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DIPHYLLOBOTHRIUM NIHONKAIENSE SP. NOV. (CESTODA : DIPHYLLOBOTHRIIDAE)—REVISED IDENTIFICATION OF JAPANESE BROAD TAPEWORM—

(cestode/ *Diphyllobothrium* /new species)

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The so-called broad tapeworm, which had been treated as *Diphyllobothrium latum* in Japan since 1889, was revised taxonomically and described as a new species, *Diphyllobothrium nihonkaiense* sp. nov., being distinguished from the original *Diphyllobothrium latum* in Finland. The taxonomic status of the new species among related species of the genus *Diphyllobothrium* is briefly discussed.

The scientific study of the so-called broad tapeworm in Japan originated from the experiment by Iijima (1), who infected himself with the broad tapeworm by taking orally some plerocercoids obtained from a masu salmon caught in Tonegawa-river. Then Eguchi (2,3) followed with more extended and careful investigations on the life cycle of the tapeworm. Since around 1970 human cases of diphyllobothriasis rapidly increased in this country and roused interest of many parasitologists, clinicians and public health specialists.

Kamo et al. (4) proposed that it is necessary to review the

taxonomy of diphyllobothriid cestodes. Further Kamo (5,6) stressed the importance of the studies by investigators of Baltic countries and Alaska (USA) on the taxonomical features of *Diphyllobothrium latum* (Linnaeus, 1758) of the North European origin and on the diphyllobothriid taxonomy (7,8,9,10,11). To establish the taxonomical criteria for the diphyllobothriid cestodes of the Japanese origin, these studies were reviewed carefully. In the meantime some cases of human infection with some marine species of the genus *Diphyllobothrium* were reported and the establishment of accurate taxonomic criteria became more and more necessary (12,13,14).

Since 1978 the Institute of Parasitology, Åbo Academy, Finland, Tottori University School of Medicine and Shimane Medical University have pursued a cooperative study on the taxonomy of cestodes by comparing the so-called broad tapeworm in Japan with the original *Diphyllobothrium latum* in Finland.

In result the so-called broad tapeworm in Japan was recognized as a species different from the original *Diphyllobothrium latum* in Baltic countries, and herein we report it as a new species, *Diphyllobothrium nihonkaiense*.

MATERIALS AND METHODS

Plerocercoids of the Japanese broad tapeworm were collected from the musculature of masu salmons (*Oncorhynchus masou*), and pink salmons (*Oncorhynchus gorbuscha*), both caught in Hokkaido, Japan.

Plerocercoids of the original *Diphyllobothrium latum* in Finland were collected from the musculature of pike (*Esox lucius*) caught in Lake Pyhäselkä, Finland.

All these plerocercoids were removed alive from the fish, and placed in Hanks' solution. A part of them, eight plerocercoids, were directly intubated into the stomach of a golden hamster (*Mesocrisetus auratus*), which was slightly anaesthetized with Fluothane. The hamsters were autopsied 20 days postinfection, and the strobilae which were collected from the intestine were transferred into tap water with a few drops of chloroform and placed in a refrigerator at 4 C for 3 h so that they were completely relaxed and dead. After a morphometric study the strobilae were fixed in 70% alcohol or 5% formalin in preparation for the histological study (8).

Some plerocercoids were fixed in 1% saline containing 4% formalin (formol-saline) at 4 C for 24 h for the purpose of morphological observation and collection of morphometric data (7). These plerocercoids and parts of the strobila were embedded in paraffin, cut into 7μm sections and stained with Heidenhein's Azan or trichrom stain solution. Histological measurements were made on the 10 sections cut from each plerocercoid (9).

Some plerocercoids, some mature proglottides and some eggs were put into 5% glutaraldehyde with phosphate buffer or 4% formol-saline, and were fixed in 1% osmium tetraoxide for 2 h at 4 C. Then they were dehydrated through graded series of ethanol, transferred into amylacetate, and dried by the critical point method. Finally they were coated with gold, and were examined under a Hitachi MSM-III, and a MSV-V type scanning electron microscope.

After the eggs were taken off from the uteri of gravid proglottides, they were cultured in fresh water at the room temperature for 14 days in a dark room. These eggs were hatched by light exposure, and the coracidia were placed at the room temperature for 24 h and fixed with a drop of ammonium picrate-glycerol (15). Observations were made under a Leitz Orthoplane microscope equipped with a Heine phase contrast condensor. The embryonal hooks were measured and drawn with a Leitz drawing prism (60° prism) using a X 90 oil immersion objective and X 25 eyepieces (10).

DIPHYLLOBOTHRIUM NIHONKAIENSE SP. NOV.

Diagnosis

Adult stage(Figs.15 - 28): Adults experimentally reared in golden hamster.

The mean strobilar length is 693 mm (645 - 741 mm), with about 633 segments (631 - 665); the maximum width, attained in gravid segments, is 6.9 mm. All the strobilae are attenuated with segmental margins indented. The mean width and length of gravid segments are 6.8 mm (6.7 - 6.9 mm) and 1.9 mm (1.9 - 2.1 mm), respectively. The number of segments from the first segment to the primordium (A) is 119(117 - 120); the number of segments from the first segment to mature proglottid (B) 444 (437 - 450), so the difference (B-A) is 325 (320 - 330).

In each segment the width is larger than the length, and the length increases as the segment becomes closer to the end of

strobila. The length/width ratio of immature segments is about 1 : 7, and that of gravid segments 1 : 3.5.

The scolex is mostly 2.6 mm (2.4 - 2.8) long, 1.40 mm (1.2 - 1.5) wide, and nearly spatulate in its shape. There are numerous pits at the top of the scolex.

The bothria is well-developed, deep, and as long as the full length of the scolex. The neck is well developed, mostly 15.6 mm (14.4 - 16.8) long and 1.22 mm (1.16 - 1.28) wide.

The genital pore is situated ventrally on the midline at 260 - 340 μm posterior from the anterior margin of the segment which is 1.9 - 2.1 mm in length. The uterine pore opens at 200 - 350 μm posterior from the genital pore.

The cirrus sac is oval and large, 420 - 480 μm in length by 390 - 400 μm in diameter, opening obliquely into the genital atrium. The seminal vesicle is also large and elliptical, 250 μm in length by 100 μm in diameter. It has a thick wall (about 50 μm thick) and is connected to the back of the cirrus sac making a sharp angle. The spherical testes, which are bulbs of 35 - 45 μm in diameter, are arranged in a single layer in the medullary parenchyma, and are covered with a transverse muscle layer of 5 - 10 μm in thickness. The testes are not distributed astride the boundary of segments.

The uterine loops extend laterad (usually there are 6 - 7 loops in each side), with peripheral loops opening into the uterine pore, not extending beyond the anterior margine of the genital atrium. The ovary is renal-formed, situated in the posterior margin of the segment, having neither anterior nor posterior horns.

Eggs in the uterine loops of strobilae reared in golden hamsters, are ellipsoidal with an operculum, measuring 55.2 ± 1.3 μm in length by 38.2 ± 1.5 μm in diameter on an average, the length/width ratio being 1.45 ± 0.06. Eggshells exhibit shallow pits distributed sparsely on the smooth surface.

The embryonal hooks are relatively short. The lengths of the 1st, 2nd and 3rd hooks are 11.06 μm, 12.2 μm and 12.35 μm, respectively. The ratio of blade length to total length of the hook is relatively large, 39.6% in the 1st hook, 36.2% in the 2nd and 37.4% in the 3rd. The curvature of the 2nd and 3rd hook blades is deep and prominent, and the transitional slope from the blades to the guards is gentle. The hook guards are cylindrical, prominent, and projecting at right angles (90°) from

the handles.

Host: *Homo sapiens*
Habitat: In the small intestine
Type locality: Japan
Type: A strobila expelled from a man, fixed and preserved in the 5% formalin solution ; prepared slides bearing short series of segments and serial sections of several portions of strobila have been deposited as a holotype (No. HLS7903) and two strobilae experimentally reared in a golden hamster have been deposited as paratypes (No. PSH8509-1,PSH8509-2) in the Department of Medical Zoology, Tottori University School of Medicine, Yonago City 683, Japan.

Plerocercoid stage(Figs.1 - 14): Plerocercoid collected from masu salmons, fixed in 4% formol-saline.

The plerocercoids are yellowish white and cylindrical. On the average they are 8.2 mm (6.2 - 10.7) long x 1.1 mm (0.9 - 1.3) wide x 0.7 mm (0.6 - 0.8) high. The body tapers slowly toward the tail, somewhat flattens dorso-ventrally. The width is maximum at the middle of the body. The scolex is evaginated with dorsal and ventral median grooves like lips. The tail is concaved concentrically. The bothrium extends dorso-ventrally. The tegument is covered with regular transverse wrinkles which are 240 - 400 μm in width. Microtriches, 3.3 μm long (2.5 - 10.0 μm), cover the tegumental surface. The tedument is 14.6 μm thick (12.5 - 17.5), and the subtegumental, longitudinal muscle layer is 9.2 μm thick(7.5 - 12.5) on the average. The average number of muscle bundles within 50 μm space is 31.6 (24 - 43).

There are well-developed parenchymal longitudinal muscles, 85.7 μm thick (62.5 - 138.6). Their muscle bundles are arranged densely---119 (90 - 161) within 50 μm space. The subtegumental cell layer is extremely well-developed---36.8 μm thick (25.0 - 62.5). Parenchymal cells are also densely arranged---34 (21 - 48) in 50 μm^2 area. The frontal glands are distributed locally and concentrated in the top region, occupying 6.4% of the whole body.

Host: Masu salmon (*Oncorhynchus masou*), Pink salmon (*O. gorbuscha*)
Habitat: In the musculature

Type: Plerocercoids fixed in 5% formalin prefixed in 4% formol-saline, and serial sections of larvae have been deposited in Department of Medical Zoology, Tottori University School of Medicine, Yonago City 683, Japan (No.PS7901).

DISCUSSION

Morphological features of strobila of *Diphyllobothrium latum* had been described by Magath (16), and Wardle and McColl (17), but these descriptions could not lead to the establishment of the taxonomical criteria of this cestode.

Kuhlow (18) described precisely taxonomic features of *Diphyllobothrium latum* plerocercoid and built the criteria by which we can discriminate *D. latum* from similar species belonging to the genus *Diphyllobothrium*.

Subsequently many detailed studies on the larval and adult stages of cestodes appeared and systematized the morphological differences between species (7,19,20,21,22,23,24,25,26). As to the strobilar features in particular Rausch and Hilliard (11) and Andersen (8,27,28) reviewed critically the former descriptions offered us reliable taxonomical criteria by studying host-parasite relationship. The results of these taxonomical studies in Baltic countries, Alaska and USSR stimulated the advancement of the ecological and public health phase in the study on cestodes.

Further, studies by chemo-taxonomical methods such as protein profiles analysed by isoelectric focusing (29) and immunotaxonomical methods such as coracidia precipitation reaction (30,31) contributed to introducing more valid criteria into the taxonomic study. In our study the protein profiles, isoenzyme pattern, aminoacid composition, trace elements composition, biological features in embryonation and initial growth pattern in hosts were determined. We found species-specific differences in each experimental results, but they will be described in our future reports.

Andersen (28) pointed out that in the strobila of cestodes the best morphological indices that tell differences between species are length of the neck, type of the boundary between segments, position of the cirrus sac, conjunctive angle of the cirrus sac to the seminal vesicle and the ovary shape, regardless of the host species and the worm population size. She also

described that, if the host species is the same and the worm population is of an equal size, length and width of each segment, length of the whole strobila, total number of segments could be supporting characters for differentiation to some extent.

Comparing with the four diphyllobothriid species in Baltic countries (*D. latum, D. dendriticum, D. ditremum, D. vogli*), the strobila of *D. nihonkaiense* n. sp. has thinner and more translucent segments which widen gradually posteriad. The strobila of *D. latum* is also thin and wide, but that of *D. nihonkaiense* is so thin that you can see through the inside of uterine loops. The strobila of *D. nihonkaiense* n. sp. has a spatulated scolex like *D. latum* and *D. vogli*, but its scolex is larger. Its dorso-ventral height is also larger and the bothrium better-developed than those of the strobila of *D. latum*. Its frontal pits are similar to those of the four species from Baltic countries except that they have no small papillae at the bottom of the pits. Its neck is also longer and wider than the northern species except *D. latum*. In the three northern species the neck is about 2 mm. The distance between the genital atrium and the uterine pores is similar to those of *D. dendriticum* and *D. vogli*, but longer than that of *D. latum* (11).

Genital papillae of *D. nihonkaiense* are flat and ellipsoidal like those of *D. latum* and *D. dendriticum* (they are prominent and hemispherical in *D. ditremum* and *D. vogli*), but they are not so densely arranged and not so well-developed as in *D. latum* (32,33).

The discontinuity of testes between neighboring segments is not so conspicuous in *D. nihonkaiense* as in *D. latum*. The cirrus sac is situated obliquely in *D. nihonkaiense* as in the northern species except *D. latum*. The seminal vesicle is situated posterior-dorsally to the cirrus sac like in *D. latum*, but the size of the seminal vesicle is relatively large.

D. ursi discovered in Alaska is a large cestode, attaining the length of as much as 11 m and the maximum width of 23 mm (34). Compared with *D. nihonkaiense* it has a larger, more massive scolex lacking a neck, and a different form of strobila, which is muscular, and relatively opaque. The uterine pore opens just posterior to the genital atrium. The plerocercoids parasitize only in red salmon, *O. nerka* (Walbaum), and are usually encysted on the serous membrane of ventriculus. The tail of plerocercoid of *D. ursi* terminates bluntly. Surface furrows of

the tegument are few or none existent. The tegument, 4 - 5 μm in thickness, is thinner than that of *D. nihonkaiense* and the microtriches are rather longer, 8 - 9 μm in length.

As the differential diagnosis between the strobilae of *D. nihonkaiense* and *D. latum* expelled from men is not so easy as in the case of specimens reared experimentally, because of the considerable variance caused by the complex microecological environment in the host and various conditions in the process of treating specimens, we are describing here the strobilae of both species reared experimentally in golden hamsters.

As for the plerocercoids, Kuhlow (18) described that the external appearance, microtriches length, arrangement of longitudinal muscle bundles, distribution and size of calcareous corpuscles, distribution of frontal glands are reliable species specific criteria. Halvorsen (7) added to these the thickness of subtegumental longitudinal muscle layer and the external appearances of the head and tail. Bylund (9) pointed out that features in the plerocercoid stage show most distinctly the differences among species of *Diphyllobothrium*, and stressed the importance of comprehensive analysis based on observations of the whole species specific features ever reported.

Plerocercoids of *D. nihonkaiense* can be easily differentiated from those of *D. vogli*, *D. ditremum* and *D. dendriticum* by their external appearance and size. Also they can be separated from plerocercoids of *D. latum* by the following characteristics: cylindrical external appearance, the maximum width being at the middle of the body, gradually tapering body toward the tail, evaginated head having a labial appearance, and concaved tail.

The histological findings such as tegumental thickness, thickness of parenchymal longitudinal muscle layer, number of muscle bundles, thickness of subtegumental cell layer and number of parenchymal cells in 50 μm^2 are also the points which differentiate *D. nihonkaiense* from *D. latum* and other similar species. The localization of frontal glands in the head region is also a feature characteristic to *D. nihonkaiense*. The plerocercoids of *D. nihonkaiense* and *D. ursi* are distinguishable from each other by morphological features, hosts parasitized and habitat in the body of the host. The species specific features of plerocercoids of *D. ursi* are excysted larvae which are 5 to 20

mm long and oval or cordate in the lateral view, and the body tapering toward head and tail. The intermediate host of *D. ursi* is red salmons, *Oncorhynchus nerka* (Walbaum), which migrate from the north side of Alaska Peninsula to the Pacific Ocean along the seashore of south eastern Alaska, but masu salmons, *Oncorhynchus masou*, which are the host of *D. nihonkaiense*, are distributed only in the Japan Sea and the Sea of Okhotsk, and spawn in the rivers of the northern Japan. The distribution of the two species of salmons can not overlap each other (35). The results of an ecological study on the 2nd intermediate hosts of *D. nihonkaiense* will be described in our future report. Here the geographical isolation of the two species of salmons is important as one of the taxonomical criteria (36).

The morphology and measurements of embryonal hooks proposed by Fraser (37) and Bylund (10) are one of the useful differential criteria. The embryonal hooks of *D. nihonkaiense* are differentiated from those of *D. latum* and of other freshwater species of *Diphyllobothrium* in the following points: shortness of the total length, high ratio of the blade length to the total length , blade curvature in the 2nd and 3rd hooks, the form of transitional part from blade to guard, the form of guard and the angle between guard and handle.

As for the taxonomic value of the egg size, Meyer (38) reported its species specific significance, but Andersen and Halvorsen (39) suggested it was affected by host differences and parasite population. The matured eggs of *D. nihonkaiense* and *D. latum* from strobila experimentally reared in golden hamsters were compared in the same condition. The eggs of *D. nihonkaiense* fell within the range of 57.5 μm in length and of 42.5 μm in width, but in the case of *D. latum* the length was over 60.0 μm and the width over 45.0 μm. The eggs from the strobilae of both species collected from human cases could not be differentiated from each other and the size overlapped each other. It is therefore important to compare the eggs as well as the strobilae of both species experimentally reared in golden hamsters.

Hilliard (40) examined the ultrastructure of eggshell surface under a scanning electron microscope and reported that the depth, form and density of surface pits were species specific, especially valid in differentiating species having marine and freshwater intermediates and final hosts. The sparsely distributed, shallow pits and the smooth surface of eggs

of *D. nihonkaiense* are similar to the features of eggs of *D. latum*. The surface pattern of eggs could be one of the differential criteria of *Diphyllobothrium* species such as a human cestode, *Diphyllobothrium yonagoense* reported by Yamane et al. (14), and marine mammal cestodes, *Diphyllobothrium fuhrmanni* reported by Yazaki et al.(41) and *Diphyllobothrium macroovatum* reported by Kamo et al. (42).

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42) Kamo, H., Maejima, J. and Hatsushika, R. (1980) First record of *Diphyllobothrium macroovatum* Jurachno, 1973 from mink whale, *Balaenoptera acutorostrata* Lacépede, 1804 (Cestoda; Diphyllobothriidae) in Japan. Jpn. J. Parasit., 29, 499 - 505 (in Japanese)

LEGENDS

Plate 1. Plerocercoids of *Diphyllobothrium nihonkaiense* (left) and *Diphyllobothrium latum* (right).
Figs. 1,2. Plerocercoids fixed in 4% formol-saline. (X 8, X 15)
Figs. 3,4. Cross sections of the middle part of plerocercoids (Heidenhein's Azan stain). (X 55, X 110)
Figs. 5,6. Cross sections showing the subtegumental cell layer (SC) and the parenchymal longitudinal muscle layer (LM) (Heidenhein's Azan stain). (X 400)

Plate 2. SEM photos of plerocercoids of *D. nihonkaiense* (left) and *D. latum* (right).
Figs. 7,8. Whole-length plerocercoids. (X10, X15)
Figs. 9,10. Head part of plerocercoids showing bothria (arrows). (X 50)
Figs. 11,12. Tail part of plerocercoids showing the concaves (arrows). (X50, X70)

Plate 3. Frontal glands , embryonal hooks and eggshells of *D. nihonkaiense* (left) and *D. latum* (right).
Figs. 13,14. Head part of plerocercoids showing the distribution of frontal glands (arrows) (Trichrome stain). (X20)
Figs. 15,16. Three pairs of embryonal hooks in coracidia. (X 1,500)
Figs. 17,18. SEM photos of eggshells showing pits and opercular sutures (arrows). (X 3,000)

Plate 4. Adult stage of *D. nihonkaiense* (left, DN) and *D. latum* (right, DL).
Figs. 19,20. Whole strobilae collected from the golden hamsters.
Figs. 21,22. Scoleces and necks of adult worms. (X2, X10)
Figs. 23,24. SEM photos of proglottides showing cirrus (C), genital atrium (GA), uterine pore (UP) and genital papillae(GP). (X 100)

Plate 5. Proglottides of *D. nihonkaiense* (left) and *D. latum* (right).
Figs. 25,26. Whole-mounted specimens of segments showing uterus (U) and cirrus sac (CS). (Carmin stain). (X 25)
Figs. 27,28. Sagital sections of mature segments showing cirrus (C), cirrus sac (CS), seminal vesicle (SV) and uterus (U). (Trichrome stain). (X 50)

Plate 1.

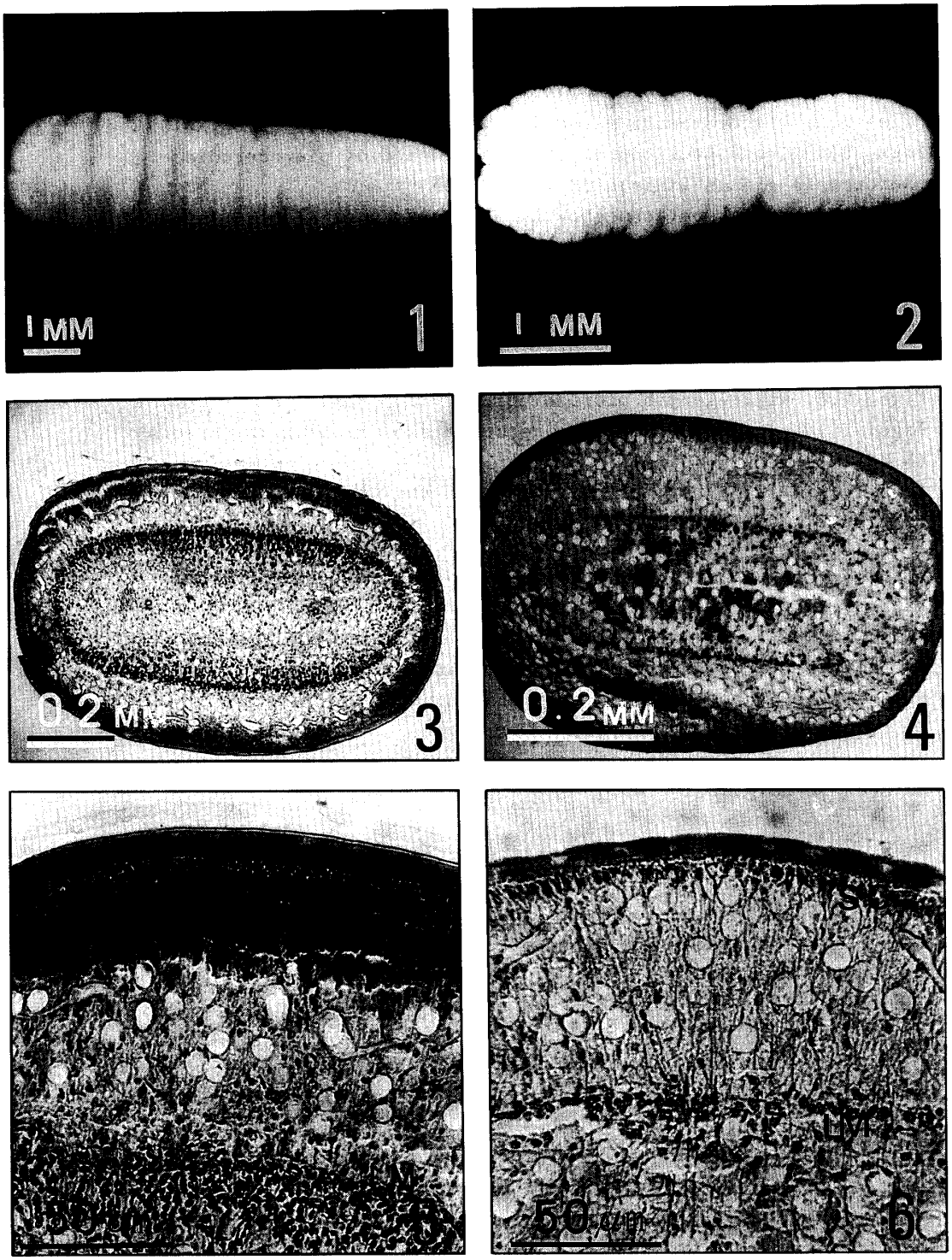

Plate 2.

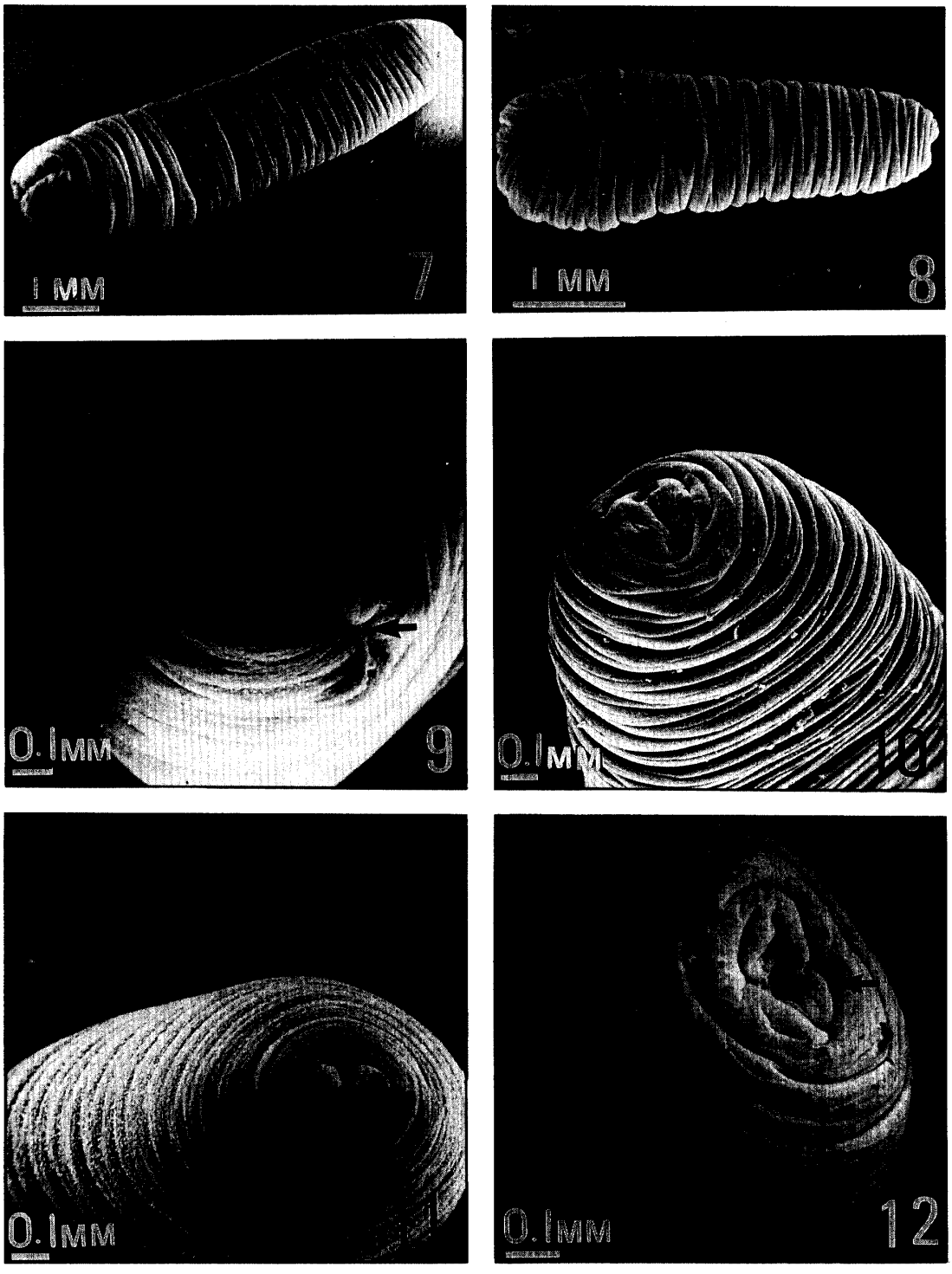

Plate 3.

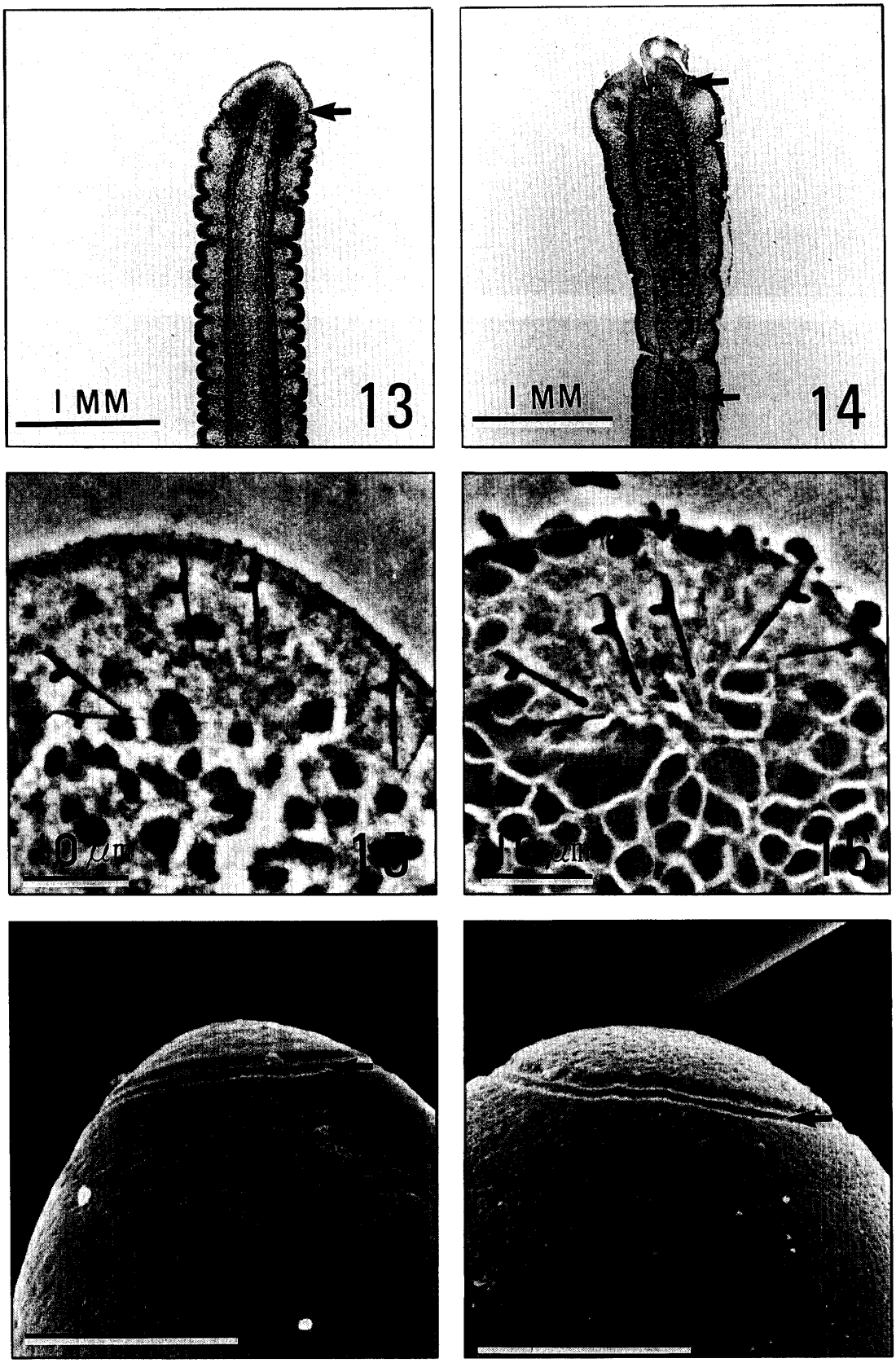

Plate 4.

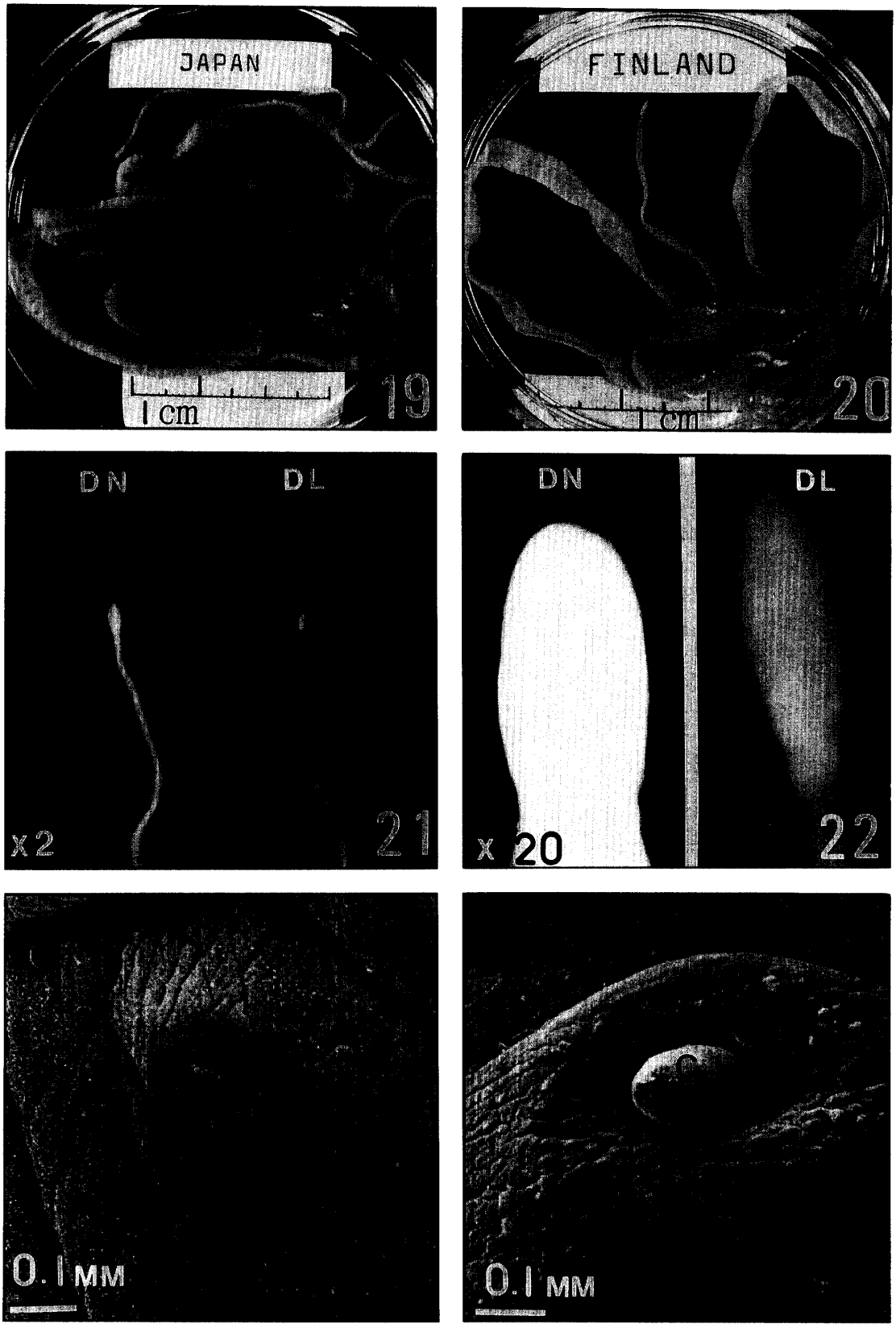

Plate 5.