μm in *Raphidascaris*, and 1.68 ± 0.056 μm in *Contracaecum* type A (Table 1). The differences between these means are significant ($p < 0.05$). The variance of *Anisakis* type I was greater than that of the other two larval types, regardless of the portion.

Each histogram of DBTS of the three worm types fitted a normal distribution using the Kolmogorov-Smirnov test. Transformation into a normal distribution was achieved by using the mean and its standard deviation for each portion. DBTS transformed into normal distributions of the head, middle and tail portions, and all portions cumulated (Fig. 9). DBTS of *Anisakis* type I, *Raphidascaris* and *Contracaecum* type A were distributed approximately between 1 to 10 μm, 1 to 5 μm and 0 to 3 μm, respectively. The variance of each genus was considerably large, and the DBTS overlapped (Fig. 9).

The mean of DBTS slightly differed between the head, middle and tail portions in larval *Anisakis* type I: DBTS was wider in the middle portion than in the tail or head portion. This tendency was also observed in the other two genera. There was no significant difference between the mean for the head and tail of larval *Anisakis* type I and *Raphidascaris*. On the other hand, a significant difference ($p < 0.05$) was found

Table 1 Mean and confidence limit, standard deviation, and coefficient of variation of the diameter of worm trunk and the distances between transverse striations of three larval anisakids (measurements in μm)

Genus	Portion	DOWT		DBTS			
		n	Mean CL	n'	Mean CL	SD	CV
Anisakis type I	Cumulative	15	268.1±37.73	783	5.45±.125	1.784±.328	
	Head	7	220.9±47.54	376	5.00±.185	1.834±.367	
	Middle	6	336.7±13.17	301	6.12±.185	1.633±.267	
	Tail	2	227.3±10.71	106	5.11±.268	1.389±.272	
Raphidascaris	Cumulative	20	112.8± 9.98	996	2.92±.051	.823±.281	
	Head	6	99.7±25.04	300	2.59±.085	.749±.289	
	Middle	9	125.5±13.19	439	3.27±.077	.826±.253	
	Tail	5	105.7±20.35	257	2.73±.081	.662±.242	
Contracaecum type A	Cumulative	12	73.8±16.51	417	1.68±.056	.580±.344	
	Head	4	53.9±32.05	110	1.38±.069	.362±.263	
	Middle	4	89.4±34.09	149	1.98±.092	.572±.289	
	Tail	4	79.3±25.95	153	1.62±.092	.583±.359	

DOWT: The diameter of the worm trunk; DBTS: The distances between transverse striations; n: Number of specimens; n': Number of DBTS measured; CL: Confidence Limits ($p < 0.05$); SD: Standard Deviation; CV: Coefficient of Variation.

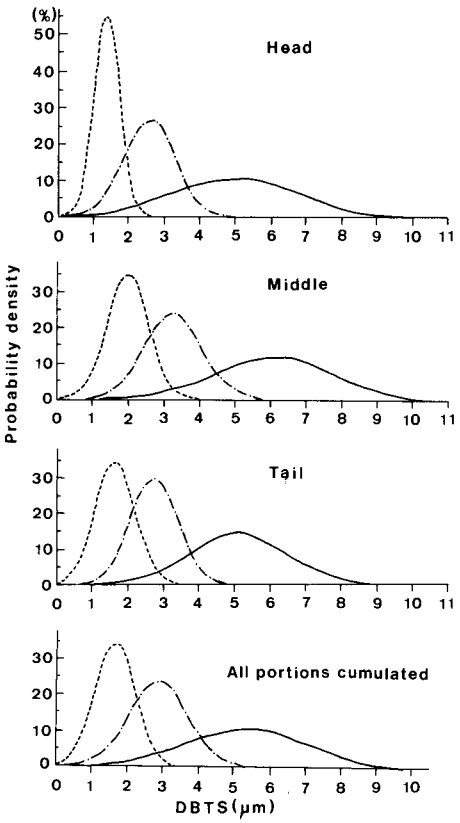

Fig. 9 The distances between transverse striations (DBTS) transformed into normal distribution of the head, middle and tail portions and all three portions cumulated. *Anisakis* type I, ——; *Raphidascaris*, —·—; *Contracaecum* type A, ------.

between the middle and tail portions, and between the head and middle portions. In *Contracaecum* type A, significant differences ($p < 0.05$) of the mean were found between the head, middle and tail portions.

The correlation between DOWT and DBTS. The relationship between DOWT and DBTS is shown in Fig. 10. The regression equations and correlation coefficients (r), which were calculated by the least squares method, are shown in Table 2. *Anisakis* type I showed the highest correlation coefficient ($= 0.7977$); the correlation was statistically significant ($p < 0.01$).

Table 2 Regression equations and correlation coefficients between the diameter of worm trunk and the distances between transverse striations of three larval anisakids

Genus	n	Relationship (μm)	r
Anisakis type I	17	S = .016 D+ 1.255 D = 40.730 S+44.672	.7977**
Raphidascaris	18	S = .024 D+ .258 D = 23.722 S+43.012	.7514**
Contracaecum type A	12	S = .009 D+ 1.002 D = 20.897 S+39.728	.4269

n: Numbers of specimens; r: Correlation coefficient; S: The distances between transverse striations (DBTS); D: The diameter of worm trunk (DOWT). ** $p < 0.01$.

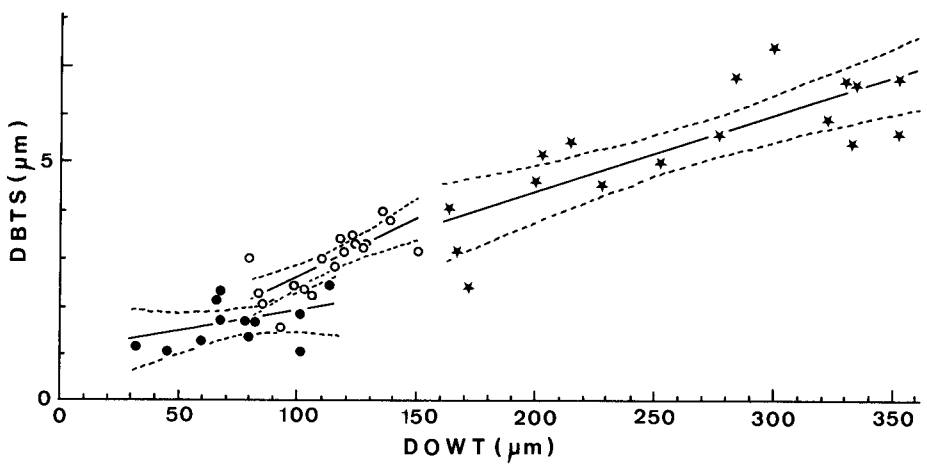

Fig. 10 Relation between DOWT (the diameter of worm trunk) and DBTS (the distances between transverse striations). Solid line is the regression of *Anisakis* type I (asterisks), *Raphidascaris* (open circles), and *Contracaecum* type A (closed circles). Broken line shows the confidence interval of each regression line at the 95% level.

The correlation coefficient of *Raphidascaris* was 0.7514, and the correlation was significant at the $p < 0.01$ level. However, the correlation coefficient of *Contracaecum* type A was 0.4269, and the correlation was significant at only the < 0.2 level.

Discussion

External morphology. The morphological features of several anisakid larvae as observed by light microscopy were reported by Koyama *et al.* (1), but details of the boring tooth and mucron were not reported. The ultrastructure of these organs has been described by several authors (5-9). Weerasooriya *et al.* (9) gave a full account of the anterior and posterior extremities of anisakid L3 larva (*Anisakis* type I, *Pseudoterranova decipiens*, *Contracaecum* type B = *Contracaecum* type A in our study, *Hysterothylacium* sp). Furthermore, they compared L4 larva with L3 larva of *Anisakis* type I, and *P. decipiens* and pointed out differences in detail. We have studied the external morphology of L3 larvae of *Raphidascaris*, L3 and L4 larvae of *Thynnascaris* (types A and B), L3 larvae of *Anisakis* type I and L3 larvae of *Contracaecum* type A. According to Weerasooriya *et al.* (9), the surface of the mucron of *Anisakis* type I is not smooth, and many transverse striations may be seen on its surface. In the present SEM study, the striations on the mucron appeared to be a continuation of those on the body. The mucron must be flexible, because both contracted (Fig. 1B) and expanded mucrons (Fig. 2A) were observed. On the other hand, the tail-tip of larval *Thynnascaris* type B (L4) was provided with about 100 small spines instead of a mucron (Fig. 7B).

Larval *Raphidascaris* and *Contracaecum* type A had a large lip on the posterior side of the anal opening. These worms may open or close their anus, or cover it with this lip. Both *Anisakis* type I and type II, and *Thynnascaris* type A probably have a different mechanism, because the anus lacks an obvious lip.

Heads bearing a boring tooth have been observed in larval *Anisakis* type I by other workers using SEM (5-9). Although it is called a "boring tooth", it appears to be too small to bore a hole in host tissue.

An excretory pore opened on the ventral side near the boring tooth in larval *Anisakis* (Fig. 1A) and *Contracaecum* (3, 6, 7, 9, 17). The ultrastructure of the excretory gland as revealed by transmission electron microscopy was described by Lee *et al.* (18). They reported that this gland functioned not only for excretion but also for secretion of histolytic enzymes which are released through the excretory pore. The presence of an excretory pore near the boring tooth (Fig. 1A) might relate to the burrowing ability of the larvae. However, in larval *Raphidascaris* and *Thynnascaris* the excretory pore opens near to the nerve ring, *i.e.*, some distance from the mouth (1-3, 7, 16-18). The excretory system may have functions additional to those mentioned above. In the present study, the orifice of the excretory pore was clearly visible only in larval *Anisakis* type I.

Measurements: DBTS and DOWT. Identification of a fragment of an anisakid larva is often required clinically after the larva has been extracted with an endoscope. However, it is difficult to identify morphologically the larva from a fragment in most cases. Morphometric investigations by light microscopy have been made by several authors (1-3, 12, 16). Fujino *et al.* (8) and Weerasooriya *et al.* (9) stated that the form of transverse striations of *Anisakis* type I differed in each larval stage: the DBTS was irregular in L3 larva but rather regu-

lar in L4 larva, in the range 10-13 μm. In the present study, the larvae were used after fixation because clinical materials are usually fixed. The larvae were classified statistically according to the DBTS. The DBTS of larval *Anisakis* type I was 5-6 μm. Thus, together with features of the head, we consider this *Anisakis* type I to be L3 larva. The DBTS in larval *Raphidascaris* was fairly regular even in L3 larva. It is probable, therefore, that the DBTS in each stage varies with worm type.

Because larval *Anisakis* type I obtained from fishes is mainly L3 larva (17) and most anisakiasis is contracted by eating fish (8), it is relevant to concentrate the discussion on L3 larva.

The variance of the mean of DBTS of larval *Anisakis* type I was greater than that of the other two larval types, *i.e.*, *Raphidascaris* and *Contracaecum* type A. This means that the DBTS of *Anisakis* type I is irregular, because the striations are often bifurcated (Fig. 8A). On the other hand, the DBTS of *Raphidascaris* was regular (Fig. 8B). This variance in the DBTS of larval *Anisakis* type I may be diagnostic.

Although the means of the DBTS significantly differ among the three types ($p < 0.05$), the range in each type is appreciably wide, and overlaps in the DBTS occur (Fig. 9). Based upon the cumulative values of all three portions (head, middle and tail), it is considered that a worm having a DBTS wider than 5 μm is *Anisakis* type I, and that a worm having a DBTS narrower than 1 μm is *Contracaecum* type A. However, it is difficult to identify a larvae with DBTS between 1 and 5 μm. Accordingly, a method for obtaining the frequency distribution of DBTS of the three larval types was devised. A probability density for each portion of the three types is calculable from the value of the DBTS (Fig. 9). Formulae for obtaining the probability that a given worm is larval

Table 3 Formula to obtain the probability of three type worms about every DBTS (fa = *Anisakis* type I, fr = *Raphidascaris*, fc = *Contracaecum* type A)

Portion	Formula
Cumulated	$fa(x) = 11.18/\exp[(x-5.45)^2/6.37]$
	$fr(x) = 24.24/\exp[(x-2.92)^2/1.35]$
	$fc(x) = 34.39/\exp[(x-1.68)^2/0.67]$
Head	$fa(x) = 10.88/\exp[(x-5.00)^2/6.73]$
	$fr(x) = 26.63/\exp[(x-2.59)^2/1.12]$
	$fc(x) = 55.10/\exp[(x-1.38)^2/0.26]$
Middle	$fa(x) = 12.22/\exp[(x-6.12)^2/5.33]$
	$fr(x) = 24.15/\exp[(x-3.27)^2/1.36]$
	$fc(x) = 34.87/\exp[(x-1.98)^2/0.65]$
Tail	$fa(x) = 14.36/\exp[(x-5.11)^2/3.86]$
	$fr(x) = 30.13/\exp[(x-2.73)^2/0.88]$
	$fc(x) = 34.21/\exp[(x-1.62)^2/0.68]$

Anisakis type I, *Raphidascaris* or *Contracaecum* type A are shown in Table 3, where "x" is the mean of DBTS (μm). The probabilities of the three types of worms at "x" are as follows: fa(x)/@% for *Anisakis* type I, fr(x)/@% for *Raphidascaris*, and fc(x)/@% for *Contracaecum* type A, where @ = fa(x) + fr(x) + fc(x). When the region of the worm body from which a fragment came is known, the formula for that fragment should be used. When the region is unknown, then the formula for all portions cumulated should be used. It is possible to obtain the probabilities of the three larval types by using the above calculation, and to identify a worm obtained operatively from a patient.

Each histogram had a wide range when obtained by the above-mentioned method, in which all DBTS for individual specimens are treated. Only the maximum DBTS of individual specimens was treated and analyzed in the same way, but the results were almost the same.

In clinical cases of anisakiasis, *Anisakis* type I is usually responsible. Once a probability of occurrence for each species is

obtained from established clinical cases, we will be able to predict the worm species more exactly by the formula shown in the present study.

Both DBTS and DOWT of the middle, tail and head parts of all worms were in the order of wide to narrow. A correlation between DOWT and DBTS in *Contracaecum* type A was recognized only at the 80% confidence level, probably owing to the large variance and few specimens examined. In *Anisakis* type I and *Raphidascaris*, correlation coefficients between DOWT and DBTS were significant at the 99% level. In general, worms having the greatest diameter had the widest DBTS, even in *Contracaecum* type A.

In conclusion, worms may be identified by their mean DBTS even when only a fragment is obtained from a patient. If the diameter is unknown, it can be predicted from the regression lines of DBTS and DOWT given here. Furthermore, it is possible to estimate the size and developmental stage of the worm.

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