STEREO AND SCANNING ELECTRON MICROSCOPIC STUDIES OF THE THIRD STAGE LARVAE OF *ANISAKIS SIMPLEX*

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Abstract. This study was to demonstrate the surface anatomy of the third stage larvae of *Anisakis simplex* in marine fish using stereo and scanning electron microscopes (SEM). The round worm is slender, elongated and of cylindrically shaped. The head of this worm is a globular structure. The mouth is triangularly shaped and surrounded by three lips. A boring tooth projects dorsally at the anterior end. There are four pairs of tactoreceptors, the labial papillae, enclosing the lips. The tail end is blunt and acquires a distinct slender process, the mucron. Stereomicroscopy revealed the esophagus is elongated, bulbous and club shaped, subdivided into an anterior muscular part and a posterior glandular part or ventriculus. The intestine is a long straight tube where the digestion and absorption occur. Waste pass through the intestine and is stored in the rectum until excreted via the anus. A SEM is a powerful tool in distinguishing worm species, as was seen when examining that the mouth of *Anisakis simplex*, which is triangular shaped and enclosed by three lips with one boring tooth; other species are different. The mucron projection at the distal end is another distinctive structure revealed by SEM.

Keyword: L3 *Anisakis simplex*, surface anatomy, SEM

INTRODUCTION

Anisakis simplex, a marine nematode, can be found in the body cavity, liver, muscle and especially gastrointestinal tract of various marine fishes, birds and fish-eating mammals, such as whales and dolphins (Muller, 2002). The third stage larva causes an infection in humans who contract it by consuming raw or undercooked seafood, such as sushi and sashimi (Anderson, 2001). Anisakid infections have been frequently reported in Europe, America and Asia (Miller and Harley, 2005). Symptoms include acute severe abdominal pain, nausea, vomiting, hyperemia and bleeding which begin 1-12 hours after ingestion of infected fish (Tsieh, 1993). Anisakid larvae are not found in human feces, diagnosis is made by history of ingesting infected fish along with abnormal findings on endoscopy (Marquardt, 2000). There is no drug for the treatment of infected patients. Patients are treated with endoscopic removal of the worm using biopsy forceps; the pain disappears within a few hours of treatment (Bogitsh *et al*, 2005).

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Abollo and Pascual (2002) studied the morphology of *Anisakis brevispiculata* and *Pseudoterranova ceticola*, parasites of the pygmy sperm whale, using scanning electron microscopy (SEM). There was another recent report using SEM to identify the morphology of L_3 *Anisakis simplex* and *A. pegreffi* (Quiazen *et al*, 2008). The SEM study revealed the surface topographic features using 3-D pictures, some of which were different from those features described previously. All those SEM studies had limited coverage of the anterior or posterior ends of the parasite. In this study we used the SEM to evaluate the characteristics of infective stage *Anisakis simplex* compared with pictures from a stereomicroscope and suggest the unique structures, which can be clearly used to differentiate this parasite from others.

MATERIALS AND METHODS

Specimen collection

Third-stage larvae of *Anisakis simplex* were removed from the peritoneal cavity and muscles of marine fish, washed several times in 0.85% NaCl and fixed in 10% formalin for stereomicroscopic study and fixed in 2.5% glutaraldehyde for SEM study.

Stereomicroscopic preparation

For stereomicroscopic study, after formalin fixation, specimens were washed several times in distilled water and dehydrated in graded ethanol from 70% to absolute alcohol for 10 minutes at each step and cleared twice with xylene for 10 minutes; all steps were performed at room temperature. The specimens were then examined with a stereomicroscope. Photographs were taken with a digital camera.

SEM preparation

For ultrastructural study of third stage larvae (L3) of *A.simplex* by SEM, after specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4ºC, they were washed three times with the same buffer and post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer, pH 7.2, at 4ºC for one hour and washed three times with the same buffer. Specimens were further processed by dehydrating in a graded series of ethanol from 70% to 100% for 10 minutes at each step at room temperature. They were dried in a critical point drier (CPD), mounted on a stub with double stick scotch tape, and coated with gold mixed with palladium. Finally, the worms were observed under a SEM.

RESULTS

Stereomicroscopic study of L3 *Anisakis simplex*

The worms were studied with a stereomicroscope and found to be slender, elongated, cylindrical with tapering head and tail ends. The lengths varied (31.50 ± 6.97 mm), the widest part of the body ranged from 0.3 to 0.5 mm. At higher magnification, the cuticle showed delicate transverse striations at the anterior and posterior regions, whereas the middle region had longitudinal striations. The head was globular (Fig 1A). A boring tooth, was seen dorsally on the head (Fig 1B). There were 3 poorly developed lips, one dorsal and two ventrolateral (Fig 1C). At a distance of 2-4 mm from the head end, the esophagus was surrounded by a nerve ring (Fig 1B). The length of the esophagus was about 1.5-3 mm with a bulbous, club shaped section toward the esophago-intestinal junctions but slightly tapered toward the base of the

Fig 1A–Stereomicrograph of the anterior part of the third stage larvae of *Anisakis simplex*.

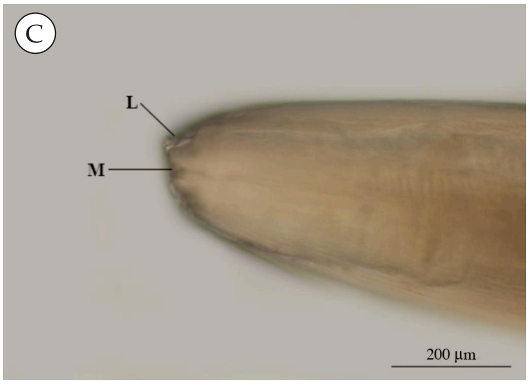

Fig 1C–Higher magnification of the head; the mouth (M) is surrounded by three lips (L).

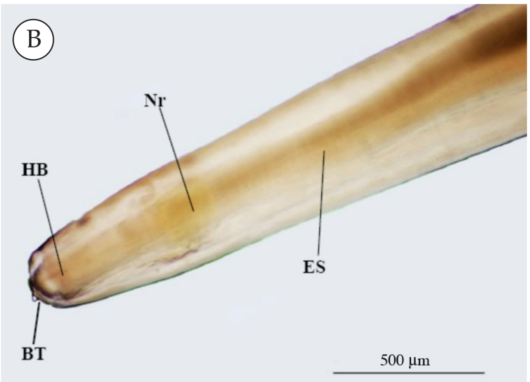

Fig 1B–Stereomicrograph shows the head is globular (HB), a boring tooth (BT) is found slightly dorsally at the anterior end and the proximal part of the esophagus (ES) is surrounded by a nerve ring (Nr).

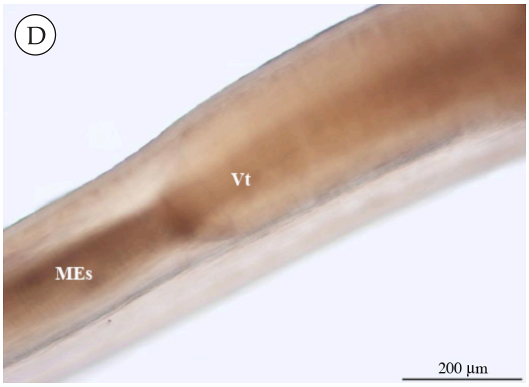

Fig 1D–Stereomicrograph shows an esophagus which consists of an anterior muscular part (MEs) and a posterior glandular part or ventriculus (Vt).

head bulb. The esophagus consisted of two parts: an anterior muscular part and a posterior glandular part (Fig 1D). The muscular esophagus depends on the size of the larva. The glandular part, called the ventriculus, was 0.6-0.8 mm long and 0.2 mm wide and joined with the intestine. The intestine was a long tube; the distal end connected to the rectum. The rectum formed a short oblique canal that joined the anus, with a thick cuticular layer. The anal glands were pear-shaped, situated adjacent to the rectum (Fig 1G).

In the middle region of the body, there were grooves and ridges (Fig 1E). The tail was short and with a blunt end (Fig 1F) and a clear process called a mucron (Fig 1G, 1H).

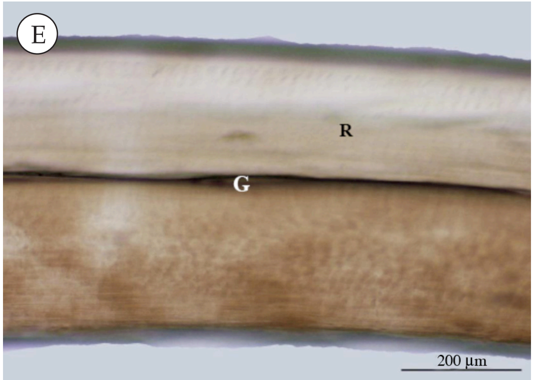

Fig 1E–Stereomicrograph shows the middle part of the body composed of grooves (G) with intervening ridges (R).

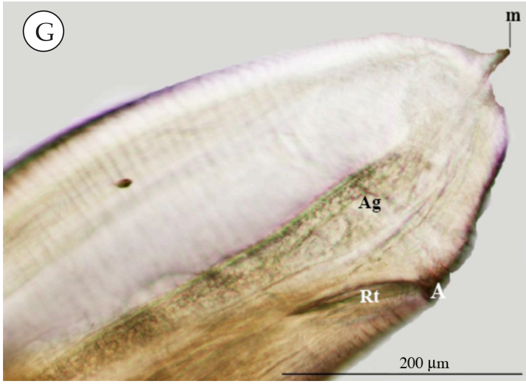

Fig 1G–Higher magnification of the lateral aspect of the posterior end. The rectum (Rt) forms a short canal that obliquely joins the anus (A). The anal glands (Ag) are found as grouped cells adjacent to the rectum. A rounded tip with a clear process is called a mucron (m).

Fig 1F–Stereomicrograph shows the posterior part of L_3 *Anisakis*. The tail is short with a blunt end.

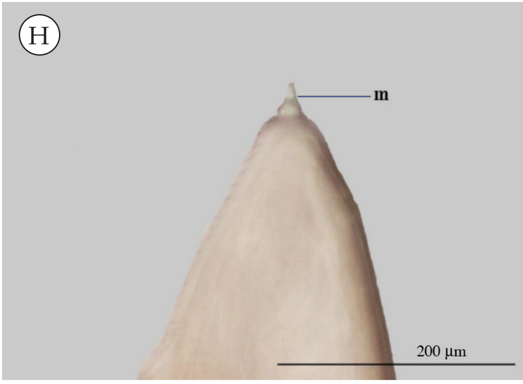

Fig 1H–Higher magnification of the dorsal aspect of the posterior end. The mucron (m) is a clear process at the tail end.

Scanning electron microscopic study of L3 *Anisakis simplex*

The whole surface of the L3 *A. simplex* was studied at the anterior, middle and posterior parts with a SEM.

The anterior part of the worm had a globular head (Fig 2A). The mouth was triangular shaped and surrounded by three lips, one dorsal and two ventrolateral. The thickness of each lip was approximately 6.16 ± 1.52 m (Fig 2B). A boring tooth; 10-11 m long, projected dorsally at the anterior end (Fig 2C, 2D). There was an opening of an excretory pore, 4-6 m in diameter, on the ventral surface, posterior to the angle between the ventrolateral lips (Fig 2E, 2F). The lips were surrounded by four pairs of dome shaped labial papillae 20-40 m in diameter (Fig 2B, 2E). There were transverse cuticular striations pos-

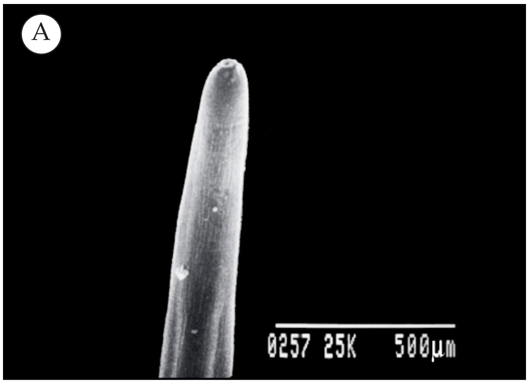

Fig 2A–Scanning electron micrograph shows the anterior part, which is a globular head.

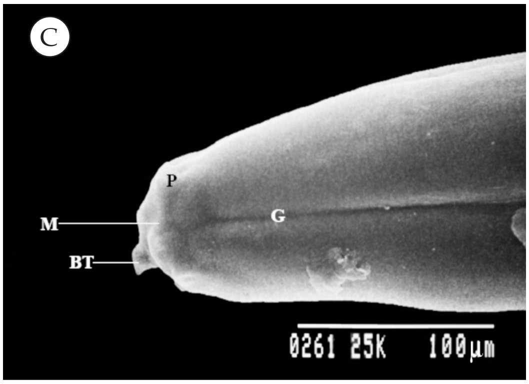

Fig 2C–Scanning electron micrograph shows the ventral surface of anterior part of the worm; a groove (G) is located midline on the ventral aspect. A mouth (M), boring tooth (BT) and papillae (P) are shown.

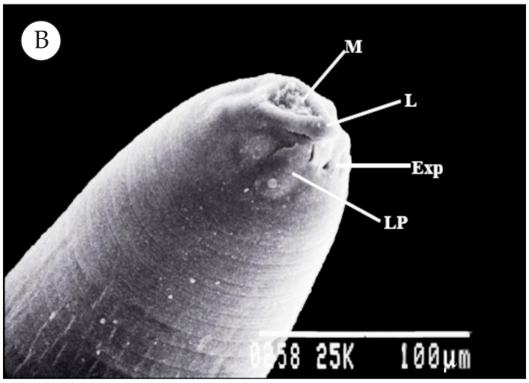

Fig 2B–Scanning electron micrograph shows the head; there are three lips (L) surrounding the mouth (M) which is triangular in shape. An excretory pore (Exp) is located along the ventral surface inferior to the lip. The labial papillae (LP) are found around the head of the worm.

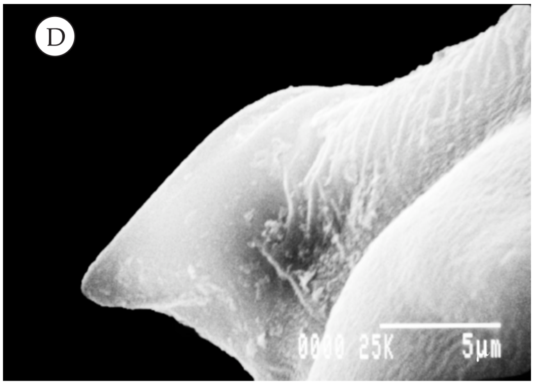

Fig 2D–Scanning electron micrograph shows a higher magnification of the boring tooth.

terior to the labial papillae. The distance between each anterior transverse cuticular striation was 0.5-1.7 m. Amphids were seen as a pair of anterior depressions in the cuticle near the cephalic end; they functioned as chemoreceptors.

In the middle region, longitudinal grooves with ridges appeared on the outer surface of the body (Fig 2G, 2I), all ridges had several linear striations (Fig 2H). The distance between each linear cuticular striation was 3-7 m.

The posterior end was blunt and transverse cuticular striations were on its surface (Fig 2L). The distance between the posterior transverse cuticular striations were 2-4 m. There were button-like papillae on the mid dorsal surface of the

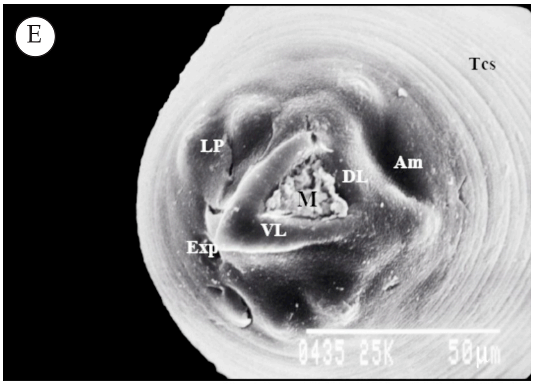

Fig 2E–Scanning electron micrograph shows the top of the anterior end. The mouth (M) is triangular shaped surrounded by 3 lips: a dorsal lip (DL) and two ventrolateral lips (VL). The opening of an excretory pore (Exp), labial papillae (LP), amphid (Am) and transverse cuticular striations (Tcs) are shown.

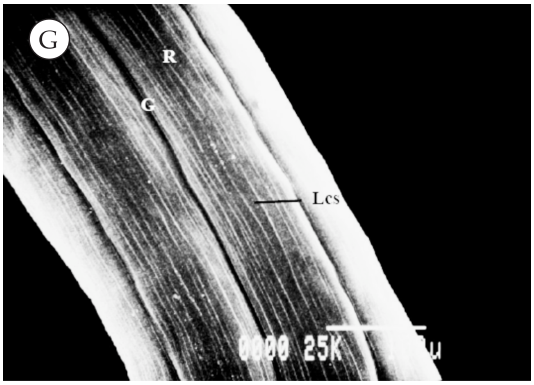

Fig 2G–Scanning electron micrograph shows the surface of the middle part. The grooves (G) and ridges (R) are present on the outer surface of the body.

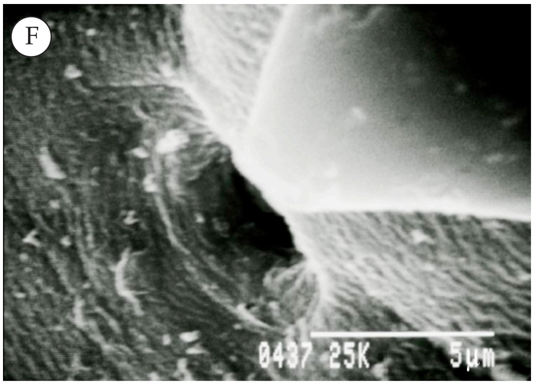

Fig 2F–Scanning electron micrograph shows the opening of an excretory pore at the tip, at the joining of the ventrolateral lips.

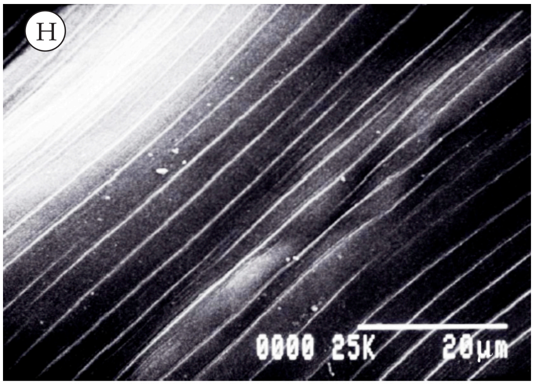

Fig 2H–Scanning electron micrograph shows the middle part of the larvae. There are several longitudinal linear cuticular striations on each ridge of the worm body.

worm (Fig 2J, 2K), which were assumed to be sensory receptors. Each papilla appeared as a small dome or button-like structure with a diameter of 13-15 m (Fig 2K). There was an anal opening on the ventral surface of the tip of the tail, triangular in shape (Fig 2L, 2M). The width of the anal opening was 14-16 m.

The tail was round with a prominent mucron structure. The mucron length was 8 m, small and conical shaped (Fig 2N, 2O, 2P). No papillae were found around the distal part of the tail of the worm. The phasmids appeared as small depressions that originated near the anus and function as chemoreceptors (Fig 2L).

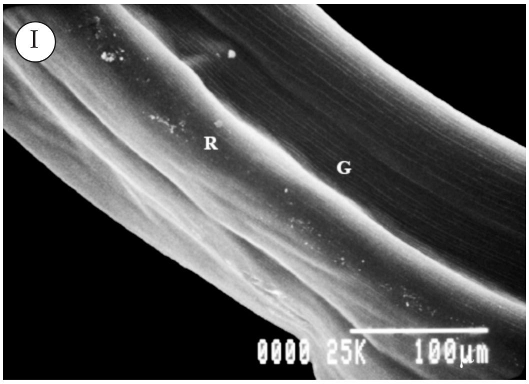

Fig 2I–Scanning electron micrograph shows the posterior part of the body. The grooves (G) with ridges (R) are present on the outer surface of the body.

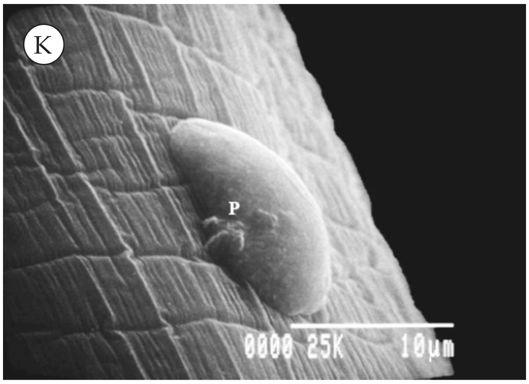

Fig 2K–Scanning electron micrograph shows a higher magnification of a papilla (P).

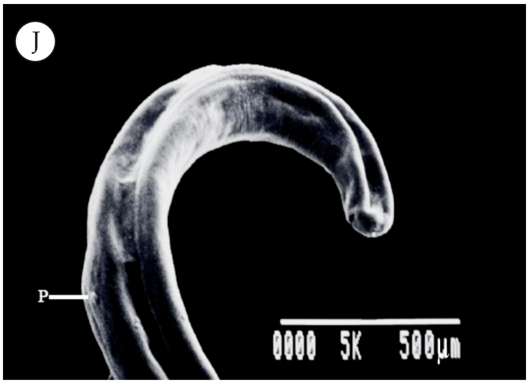

Fig 2J–Scanning electron micrograph shows the posterior part of the worm. The posterior end is blunt with a papilla (P) on the dorsal surface.

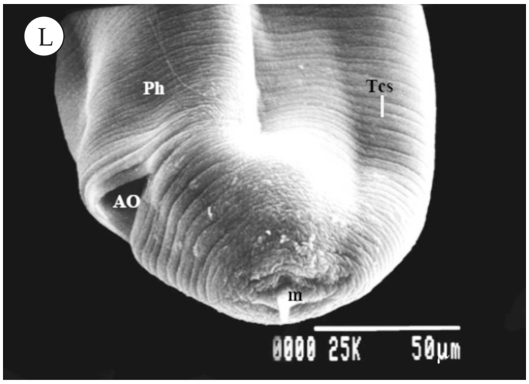

Fig 2L–Scanning electron micrograph shows a higher magnification of the posterior end. There are several transverse cuticular striations (Tcs) around the tail. There is a triangular anal opening (AO) near the tip of the tail. The tail is round with a mucron structure (m). The phasmids (Ph) appear as small depressions near the anus.

DISCUSSION

The stereomicrographs show the general morphology of L3 *A. simplex*, which is, more or less, similar to other nematodes, such as *Ascaris lumbricoides, Ancylostoma duodenale*, *Capillaria philippinensis* and *Strongyloides stercoralis*. It is slender, elongated, whitish yellow, has bilateral symmetry, is cylindrically shaped and tapering at both ends. The digestive tract is a linear complete tube consisting of a mouth, esophagus, intestine, rectum and anus. The mouth is surrounded by lips. The esophagus is a long tube; it joins the mouth to the intestine and consists of an anterior muscular part and a posterior glandular part or ventriculus. Food material is sucked into the esophagus when the muscle in the anterior part contracts

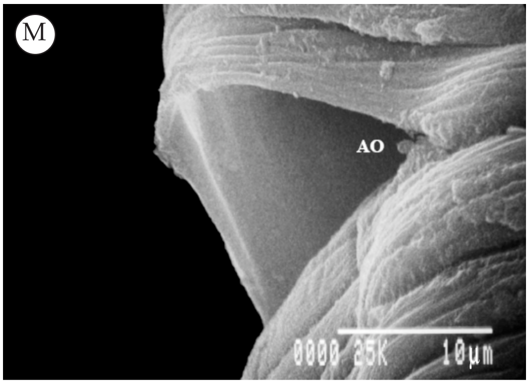

Fig 2M–Scanning electron micrograph shows the opening of the anus (AO) which is triangular in shape and found on the ventral aspect of the posterior part near the end of the tail.

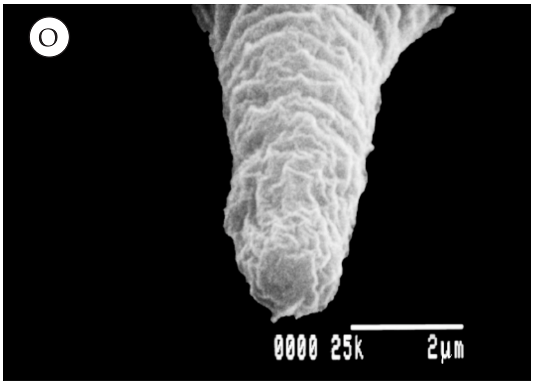

Fig 2O–Scanning electron micrograph shows the mucron, which is conical shape, located at the tip of the tail.

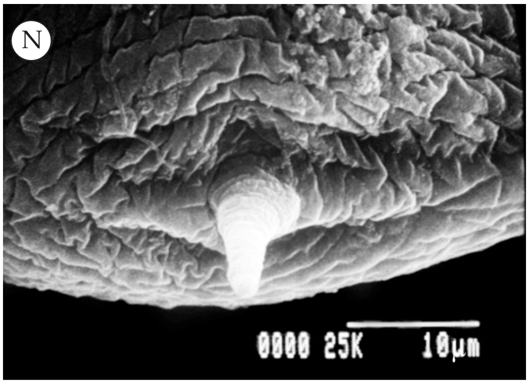

Fig 2N–Scanning electron micrograph shows the tail of the worm.

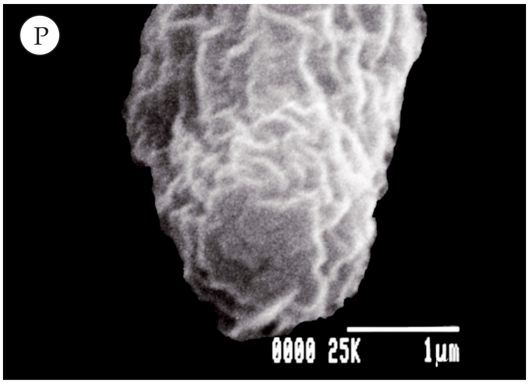

Fig 2P–Scanning electron micrograph shows the tip of the mucron.

rapidly and opens the lumen, while the posterior part secretes various enzymes for digestion. The intestine is the longest straight tube in the body, where the digestion of food and absorption of nutrient materials occurs. Waste travels though the intestine and is stored in the rectum until excreted via the anus. The rectum is a short canal, courses obliquely from the intestine to the anus and is surrounded by several anal glands. These glands secrete mucous fluid for lubrication when defecating. The tail is short, blunt, with a prominent process called a mucron. The mucron is a clear conically shaped structure with an unknown function.

The scanning electron micrographs reveal the mouth is triangular in shape and surrounded by three lips, one dorsal and two ventrolateral. This is similar to a previous study by Shih *et al* (2010) but the amphid and the boring tooth in our SEM pictures are more distinct. The mouths of *Ascaris lumbricoides* and *Ancylostoma duodenale* are circular in shape. *Anisakis simplex* has one boring tooth, while *Ancylostoma duodenale* has four teeth. The tail has a mucron, similar to *Pseudoter-*

ranova decipiens (Chai *et al*, 1995) and *Angiostrongylus cantonensis*, unlike *Anisakis brevispiculata* and *Pseudoterranova ceticola* (Abollo and Pascual, 2002), which do not have a mucron. The mucron of *A. simplex* is distinctive. The anterior and posterior parts of the worm have transverse cuticular striations similar to other nematodes, such as *Pseudoterranova decipiens*, *Pseudoterranova ceticola, Anisakis brevispiculata, Angiostrongylus cantonensis*, *Ascaris lumbricoides* and *Ancylostoma duodenale*, while the middle region has longitudinal striations and rugae.

SEM can be used to observe the ultrastructure in more detail than stereomicrographs. Ultrastructural features, such the boring tooth, mucron, mouth, lips, striations and other organs can be studied in more detail than with stereomicrographs. The papillae, amphids, phasmids and opening of an excretory pore cannot be seen with a stereomicrograph. The intestinal tract, including esophagus, intestine, rectum and anal glands can only been seen in stereomicrographs. SEM can be used to confirm worm species by observing morphological differences, such as the lips, the tooth and the mucron.

In conclusion, we studied the surface structure of *Anisakis simplex* with a stereomicroscope and a SEM. A SEM is a powerful tool for distinguishing *Anisakis simplex* from other L3 nematodes which may have the same size and appearance under light microscopy. This study clearly shows the difference in mouth, lips, boring tooth and mucron structures. *Anisakis simplex* has a triangular mouth, surrounded by three lips, with one boring tooth, while the mouths of *Ascaris lumbricoides* and *Ancylostoma duodenale* are circular and *Ancylostoma duodenale* has four teeth. The end of the tail has a mucron, similar to *Pseudoterranova decipiens* and *Angiostrongylus cantonensis*, while *Anisakis brevispiculata* and *Pseudoterranova ceticola* lack a mucron. There are several ways to distinguish worm species; SEM is useful to disguise between species.

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