[Jpn. J. Parasitol., Vol. 35, No. 3, 191–199, June, 1986]

The Longitudinal Cuticular Markings of *Dirofilaria immitis* Adult Worm

SHIGEHIKO UNI AND SUEHISA TAKADA

(Received for publication; November 16, 1985)

Key words: *Dirofilaria immitis*, *Dirofilaria ursi*, longitudinal cuticular markings, longitudinal ridges, scanning electron microscopy, metallurgical microscopy

Introduction

Cuticular markings, especially longitudinal ridges, were suggested as one of the reliable morphological characteristics of identification and grouping of species in the genus *Dirofilaria* (Chandler, 1942; Anderson, 1952). The ridges varied in shape and distribution from species to species of the subgenus *Nochtiella* (Faust, 1937; Orihel and Beaver, 1965, and Wong and Brummer, 1978).

So far, the cuticular markings of *D. immitis* (Leidy, 1856) have not been intensively examined, though Tulloch *et al.* (1972) and Wong and Brummer (1978) described the cuticular ridges on the posterior part of the male worms under SEM. Recently, one of us reported briefly the longitudinal markings at the midbody of male and female specimens of *D. immitis* by SEM (Uni, 1978).

In the present paper we will report that the longitudinal markings can be seen on the cuticular surface of *D. immitis* adult worms not only by SEM, but also by special kinds of light microscopies and transmission electron microscopy (TEM). And then, we will compare the markings of *D. immitis* with those of *D. ursi* from bears in Japan.

Department of Medical Zoology, Osaka City University Medical School, Osaka 545, Japan.

Materials and Methods

To observe the fresh worms, living worms (82 specimens) of *D. immitis* were collected from the right ventricles of the hearts of five dogs (*Canis familiaris*) in the animal unit of our medical school. The dogs were killed after anesthesia by the intravenous injection of sodium pentobarbital or air. Other worms (31 specimens) examined in this study were those collected from dogs from different localities in Japan; Aomori, Kanagawa, Oita and Nagasaki, and from seals (*Phoca vitulina* and *Pusa hispida*) and sea lions (*Eumetopias jubata*) kept in the animal park of Hokkaido (Kamiya and Kagoshima, 1977). Specimens from Aomori, Hokkaido and Nagasaki had been preserved in 5% formalin, Kanagawa in 70% alcohol, Oita in 3% formol saline, respectively. In addition to the worms from Japan, we examined the material (8 specimens in 70% alcohol) from dogs of Lousiana and California, and from a nutria (*Myocastor coypu*) of Louisiana.

D. ursi preserved in 70% alcohol were examined in our study as comparative material. The specimens were taken from the perirenal adipose tissue of the Japanese black bear (*Selenarctos thibetanus japonicus*) in Kyoto (Uni, 1983).

Preparation for SEM

After being washed in saline, the living worms of *D. immitis* were prefixed in 5%

glutaraldehyde in Millonig's phosphate buffer (0.2 M, pH 7.2) at 5°C for 12 hr cut into small pieces in the buffer, postfixed in 1% osmium tetroxide in the buffer for 1 hr, then washed in the buffer, dehydrated with a graded series of ethyl alcohol, treated with isoamyl acetate, dried with liquid CO_2 in a critical point apparatus (Hitachi, HCP-1) and finally coated with gold by sputtering in an ion bombardment apparatus (Eiko, IB-3). The specimens were examined by a JEOL F-15 field emission SEM with a high resolution, operated at 15 kV.

In order to compare the effects of fixatives on the surface architecture of the worms, the following solutions were used for prefixation; glutaraldehyde (1%, 2.5%) at different temperatures (5°C, 20°C, 37°C or 60°C), osmium tetroxide (1%), Karnovsky solution, formalin (1% in the phosphate buffer or saline, 10% in water), ethyl alcohol (70%), and glacial acetic acid (Berland, 1961).

Other specimens examined were the worms which had been prefixed in formol solution, alcohol or glacial acetic acid. They were washed in saline, postfixed with osmium tetroxide, and treated in the same ways as described above.

Preparation for TEM

The fragments of the midbody of male specimens were fixed in 5% glutaraldehyde, and postfixed in 1% osmium tetroxide. They were dehydrated, and embedded in Epon 812. The blocks were cut with a Porter-Blum ultramicrotome. The thin sections stained with uranyl acetate and lead acetate were examined with a Hitachi HS-8 electron microscope.

Preparation for metallurgical microscopy (MM)

Living or fixed worms were directly observed without cover glasses, quickly to avoid drying, on the glass slides by MM with a vertical illuminator.

Preparation for Nomarski differential interference contrast microscopy (NDICM)

Pieces of the fresh worms were embedded in the compound (Cryo-M-Bed, Bright Co., for frozen sectioning), sectioned in the freezing microtome (Bright cryostat), and mounted in glycerin. The sections were observed by NDICM.

Preparation for light microscopy (LM)

The frozen sections mentioned above were fixed in ethyl alcohol, stained by Harris's hematoxylin, and observed by LM. We also examined histological sections made by routine methods and stained with hematoxylin and eosin (HE) (Uni *et al.*, 1980).

Results

Examinations by SEM

Fine striations were seen on the heads of all the male and female specimens of *D. immitis* (Fig. 1). Only transverse striations (1 μm apart)

Figs. 1-9 Scanning electron micrographs of *Dirofilaria* species: Figs. 1-8. *D. immitis* and Fig. 9. *D. ursi*. Scale bar unit: μm.

Fig. 1 Striations at head of male. Amphid (*), Papilla (P). ×800.

Fig. 2 Solitary cuticular elevations (arrows) at anterior part of male. ×1,000.

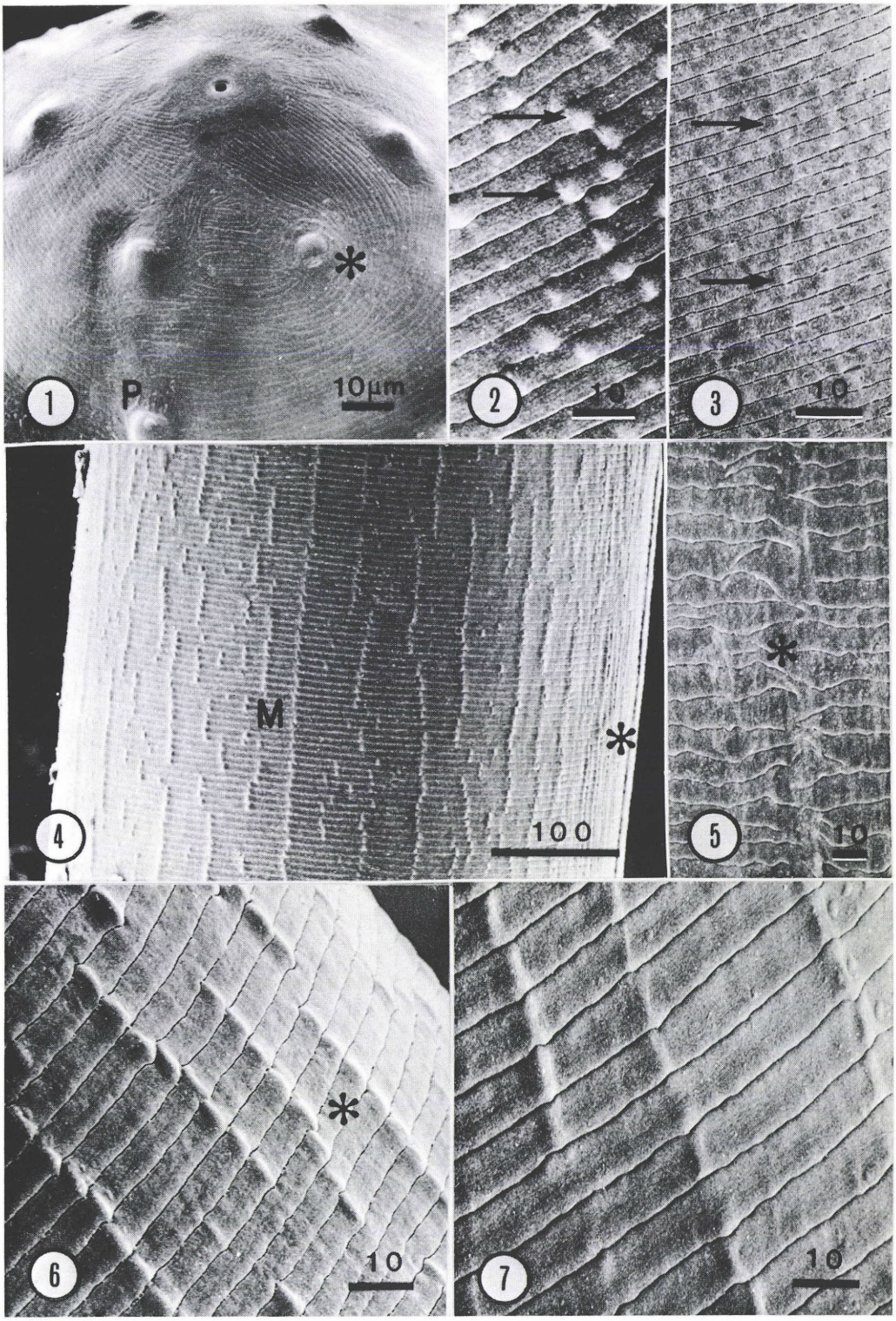
Fig. 3 Faint cuticular elevations (arrows) at anterior part of female. ×1,000.

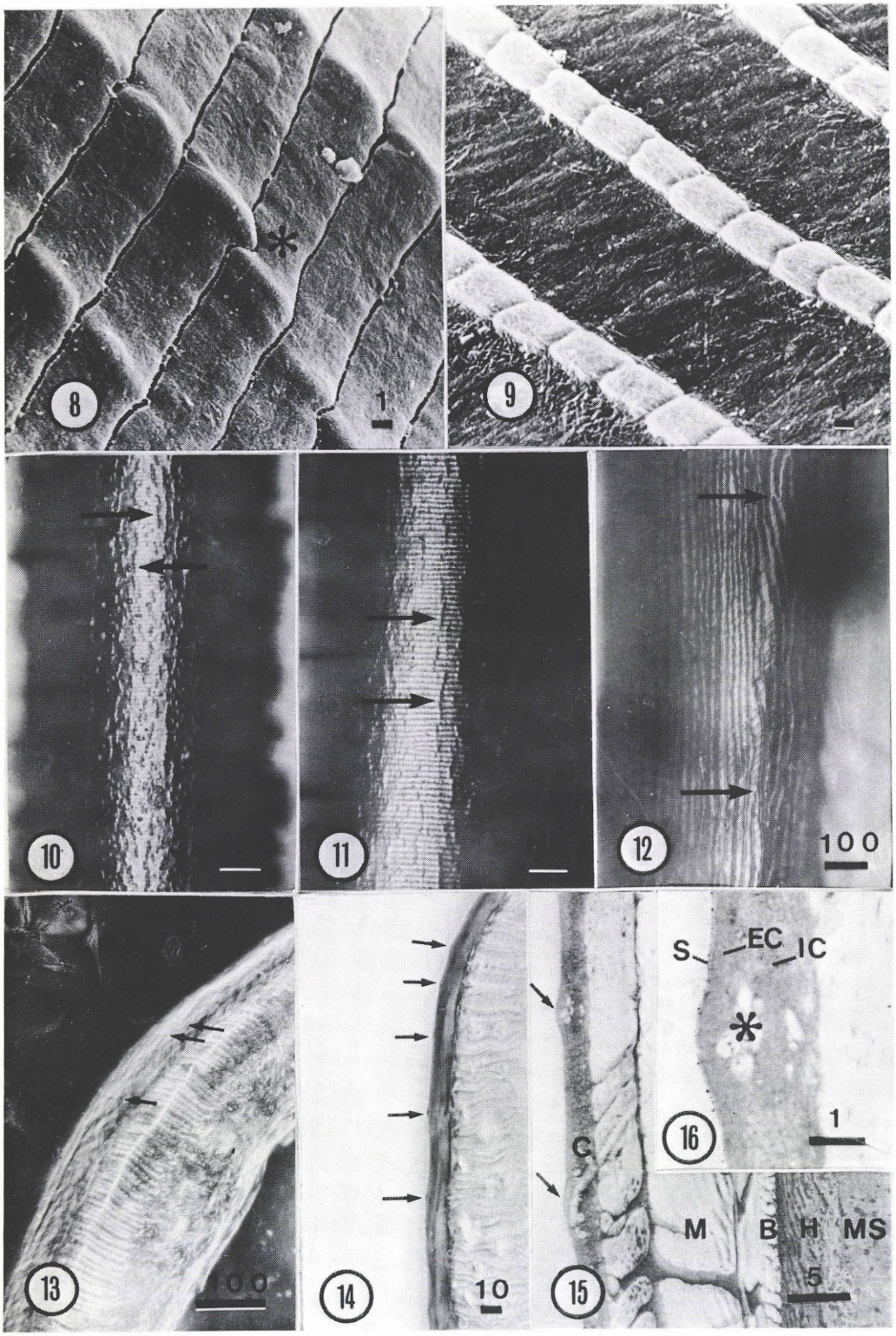
Fig. 4 Longitudinal cuticulart markings at midbody of male. Median line area (M), Lateral line area (*). ×200.

Fig. 5 Lateral line area (*) of Fig. 4. Transverse striations disappear. ×500.

Fig. 6 Longitudinal markings (*) at midbody of male. ×1,000.

Fig. 7 Longitudinal markings at midbody of female. ×1,000.


1
P
10μm
2
3
4
M
100
5
6
7


8
1
9
10
11
12
100
13
00
14
10
15
S
EC
IC
C
16
1
M
B
H
MS
5

were recognizable in the part anterior to the deirids. Small elevations were dispersed on the cuticular bands between transverse striations around the deirid level of males (Fig. 2). At the vulva level of females, the elevations were not so prominent as those of the anterior part of males (Fig. 3, arrows).

At the midbody of males, the longitudinal markings were found, which looked like beads or ridges of short length (Fig. 4). The markings were longer on the dorsal and ventral median line areas (Fig. 4, M). They were not observed near the lateral line areas where the transverse striations were irregular and disappeared (Fig. 5). The smallest male (body length: 5 cm) showed 42 lines of ridges (markings) around the midbody, and the markings were constituted of a few elevations. The males (7 specimens, 12–15 cm long) had 52 (40–70) lines of ridges around the midbody, and the markings were composed of 6 (4–11) elevations (Fig. 6). A single cuticular elevation was 4–5 μm long and 1.3–1.7 μm wide in male worms (Fig. 8). Some elevations extended longitudinally and disfigured the regular arrangement of transverse striations (Fig. 8,*).

At the midbody of female worms, the markings were less prominent in shape and the lines of the ridges were less in number than those of male worms. A smaller female (20 cm long) had only a few lines of the ridges around the midbody, but longer females (7 specimens, 24–31 cm long) showed 34 (24–45) lines of the ridges, each of which consisted of 4 (2–5) elevations (Fig. 7). The single elevation was longer almost twice than that of male worms.

As for the markings of the posterior part of females, the majority of them became fainter near the postdeirid level and then disappeared caudally. In the posterior part of males, the cuticular elevations of the markings became isolative on the ventral aspect just anterior to the coiled part and then they turned into rather distinct beaded appearance and finally led to broad ventral ridges (10–11 lines) posteriorly ("ventral ridges" by Chitwood and Lichtenfels, 1972). On the dorsal aspect the markings remained to be continuous slender lines of less number.

Out of the fixatives used in the present SEM study, 5% glutaraldehyde at 5°C–37°C resulted in no wrinkles on the cuticle of the

Fig. 8 Longitudinal markings, enlarged of Fig. 6 (*). x3,000.

Fig. 9 Longitudinal ridges at midbody of male *D. ursi.* x3,000.

Figs. 10-12 Metallurgical micrographs.

Fig. 10 Longitudinal markings (arrows) at midbody of living male *D. immitis.* x60.

Fig. 11 Longitudinal markings (arrows) at midbody of living female *D. immitis.* x60.

Fig. 12 Longitudinal ridges at midbody of female *D. ursi.* Arrows showing discontinuation of ridges. x60.

Fig. 13 Cuticular markings (arrows) at frozen sections of midbody of female *D. immitis* by Nomarski differential interference contrast microscopy. x100.

Fig. 14 Small projections (arrows) at stained cross-sections of midbody of male *D. immitis* by light microscopy. x300.

Fig. 15 Cuticle at cross-sections of midbody of male *D. immitis* by transmission electron microscope. Cuticular elevations (arrows), Cortical layer (C), Median layers (M), Basal layer (B), Hypodermis (H) and Musculature (MS). x1,900.

Fig. 16 Cuticular elevation, enlarged of Fig. 15. Sheet (S), External cortical layer (EC), Internal cortical layer (IC) and Electron lucent spaces (*). x8,400.

worms and allowed us to examine the cuticular markings of the worms in detail. The same results were also obtained in Karnovsky solution. The cuticular markings, however, were recognized on the specimens fixed in formalin or alcohol, unless the worms were intensively damaged by rough treatment, such as long term preservation or replacements in solutions with a distinctly different pH. On the other hand, glacial acetic acid obscured the recognition of the markings by producing a slight unevenness and small wrinkles on cuticular bands all-over the surface.

The longitudinal cuticular markings were recognizable on the specimens from dogs, seals and sea lions from different parts in Japan, and from dogs and a nutria of North America, in spite of different prefixations.

The specimens of *D. ursi* exhibited prominent longitudinal ridges on the cuticle at the midbody. The ridges were divided into small elevations by transverse striations. Sixty to sixty-five ridges were counted around the midbody of the male worms and each elevation had a length of 3–4 μm and a width of 2–3 μm (Fig. 9). In female worms, seventy-five to seventy-seven ridges were counted at the midbody level and each elevation was 4–5 μm long and 3–4 μm wide. At the anterior part of male and female worms, the ridges were faintly seen. Nineteen lines of the ventral ridges with sharp crests were seen at the ventral area of the posterior part of the males.

Examinations by the different kinds of light microscopies

The longitudinal markings, in addition to transverse striations, were directly found on the cuticle at the midbody of living worms of *D. immitis* by MM. Here the minute markings were clearly observable on the cuticle by the reflection of vertical illumination. The markings were more prominent in males than in females (Figs. 10 and 11).

The markings were clearly visualized on the fixed worms for LM by MM. On the surfaces of the specimens of *D. immitis* from different localities of Japan and of North America, the similar markings were found by MM. On the specimens of *D. ursi* the straight longitudinal ridges were found on the cuticle at the midbody by MM (Fig. 12).

The markings could be seen on the cuticle of frozen sections of a fresh *D. immitis*, without using any fixatives, by NDICM (Fig. 13). On the stained cross-sections of the frozen material, small elevations of the markings were recognized at the midbody by LM (Fig. 14). The elevations were very minute and much lower in comparison with those of the ridges of *D. ursi*, although they could be recognized on the common histological sections of the midbody specimens of *D. immitis*, when they were deeply stained by HE and carefully examined.

Examinations by TEM

The cuticle of a male *D. immitis* showed five layers; one cortical, three median and one basal layers, at the cross-sections of midbody (Fig. 15). The cortical layer appeared further to be subdivided into external and internal cortical layers. The cuticle was covered with a thin sheet (Fig. 16). At the cuticular elevations there found electron lucent vacuolar spaces under the external cortical layer.

Discussion

SEM is one of the most useful techniques to reveal the appearance of the cuticular markings of the worms (Hell and Blix, 1973; Shoho and Uni, 1977). Therefore, the specimens which had already been fixed for LM have been frequently observed for SEM study, especially, when they were rare specimens. However, the differences in the prefixation used for SEM study sometimes resulted in the different SEM images (Tulloch *et al.*, 1972; Wong and Brummer, 1978; Uni, 1978).

In the present examinations, we tested the various kinds of fixatives which were usually used for LM study to know if they could be used as prefixations for SEM. Our results

showed that formalin and alcohol could be used for the examination of the cuticular markings of *D. immitis*, but glacial acetic acid damaged the fine structure of the cuticle.

The present studies by SEM and MM first disclosed the distribution and structure of the longitudinal cuticular markings of *D. immitis* adult worms in detail. The number and the length of the markings around the midbody were correlated to the size or sexes of the worms.

Our results seem to be comparable to the cuticular bosses of *Loa loa*. Eberhard and Orihel (1981) made an intensive study on *Loa loa* and reported that the cuticular bosses on the body surface appeared at 40 days after the fourth molt and then 30 days later the worms became robust, covered with bosses all-over the body. The bosses appeared on the body surfaces at the level posterior to the base of esophagus in both sexes and extended posteriorly, and disappeared at the tail. The distribution of the cuticular markings of *D. immitis* and *D. ursi* adult worms seems to be relatively similar to that of *Loa loa*.

Wong and Brummer (1978) noted that the longitudinal markings were lacking at the midbody of a male *D. immitis*. According to their report, the worms were killed in glacial acetic acid and then observed by SEM. As already mentioned in our present examination, the cuticular markings of *D. immitis* were minute at midbody and were affected by glacial acetic acid. Therefore, the fine structures of the cuticle appear to be obscured in their examinations.

Since the genus *Dirofilaria* was established with six species, including *D. immitis* as type species (Railliet and Henry, 1911), more than thirty species have been known until now (Sonin, 1975; Uni, 1978). The majority of the species belongs to the group which has longitudinal ridges and is parasitic in the connective tissues (Faust, 1937; Anderson, 1952). The exceptions were *D. roemeri* (Anderson, 1959) and *D. lutrae* (Orihel, 1965) which were parasitic in the connective tissues but lacked longitudinal ridges, when they were examined by LM. However, we observed recently the fine fiber-like longitudinal markings on the cuticular surface of *D. roemeri* adult worms (Uni, 1978).

In *Nematodirus* spp. (Trichostrongyloidea), the number and distribution of longitudinal cuticular ridges (synlophe) have shown to be useful for identifying the species (Lichtenfels and Pilitt, 1982).

The present study described the characteristics of cuticular markings of *D. immitis*. The examinations of the cuticular markings appear to be inevitable for the identification and grouping of the species of the genus *Dirofilaria*.

There seems to be less variations in shape and distribution of the cuticular markings among the worms of *D. immitis* which were obtained from different localities and from different host animals.

Summary

Longitudinal cuticular markings of *D. immitis* were examined by scanning electron microscopy and metallurgical microscopy. The markings were prominent at the midbody of the both sexes of the worms. The markings were composed of some cuticular elevations and the short ridges of the markings were seen around the midbody. They were more prominent in males than in females. The longitudinal ridges of *D. ursi* were examined in addition. They were lines of conspicuous cuticular elevations in succession. The comparison between the cuticular markings of *D. immitis* with those of *D. ursi* was made. The value of the cuticular markings as a tool for identification of species of the genus *Dirofilaria* was discussed.

Acknowledgements

We are grateful to Dr. L. R. Ash, UCLA, for his contribution of North American specimens and to Drs. H. Kamiya, I. Takayasu, S. Kikuchi, Y. Kuki and C. Oda for the specimens in Japan. Our thanks are due to Dr. C. Shoho for his encouragement in this study.

References

1) Anderson, R. C. (1952): Description and relationships of *Dirofilaria ursi* Yamaguti, 1941, and a review of the genus *Dirofilaria* Railliet and Henry, 1911. Trans. Roy. Canad. Inst., 29, 35–65.
2) Anderson, R. C. (1959): The taxonomy of *Dipetalonema spirocauda* (Leidy, 1858) n. comb. (=*Skrjabinaria spirocauda*) and *Dirofilaria roemeri* (Linstow, 1905) n. comb. (=*Dipetalonema roemeri*). Canad. J. Zool., 37, 481–493.
3) Berland, B. (1961): Use of glacial acetic acid for killing parasitic nematodes for colloction purposes. Nature, 191, 1320–1321.
4) Chandler, A. C. (1942): The helminths of raccoons in east Texas. J. Parasitol., 28, 255–268.
5) Chitwood, M. B. and Lichtenfels, J. R. (1972): Identification of parasitic metazoa in tissue sections. Exp. Parasitol., 32, 407–519.
6) Eberhard, M. K. and Orihel, T. C. (1981): Development and larval morphology of *Loa loa* in experimental primate hosts. J. Parasitol., 67, 556–564.
7) Faust, E. C. (1937): Mammalian heart worms of the genus *Dirofilaria.* In: Festschrift Bernhard Nocht zum 80. Gebrutstag, Hambrug., 131–139.
8) Helle, O. and Blix, A. S. (1973): Some morphological characteristics of *Dipetalonema spirocauda* Leidy, 1858, isolated from hooded seal, *Cystophora cristata* Erxleben, 1777. J. Parasitol., 59, 217–218.
9) Kamiya, H. and Kagoshima, K. (1977): (*Dirofilaria immitis* from the sea lion and the seal, and the arrangement of the caudal papillae). J. Hokkaido Vet. Med. Assoc., 21, 192–197. (In Japanese).
10) Leidy, J. (1856): A synopsis of entozoa and some of their ectocongeners observed by the author. Proc. Acad. Nat. Sci., Philadelphia, 8, 42–58.
11) Lichtenfels, J. R. and Pilitt, P. A. (1982): Synlophe and species determination of *Nematodirus* spp. (Nematoda: Trichostrongyloidea) of domestic ruminants in North America. Mol. Biochem. Parasitol., Parasites-their world and ours, Supplement, 613.
12) Orihel, T. C. (1965): *Dirofilaria lutrae* sp. n. (Nematoda: Filarioidea) from otters in the Southeast United States. J. Parasitol., 51, 409–413.
13) Orihel, T. C. and Beaver, P. C. (1965): Morphology and relationship of *Dirofilaria tenuis* and *Dirofilaria conjunctivae.* Am. J. Trop. Med. Hyg., 14, 1030–1043.
14) Railliet, A. and Henry, A. (1911): Sur une filaire péritonéale des porcins. Bull. Soc. Pathol. Exot., 4, 386–389.
15) Shoho, C. and Uni, S. (1977): Scanning electron microscopy of some *Setaria* species (Filarioidea, Nematoda). Z. Parasitenk., 53, 93–104.
16) Sonin, M. D. (1975): (Filariata of animals and man and the diseases caused by them. Part 3. Filarioidea: Filariidae and Onchocercidae). In: Osnovy Nematodologii, 24, (Ed. by Ryzhikov K.M.), Moscow, Academy Nauka, (In Russian).
17) Tulloch, G. S., Pacheco, G., Anderson, R. A. and Miller, F. H. Jr. (1972): Scanning electron microscopy of adult male *Dirofilaria immitis.* In: Canine heart worm disease, The current knowledge., 113–115. Proceedings of the Second University of Florida Symposium on Canine Heart Worm Disease, Jacksonville, Florida.
18) Uni, S. (1978): Scanning electron microscopic study of *Dirofilaria* species (Filarioidea, Nematoda) of Japan and a review of the genus *Dirofilaria.* J. Osaka City Med. Center, 27, 439–458. (In Japanese).
19) Uni, S., Kimata I. and Takada S. (1980): Cross-section morphology of *Dirofilaria ursi* in comparison with *D. immitis.* Jap. J. Parasitol., 29, 489–497.
20) Uni, S. (1983): Filarial parasites from the black bear of Japan. Ann. Parasit. Hum. Comp., 58, 71–84.
21) Wong M. M. and Brummer M. E. G. (1978): Cuticular morphology of five species of *Dirofilaria*: A scanning electron microscope study. J. Parasitol., 64, 108–114.

犬糸状虫（*Dirofilaria immitis*）における縦状角皮構造

宇仁茂彦　高田季久

（大阪市立大学医学部医動物学教室）

犬糸状虫の角皮表面において，微小な縦状構造（longitudinal cuticular markings）が走査電子顕微鏡による詳細な観察で見いだされた．本構造は生鮮虫体において金属顕微鏡によって，また虫体断面において透過型電子顕微鏡によっても明らかになった．本角皮構造は雌雄成虫の中央部において明瞭である．本構造はいくつかの微小な角皮の隆起からなる縦状の短かい列である．また本構造は雌虫体より雄虫体において顕著である．本構造と *D. ursi* の縦状角皮構造を比較したところ，基本的な構造および分布様式において類似していた．しかし，*D. ursi* では極めて明瞭な角皮隆起からなる，しかもほとんど連続した縦状の列である．これらの角皮構造の特徴は *Dirofilaria* 属の種の同定およびグループ分けにおいて有用な標徴になると考えられる．