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Scanning Electron Microscopic Observation of Ultrastructure of *Dirofilaria immitis* Microfilaria

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ABSTRACT. Ultrastructure of *Dirofilaria immitis* microfilaria(Mf) was imaged through scanning and transmission electron microscopies. Transverse annular striations covered all over the surface of the whole body. Two small pores on the cephalic disk and the mouth-like cavity at a ventral side of the first striation next to the cephalic disk were observed. A single triangular hook was projected from the upper palate. The excretory pore was observed at the 80th annulus from the anterior end, and the anal pore at the 90th annulus from the posterior end. The both pores were located at the ventral side of the body. This study first demonstrated that a large number of nuclear column cells were distributed in the body cavity. These cells were spherical and about 1 μm in diameter. Each of the cells contained a spherical nucleus and was connected to each other by micro-strings that were running radially. Many flattened muscle cells were located at the inside of the hypodermis of the whole body. The tail contained only a single longitudinal muscle cell.
KEY WORDS: *Dirofilaria immitis*, microfilaria, scanning electron microscopy, ultrastructure.

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The ultrastructure of microfilaria (Mf) of *Dirofilaria immitis* has been observed mainly by transmission electron microscopy (TEM) [3–6, 9], while only a few reports have shown photos of scanning electron microscopy (SEM) [1,5,7] although the quality of their photos was not so high. Of them, several reports have indicated that there are morphological differences of Mfs among filarial species, particularly on the anterior part of Mf, such as a cephalic hook, lip-like processes and cephalic ciliary channels. However, the details of the morphological features of Mfs still remain in poor understanding. The present report describes detailed features of the outer and inner ultrastructure of Mf of *D. immitis* by means of SEM and TEM.

MATERIALS AND METODS

Microfilariae: Mfs were obtained from a microfilaremic dog living in Yamaguchi, Japan, which is naturally infected with *D. immitis*, according to the procedure described by Hayasaki [2]. One volume of blood was taken into a sterilized disposable test tube and mixed with two volumes of sterilized phosphate buffered saline (PBS, pH 7.2) involving 0.5% (w/v) saponin. The mixture was mildly shaken in a water bath at 37°C for 5 min, followed by centrifugation at 55 × g for 10 min. The sediment including Mfs was washed twice in PBS and used for the observation of outer and inner structures of Mf bodies by SEM and TEM.

Scanning electron microscopy (SEM): The sediment including Mfs was fixed in 2.5%(w/v) glutaraldehyde (TAAB Laboratories, UK.) buffered with 0.05 M sodium phosphate buffer (pH 7.3–7.4) for 60 min and rinsed in the same buffer three times 20 min each. The Mfs were dehydrated with a 50–100% ascending ethanol series and dried with a JEOL JCPD-5 critical point dryer [10] or freeze-dried with a JEOL JFD-300 Freeze-drying device after the treatment with 100% t-butyl alcohol two times 2 hr each.

On the other hand, aggregates of Mfs were cut into about 1 × 1 × 5 mm pieces. These pieces were fixed in glutaraldehyde and dehydrated in an ethanol series as described above, cracked in liquid nitrogen by the method of Yukawa and Tanaka [11], and then dried with a critical point dryer.

Each dried specimen was mounted on a metallic stub, coated with gold at thickness of 20 nm using a JEOL JFC-1500 ion sputtering device, and observed by means of a JEOL JSM-6100 scanning electron microscope at 15 kV.

Transmission electron microscopy (TEM): Aggregates of Mfs were cut into about 1 × 1 × 1 mm pieces. These pieces were fixed in 2.5%(w/v) glutaraldehyde buffered with 0.1 M cacodyrate buffer (pH 7.3–7.4) for 45 min under ice water, and rinsed in the same buffer. Then the pieces were post-fixed in 2% (w/v) osmium tetoroxide (OsO_4) (Merck, Darmstadt, Germany) buffered solution for 90 min under ice water and rinsed in the buffer. These fixed specimens were dehydrated in a 50–100% ascending acetone series, and embedded in a low-viscosity epoxy resin comprising Quetol 653 (Nisshin EM, Tokyo): ERL 4206 (Polyscience, Warrington, Pa.): NSA (Polyscience): S-1 (Polyscience) at the volume ratio of 14:23:63:0.5. The resin was polymerized for 24 hr at 55°C and thereafter for 12 hr at 60°C. Then sections were cut with a glass nife on a LKB 2088 Ultrotomy ultra-microtome, and observed by means of a Philips PW6031 transmission electron microscope at 80 kV after staining with uranyl acetate and lead citrate.

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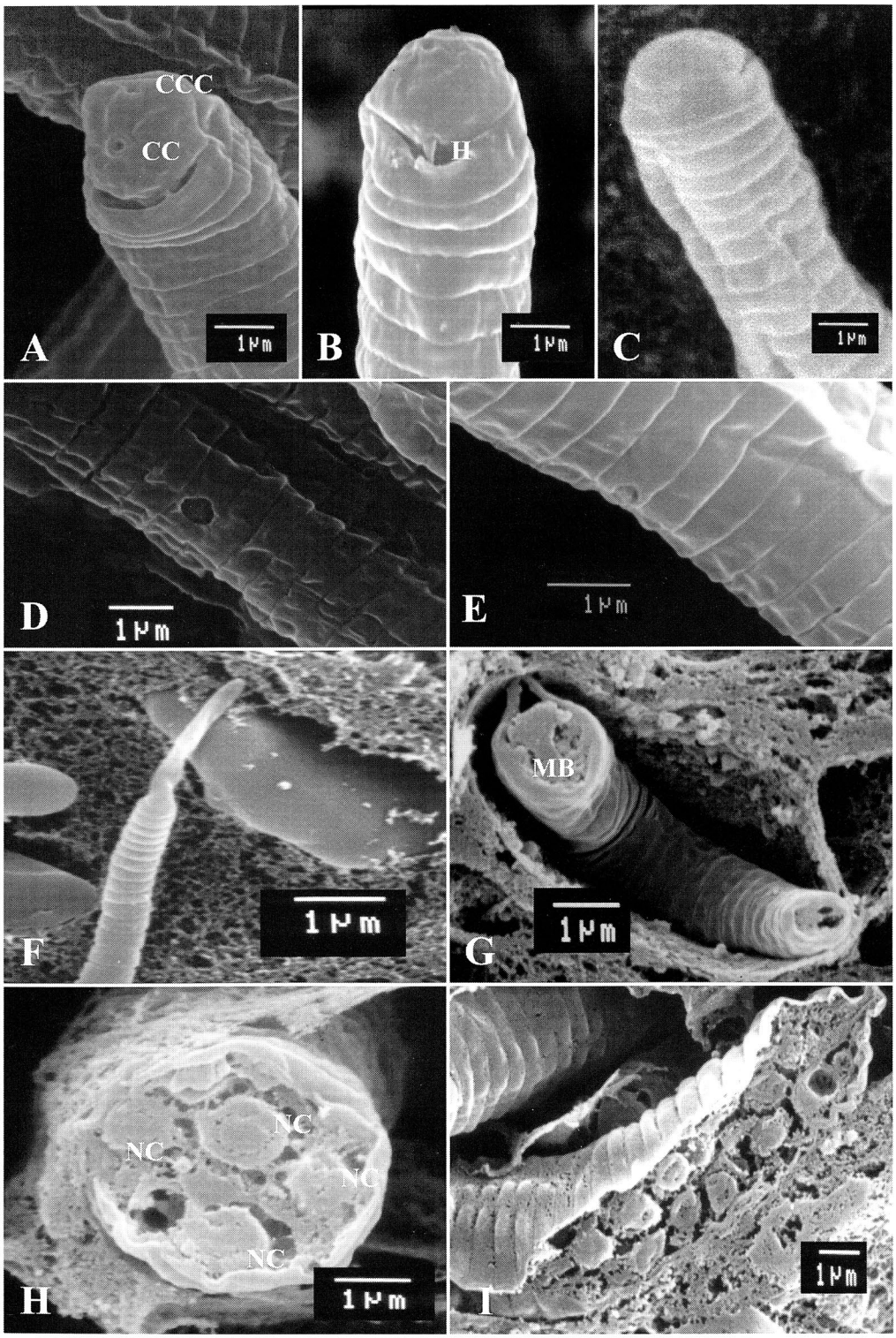
RESULTS

Ultrastructure of microfilaria by SEM: Surface of the cuticle observed by SEM was structured by transverse annular striations with interstitial distance of approximately 0.5–0.9 μm throughout the body (Fig. 1A-1G) except posterior tip. The interstitial distance of annuli in the posterior part of the body was gradually shortened. The posterior tip of the tail had a club stick-like shape, with smooth surface and about 2 μm in length (Fig. 1F). In the anterior tip, two small



pores were observed at the central and dorsal parts of the cephalic disk, which had about 1 μm in interstitial distance (Fig. 1A). These appeared to be a cephalic ciliary channel and a central canal, respectively. In the dorsal part of cephalic disk and the periphery, there was no other accessory structure (Fig. 1C). Mouth-like cavity was hemicycle in shape, and situated at the ventral side of the first striation just under the cephalic disk, with a triangular hook projected from the upper palate to the mouth-like cavity, which was 0.3 μm in length and 0.2 μm in width at the base (Fig. 1B). The lip-like edged processes encompassed the cavity (Fig. 1B). An excretory pore was located on the ventral side of approximately the 80th annulus from the anterior end, having an oval hole of 0.7 × 0.5 μm (Fig. 1D). An anal pore was

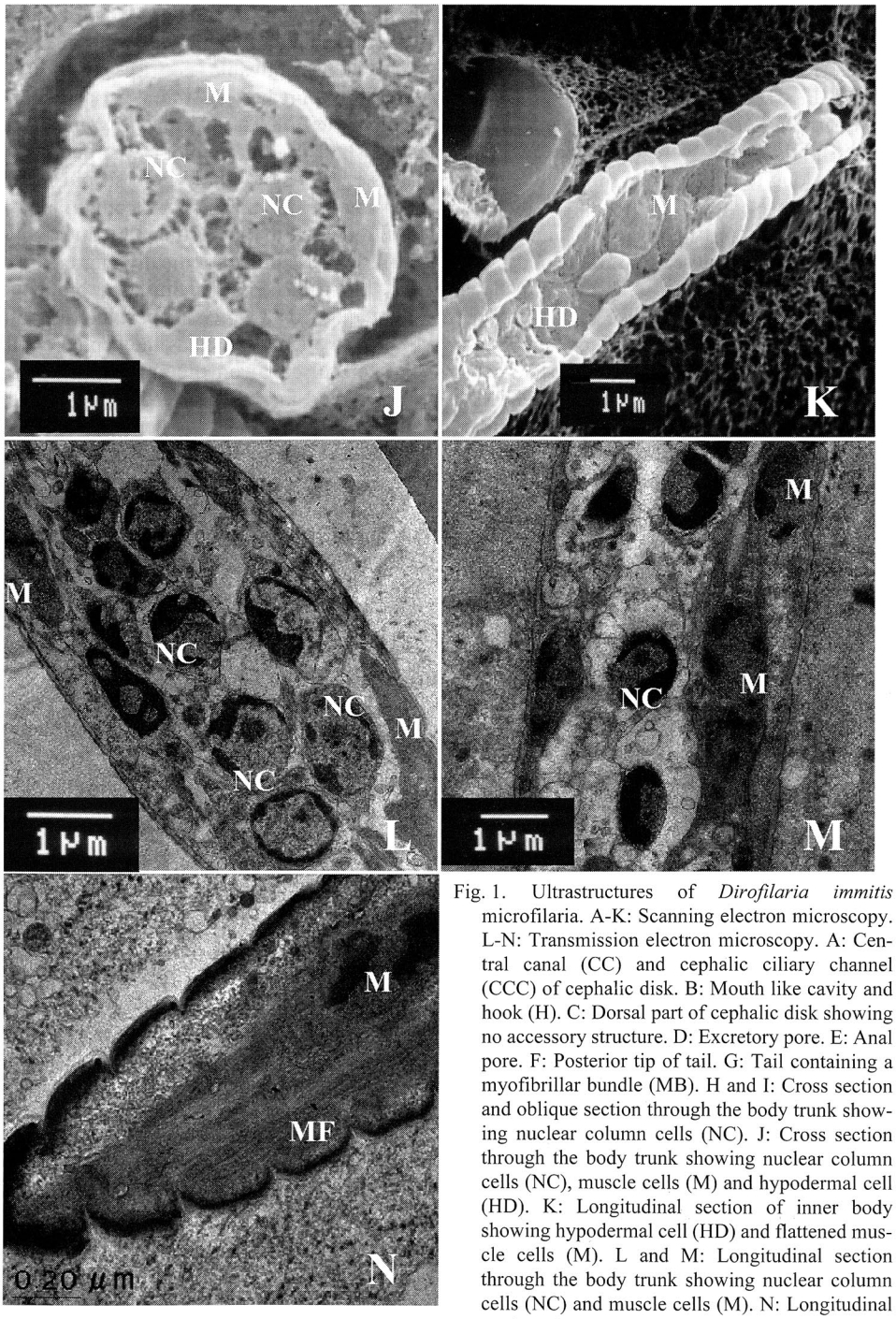


Fig. 1. Ultrastructures of *Dirofilaria immitis* microfilaria. A-K: Scanning electron microscopy. L-N: Transmission electron microscopy. A: Central canal (CC) and cephalic ciliary channel (CCC) of cephalic disk. B: Mouth like cavity and hook (H). C: Dorsal part of cephalic disk showing no accessory structure. D: Excretory pore. E: Anal pore. F: Posterior tip of tail. G: Tail containing a myofibrillar bundle (MB). H and I: Cross section and oblique section through the body trunk showing nuclear column cells (NC). J: Cross section through the body trunk showing nuclear column cells (NC), muscle cells (M) and hypodermal cell (HD). K: Longitudinal section of inner body showing hypodermal cell (HD) and flattened muscle cells (M). L and M: Longitudinal section through the body trunk showing nuclear column cells (NC) and muscle cells (M). N: Longitudinal section through the body trunk showing myofibrillar bundles (MF) and muscle cells (M).

located on the ventral side of approximately the 90th annulus from the posterior end, having a round hole about 0.4 × 0.4 μm, with a small protuberance (Fig. 1E). In the inner structure of the body, many nuclear column cells were observed in the cross section and the oblique section of the body, respectively (Figs. 1H and 1I). Each cell was spherical and about 1 μm in diameter, containing a spherical nucleus, and connected to each other by micro-strings that were running radially (Figs. 1H, 1I and 1J). Flattened muscle cells were adherent to hypodermis, as a single cell or coupling cells (Fig. 1K). Tail contained long longitudinal myofibrillar bundles (Fig. 1G).

Ultrastructure of microfilaria by TEM: The cuticle and trunk of the limited parts of Mf were observed by TEM (Figs. 1L-1N). In the longitudinal section, the cuticle consisted of high electron dense external cortical layer and low electron dense internal cortical layer (Figs. 1L and 1M). The cross sections and longitudinal sections of the body also showed many nuclear column cells in which each nucleus contained chromatin arranged peripherally. The hypodermal cells were slightly flattened fusiform and contained abundant chromatin (Figs. 1L and 1M). The muscle cells were fusiform and adjoined to the hypodermis (Fig. 1M). The tail contained longitudinally arranged long myofibrillar bundles and most of them were localized in parallel to the hypodermis (Fig. 1N).

DISCUSSION

Morphology of Mfs among several filarial species was reported [1, 3–9]. These reports indicated that the morphology of cephalic parts of Mfs was different among the species.

On the Mf of *D. immitis*, however, there are only a few reports by light microscopy [8], TEM [3,4] and SEM [1]. Thus, further morphological observations are necessary to understand detailed structure of the Mf.

The present study additionally revealed detailed morphological features of the Mf of *D. immitis* by SEM, particularly a mouth-like cavity, a lip-like process, a triangular hook, two small pores on the cephalic disk, the body wall, nuclear column cells, an excretory pore, an anal pore, and a tail.

It is clearly observed in the present study that the mouth-like cavity was situated at the first transverse annular striation between the cephalic disk and the first annulus, and structured a dead alley like a sac, indicating that it could harbor the hook. This was supported by the fact that a diluted methylenblue solution did not penetrate into the body cavity when the solution was dipped on the mouth-like cavity of Mf, and observed by light microscopy, while the solution was soaked up from the two small pores on the cephalic disk (unpublished data). The previous report also pointed out the alley structure of the mouth-like cavity [4].

Another report [1] showed different features of the Mf of *D. immitis* by SEM, in which the hook projected outside from the lateral side of cephalic disk, and the mouth-like cavity and the lip-like process were never demonstrated at any parts of the anterior end, although the photos were not clear because of fuzzy focus. These differences, however, might be caused by technical problems through the preparation procedures of the specimens.

On the other hand, TEM of the anterior part of the Mf of *D. immitis* demonstrated that the mouth-like opening was associated with the lip-like process and the internal composition consisted mainly of muscular filaments, terminating to the inside wale of cuticle [4, 6]. These findings may indicate that the mouth is movable, i.e. opens or closes, and the hook is also movable, i.e. rises up or lays down. In fact, another report has illustrated a risen cephalic hook in the study of Mf of other species, *Acanthocheilonema reconditum* [8]. These morphological structures indicated that the mouse-like cavity could be a surplus space necessary for housing the hook. The hook could play a role in holding a position itself in blood stream by hooking at blood vascular surface, and could periodically migrate between the peripheral blood vessels and the large blood vessels, although there are no data which support this supposition.

The two small pores as the cephalic ciliary channel and the central canal on the anterior end have been already known, and the latter has been considered to develop into esophagus throughout the growth to adult worm [5]. In the dorsal part of the cephalic disk and the periphery, there were no other accessory structures.

The present SEM observations in this study first revealed by the three dimensional images of a large number of nuclear column cells or spherical cells pervaded and distributed over the inside of the body and radially running microstrings connecting these cells to each other (Figs. 1H-1J). It is considered that these strings function as supporting tissues or channels relating cell-metabolism. The cuticular shell lining with hypodermis kept the original shape after cutting or slicing off into pieces throughout the SEM preparation procedure (Fig. 1K).

The tail part was filled with single stumpy muscle, meaning that Mf could move forward through vigorous and powerful kick of the tail.

Masuya [7] reported that the spherical granules with the size of 0.1–0.35 μm were found from various places in the body and these granules contained several kinds of fluorescent substances including a photo-reflecting ferritin. However, according to the photos in the report it seems that these granules merely float in the body fluids without any organized relation to other organs or tissues. Therefore, it is difficult to understand the reason why these granules exist separately without any histological connections in the body. In this study, we could not find such separated spherical granules by SEM observation. These granules, even if they exist, might be washed away throughout the procedure of pre-treatment for SEM, therefore, it could not be considered whether these granules could contribute to make a periodical behavior of Mf or not. The nature of periodical behavior is not yet revealed whether it could comply with a potential positive phototaxis or negative one, although Masuya [7] emphasized his hypothesis that such fluorescent granules

were considered to be a result of the negative phototaxis of the Mf nocturnal periodicity, because Mfs would be potentially irritated and then escape from the subcutaneous peripheral blood vessels during a daytime.

The mean body length of Mfs was 308 μm (266–359 μm, n=20) and about 300 annuli were estimated in the whole body in this study. Another report [1] has reported 306 annuli of *D. immitis* Mf in total. The excretory pore was oval and 0.7 × 0.5 μm in size, and the anal pore was round and 0.4 × 0.4 μm in size. Another report [1] has indicated a similar size of the excretory pore of 0.2 × 0.2 μm and the anal pore of 0.5 × 0.3 μm. These differences may be caused from technical artifacts due to the chemical materials for fixation.

In conclusion, *D. immitis* Mf was covered with transverse annular striations all over the surface. The central canal and the cephalic ciliary channel were located at the dorsal and ventral sides respectively on the cephalic disk. The mouth-like cavity opened at the ventral side of the first striation. The single triangular hook was projected from the palate. The excretory pore at the 80th annulus from an anterior end and the anal pore at the 90th annulus from a posterior end were located at the ventral side of the body, respectively. Many spherical nuclear column cells were located in the whole body and many flattened muscle cells were also located inside of the hypodermis of the whole body. The tail contained a thick muscle. These structures could be supportive for their vivid and dynamic movement seen in a blood smear of microfilaremia dog under the microscope.

REFERENCES

1. Aoki, K. and Katamine, D. 1975. Scanning electron microscopic observations on *Dirofilaria immitis*. *Tropical Medicine* **17**: 27–34 (in Japanese with English summary).
2. Hayasaki, M. 2001. Immunological analysis of agglutination in *Dirofilaria immitis* microfilariae. *J. Vet. Med. Sci.* **63**: 903–907.
3. Kozek, W. J. 1968. Unusual cilia in the microfilaria of *Dirofilaria immitis*. *J. Parasitol.* **54**: 838–844.
4. Kozek, W. J. 1971. Ultrastructure of the microfilaria of *Dirofilaria immitis*. *J. Parasitol.* **57**: 1052–1067.
5. Kozek, W. J. and Orihel, T. C. 1983. Ultrastructure of *Loa loa* microfilaria. *Int. J. Parasitol.* **13**: 19–43.
6. McLaren, D. 1972. Ultrastructural studies on microfilariae (Nematoda: Filarioides). *Parasitology* **65**: 317–332.
7. Masuya, T. 1976. Studies on the mechanism of the filarial periodicity: The autofluorescence in the microfilariae and their periodicity. *Jpn. J. Parasitol.* **25**: 283–313.
8. Sawyer, T. K. Rubin, E. F. and Jackson, R.F. 1965. The cephalic hook in microfilariae of *Dipetalonema reconditum* in the differentiation of canine microfilariae. *Pro. Helm. Soc. Wash.* **32**: 15–20.
9. Tongu, Y. 1974. Ultrastructural studies on the microfilaria of *Brugia malayi*. *Act. Med. Okayama* **28**: 219–241.
10. Yoshii, Z., Tanaka, S., Konishi, H., Okusa, A., Takamura, A., Kobayashi, M. and Iwasaki, A. 1977. Studies on the specimen preparation methods for scanning electron microscopy. Part III. Drying method of microbial free cell materials. 2. A contrivance in critical point drying-utilization of the small envelope of filter paper. *Yamaguchi Med. J.* **26**: 197–203.
11. Yukawa, Y. and Tanaka, S. 1979. Scanning electron microscope observations on resting sporangia of *Plasmodiophora brassicae* in clubroot tissues after alcohol cracking. *Canadian J. Botany* **57**: 2528–2532.