Doctoral Dissertation

Biology of Chaetonotids (Gastrotricha) – Taxonomy, Morphology and Culture System

By

Takahito G. Suzuki

February, 2014

Index

Abstract……1

Introduction……2

Species diversity of chaetonotid in rice paddies……3

Descriptions of four new species……8

Culture method for 3 species of chaetonotids……27

Summary……38

Acknowledgements……40

Reference……40

Biology of Chaetonotids (Gastrotricha) -Taxonomy, Morphology and Culture System

Takahito G. Suzuki

Abstract

The chaetonotids are freshwater gastrotrichs, living in the interstitial spaces of bottom sediments and superficial detritus, the surfaces of submerged plants and animals, and the water film of soil particles. Their body shapes are mainly bottle-like, flattened ventrally and arched dorsally. In chaetonotids the male is entirely absent, so the population consists of parthenogenetic females, producing unfertilized eggs. This organism has both characters of two large protostome groups, ecdysozoans and lophotrochozoans, thus it is the key taxon in understanding early protostome evolution. However, their biology remains largely unknown, because an experimental system for chaetonotids has not been established yet. In this study, the methods of *in vitro* culture systems for 3 chaetonotids, *Chaetonotus machikanensis*, *Ichthydium podura* and *Lepidodermella squamata*, were created and their strains were established. These systems enabled to obtain sufficient numbers of genetically uniform specimens.

Systematic knowledge in the Japanese chaetonotids is largely unknown. The morphological characteristics of chaetonotids were studied to establish the chaetonotid classification. Four new species of chaetonotids were described from the Kinki region, Japan. *Chaetonotus retiformis* n. sp. and *C. machikanensis* n. sp. were found in the Machikane pond of Osaka university campus (Osaka) and *Dichaetura filifurca* n. sp. and *Lepidodermella acanthofurca* n. sp. were found in the rice paddies in Mano (Shiga). There is the broad diversity of gastrotrichs in the rice paddies, in which 32 unidentified species were found.

Introduction

Gastrotricha is a small phylum of 767 marine, brackish, and freshwater species that live in the interstitial spaces of bottom sediments and superficial detritus, the surfaces of submerged plants and animals, and the water film of soil particles (Balsamo et al., 2009; Hummon and Todaro, 2010; Todaro and Tongiorgi, 2013). There are two major groups: Macrodasyida, which is marine, except for two species; and the marine and freshwater Chaetonotida. The chaetonotid body shape is mainly bottle-like, whereas macrodasyids are mainly cylindrical; both groups are flattened ventrally and arched dorsally (Fig. 1). There is an anterior head with sensory organs, brain, and pharynx. The adhesive tube is beard posterior tail like organ "furca" (mainly chaetonotids) or hole body (mainly macrodasyids). The cilia used for locomotion are restricted to the ventral surface, where they form ciliary bands. The body wall is usually composed of an external cuticle of flexible proteinaceous layers, an external lamellar layer and a basal granular layer. Most gastrotrichs have cuticular scales, spines, and hooks under the cuticle layers (Fig. 1B). The cuticular scales vary in arrangement and shape, depending on the species (Fig. 1C).

Fresh water chaetonotids are parthenogenetic, producing unfertilized eggs that produce only females. A female produces some eggs including two types of egg (Burunson, 1949). Normal (tachyblastic) eggs develop immediately, while resting (opsiblastic) eggs remain inactive for long periods and can survive dry and freezing conditions.

Freshwater gastrotrich species of Japan have been studied by some taxonomists, 33 species including 7 genera have been recorded in Japan. Kawamura (1918) reported the first species, *Polymerurus nodicaudus* (Voigt, 1901) in Shinshu, Nagano. Later, Saito

(1973) reported 26 species in the ponds of Hiroshima. Sudzuki (1971a, b) reported 11 species in lakes around Mount Fuji, Yamanashi and Shiga-kogen, Nagano. The freshwater species has not been reported from the Japan in the subsequent decades. There is limited information in freshwater gastrotrich fauna in Japan.

Molecular phylogenetic analyses suggest that protostomes are composed of two major taxa, ecdysozoa and lophotrochozoa. Affinities of the gastrotrichs have been long been debated, because they have both the characteristics of ecdysozoans and lophotrochozoans. The tissue arrangement of protonephridia of the gastrotichs resemble to that of the Kinorhyncha (Lorenzen, 1985, Ehlers *et. al.*, 1996, Nielsen 2001). On the other hand, the gastrotrichs share the syncytial epidermis with the rotifers and monociliated epidermal cells with the gnathostomulids (Calavier-smith 1998, Zrzavy *et al.*, 1998). Thus, the gastrotrichs are the key taxon in understanding protostome evolution. Current molecular analyses by use of the18SrRNA genes suggest that the gasrotrichs are related to the lophotrochozoans (Manylov, *et al*., 2004; Dunn *et. al.*, 2008), although the phylogenetic trees showed the very low bootstrap values for the branches. The phylogenetic position of gastrotrichs still remains unclear.

Studies of the taxonomy and molecular phylogeny of microorganisms are greatly restricted unless the organisms can be raised in a culture system. This is particularly true in the gastrotrichs, which are small and composed of relatively small numbers of cells. It is quite difficult to obtain DNA samples from a single individual. Sufficient numbers of genetically uniform specimens are required for molecular studies, and for this reason the laboratory culture system for gastrotrichs was established.

Species diversity of chaetonotid in rice paddies

Introuction

The chaetonotids live in freshwater can be found in plant-choked ditches and mossy ponds. In Japan, 34 chaetonotid species have been reported from lakes, ponds, and swamps (Suzuki & Furuya 2011). Only a small number of chaetonotids were reported from rice paddies after midseason drainage (Yamazaki *et al.* 2003; Yamazaki *et al.* 2004). Paddies are a typical feature of rice (*Oryza sativa*) culture in eastern and southern Asia, providing a habitat for a wide variety of aquatic organisms despite the heavy interference by rice farmers. In a previous study (Yamazaki *et al.* 2003; Yamazaki *et al.* 2004), specimens were observed in the mud or water collected from areas surrounding rice plants in the main part of the paddy. Chaetonotids do not favor such open areas without shade, so in the present study the specimens were collected from mud near paths at rice paddy edges. These paths are associated with various kinds of weeds and the leaves of which extend over, or even touch, the water. The shaded areas provided by these leaves were considered to be likely preferred habitats for chaetonotids, and indeed in the present study a large number were found living in this environment, and a high diversity was found also upon inspecting paddies before midseason drainage.

Materials and methods

Specimens were collected from 13 rice paddies at Mano (Otsu District, Shiga Prefecture, Honshu, Japan). These paddies are supplied by different water sources. According to the sources, the survey area was divided into 3 sites comprising 4 paddies

(site1-a, b, c, d; site2-a b, c, d; site3-a, b, c, d) (Table1). And an additional paddy was set in site 3 (site3-add).

Sampling took place during the period when paddies are flooded (mid-May until late June), before midseason drainage. Samples were collected once or twice per week, from bottom sediments, floating plants (*Azolla japonica, Spirodela polyrhiza*), and from fallen weed leaves lying on the under-water soil surface of the paddy. Chaetonotids were obtained from samples by gentle rinsing with water.

Specimens were collected by washing out the plants and scooping the top sediments, and chaetonotids were picked up with a pipette for viewing under a stereoscopic microscope. The recovered specimens were anaesthetized in 1% MgCl2, and fixed in 10% formalin. For light microscopy, specimens were transferred through a graded series of glycerol (50%, 100% twice) for 30 min each and mounted in glycerol on H-S slides (Shirayama et al., 1993). They were observed with a differential interference contrast microscope with Nomarski optics (Olympus BX-50, Tokyo, Japan).

Result

The present study revealed a broad diversity of chaetonotids in rice paddy habitats (Table 2), including the first reports for Japan of seven species (*Chaetonotus brevisetosus*, *C. brevispinosus*, *C. oculifer*, *C. venustus*, *Heterolepidoderma macrops*, *H. obesum* and *Polymerurus nodifurca*). Many chaetonotid species were found, together with various other aquatic organisms, including euglenas (*Euglena*, *Phacus*), ciliates (*Paramecium*, *Spirostomum*, *Stentor, Vorticella*), rotifers (*Brachionus*, *Cephalodella*, *Rotaria*), branchiopods (*Branchinella*, *Daphnia*, *Moina, Scapholeberis, Triops*),

copepods (*Cyclops*), ostracods (*Cypridopsis*, *Notodromas*), and various aquatic insects.

These small organisms were possibly introduced from the drain or inhabit originally the rice paddies. There was the high species diversity in the rice paddies. Several paddy-specific species of chaetonotids were found in the rice paddies which have the same water sauce (Table 3). Some species appeared in the same paddies every year (Site 2-b, Site 3-add: *Dichaetura filispina*; Site 3-a, b; Lake Biwa Museum, *Lepidodermella acanthorepida* and Site 2-a, *Polymerurus nodicaudus*). There are chaetonotids that appear in a certain period, for example, *D. filispina* in late June, *L. acantholepida* after the middle of June.

Discussion

Rice paddy ecology

Rice paddies were originally wetlands or flood plains, artificially adapted and constructed for rice production since around 3,000 years ago in Japan. Paddy flooding and draining during rice cultivation provides similar conditions to temporary water pools with emergent plants in still, shallow water. Drastic changes in temperature and pH occur in rice paddies, rendering this a challenging environment for aquatic organisms.

Despite being man-made constructions, rice paddies exhibit a surprisingly broad range of organisms. Segers and Sanoamuang (2007) reported a total of 135 rotifer species in a rice paddy and an adjacent pond in Laos, and Kiritani (2010) reported 5,668 species in Japanese rice paddies. However, chaetonotids were not recorded in those studies, and general surveys of life in rice paddies have recorded few chaetonotids

(Yamazaki *et al.* 2003; Yamazaki *et al.* 2004). Yamazaki *et al.* (2004) reported that rice paddy chaetonotids are found mainly in the water around rice plants after midseason drainage, when the rice plants are well established and the marginal areas are in shade. Chaetonotids favor habitats in the shade of weeds and under submerged leaves. Such areas in the shade have not been examined previously for rice paddy microfauna. In general methods of rice paddy surveys, samples are typically collected from water and filtered from mud collected from open areas but few chaetonotids live in such places. Chaetonotids may also be removed during filtration of mud and sediments so, in general, methods for other aquatic organisms are inappropriate for collecting chaetonotids.

The chaetonotid species found in the present study, other than the two new species, are also found in ponds and swamps (Schwank & Bartsch 1990). Aquatic organisms have their own characteristic pH range, so shifts in pH cause qualitative changes in the composition of aquatic life (Lacoul *et al.* 2011). *Chaetonotus oculifer* and *H. majus* inhabit acidic environments, while *H. macrops* prefers alkaline conditions (Kisielewski 1981). However, rice paddies show wide diurnal changes in pH (pH3-10; Usui *et al.* 2003). Chaetonotids living in habitats of various pH were found in the same rice paddies, suggesting that they have a wide range of pH tolerance.

Water temperatures range over 12–32°C in rice paddies (Yoshida & Hara 1977), so the upper range is relatively high for aquatic invertebrates. Okada and Uyemura (1940–1941) found chaetonotids (*Chaetonotus* sp.) in hot springs (32–35.2°C), suggesting that they may have the capacity to adapt to environmental changes, including high temperatures and a wide range of pH.

Some species, such *Dichaetura filispina*, *Lepidodermella acanthorepida,* and

Polymerurus nodicaudus, were found in the same paddies every year. These species are possibly inhabitants, which can pass the dry season by producing a resting egg.

Descriptions of four new species

Introduction

To date, 33 freshwater chaetonotids including 7 genera have been recorded in Japan. Kawamura (1918) reported the first species, *Polymerurus nodicaudus* (Voigt, 1901) in Shinshu (Nagano Japan). Later, Saito (1973) reported 26 species in the ponds of Hiroshima (Japan). Sudzuki (1971a, b) reported 11 species in lakes around Mount Fuji (Yamanashi Japan) and Shigakogen (Nagano Japan). There is no record of Japanese freshwater chaetonotids in the subsequent decades. Two new species were found from a pond in the Osaka University campus.

Although paddies have great diversity of chaetonotids, only a small number of chaetonotids were reported from rice paddies after midseason drainage (Yamazaki *et al.* 2003; Yamazaki *et al.* 2004). Samples were collected from mud near paths at rice paddy edges. Two new species were found from rice paddies in Otu (Shiga Japan).

Materials and methods

Chaetonotids were found on water plants and in bottom sediment of Machikane Pond in the Osaka University campus (Toyonaka, Osaka Prefecture, Honshu, Japan). This pond is about 10m in diameter and 0.5m in maximum depth. Rainwater is the only source of water. It is surrounded by a grove of trees, including plum, oak, and cherry. Fallen leaves accumulate in the sandy, mud bottom and it is bordered by large rocks.

The pond fauna has several introduced vertebrates, including *Trachemys scripta* (Schoepff), *Micropterus salmoides* (Lacepède), *Lepomis macrochirus* Rafinesque, and *Rana catesbeiana* Shaw. The benthic invertebrate fauna is relatively diverse and includes turbellarians, isopods, oligochaetes, nematodes, rotifers, ostracods, and snails. The isopod *Asellus hilgendorfii* Bovallius and the snail *Austropeplea ollula* Gould are found on the mud and fallen leaves at the bottom of the pond. The predominant turbellarian is the introduced freshwater planarian *Girardia tigrina* (Girard), which was originally described from North America. These species are highly tolerant of organic pollution and have been used as an indicator of water quality (Whitehurst, 1991). The presence of these species suggests that the water quality of this pond is eutrophic.

Rice paddy specimens were collected from 12 rice paddies (4 paddies each from 3 sites) at Mano (Otsu District, Shiga Prefecture, Honshu, Japan). The water source differs at each site (Table 4).

Sampling took place during the period when paddies are flooded (mid-May until late June), before midseason drainage. Samples were collected once or twice per week, from bottom sediments, floating plants (*Azolla japonica, Spirodela polyrhiza*), and from fallen weed leaves lying on the under-water soil surface of the paddy. Chaetonotids were obtained from samples by gentle rinsing with water.

Specimens were collected by washing out the plants and scooping the top sediments, and chaetonotids were picked up with a pipette using a stereoscopic microscope. The extracted specimens were anaesthetized in 1% MgCl2, and fixed in 10% formalin. For light microscopy, specimens were transferred through a graded series of glycerol (50%, 100% twice) for 30 min each and mounted in glycerol on H-S slides (Shirayama et al.,

1993). They were observed with a differential interference contrast microscope with Nomarski optics (Olympus BX-50, Tokyo, Japan).

For scanning electron microscopy, specimens were dehydrated through a graded series of ethanols for 15 min each. After dehydration, ethanol was replaced with isoamyl acetate (ethanol: isoamyl acetate = 1 : 1, then 100% isoamyl acetate twice for 1 hour each). Specimens were then dried in CO2 in a critical-point drier (HCP-2, Hitachi, Tokyo, Japan). Dried preparations were coated with gold-palladium under reduced pressure in an ion coater (IB-3, Eiko, Tokyo, Japan) and examined with a scanning electron microscope (Hitachi SU6600, Tokyo, Japan).

Measurements and drawings were made with the aid of an ocular micrometer and drawing tube (Olympus UDA, Tokyo, Japan). In the description, locations of some morphological structures along the body axis are given in percentage units (U) of total length measured from anterior end.

Holotype specimens collected from Machikane pond were deposited in Osaka University Museum and from rice paddies were deposited in Lake Biwa Museum.

Species from Machikane pond

Systematics Family Chaetonotidae Gosse, 1864

Subfamily Chaetonotinae Kisielewski, 1991

Genus *Chaetonotus* Ehrenberg, 1830

Subgenus *Chaetonotus*

***Chaetonotus retiformis* n. sp.**

[New Japanese name: Amime-itachimushi] (Figs. 2, 3)

Type locality. Japan, Honshu, Osaka Prefecture, Toyonaka, Machikane Pond (34° 80′N, 135° 45′E).

Type specimens. Holotype (OUM-GA-00001) and paratype (OUM-GA-00002) deposited in Osaka University Museum (OUM). Other specimens in the first author's collection.

Etymology. From the Greek in reference to the characteristic net-like pattern on the dorsal surface caused by arrangement of the scales.

Diagnosis. A medium-sized *Chaetonotus* of total length 180 μm including furca of 10 μm beginning at 93 U; pharyngeo-intestinal junction (PhIJ) at 41 U; head slightly three-lobed, with cephalion, hypostomion, and a pair of sensory cilia. Eye-spots absent. Body enveloped by small arrowhead-shaped, scales with a short spine in 22 longitudinal alternating rows, 38 in each. Four long spines on the base of each member of the furca at 92 U. Ventral scales between two columns of ventral cilia, keeled, oval scales with a short spine, in 2-4 longitudinal alternating rows. A pair of terminal plates covering posterior end of ventral scales is the largest in ventral scales. Juvenile length is about 60% of adult length, but head length is the same.

Description. Based on an adult specimen, 180 μm in total length. Body medium-sized tenpin-like shape with distinct head, neck, and trunk region. Widths of head / neck / trunk / caudal base are 30 / 21 / 41 / 21 μm at 08 U / 23 U / 65 U / 88 U, respectively. Length of distal furca 10 μm. Pharynx 58 μm in length, from posterior edge of mouth to junction with intestine; pharyngeo-intestinal junction (PhIJ) at 41 U.

Head slightly three-lobed, with cephalion and linear hypostomion posterior of mouth. Hypostomion with forward protrusions on each side.

Sensory organs: Seven or more isolated cephalic sensory cilia on either side of head, length around 11–12 μm. Eye-spots absent. Tactile bristles 12–15 μm in length are at 21 U on neck and 84 U caudally.

Cuticular armature: Body coverd with spined scales in alternating lines (22 longitudinal alternating rows, 38 in each), except for cephalion and furca regions. Dorsal scale arrowhead-shaped, width 3 μm, length 6 μm. Scales and spines relatively small and overlapping. Shape of all dorsal scales identical, but scales of posterior region are larger. Scales on neck region about half the size of those of trunk region. Length of spines on trunk scale 4 μm; 8 spines beneath base of each furca limb, each 8 μm at 92 U. Ventral scales covering region between two columns of ventral cilia are keeled, oval with a short spine, scale width 2 μm, length 6 μm and spine length 2 μm. Terminal plates form a pair covering posterior end of ventral scales width 6.5 μm, length 8 μm and spine length 2.5 μm; larger than other ventral scales.

Ventral ciliation: Densely packed field of cilia at posterior edge of hypostomion (0.5 U) splits into two parallel bands over most of trunk region, ending at 88 U.

Digestive tract: Mouth opening 8 μm in breadth, 6 μm in depth. Pharynx with swelling at both ends; the anterior being less obvious (11 μm) than the posterior (12 μm); central portion of fairly constant width (9.5 μm). Intestine straight, slightly wider anteriorly (21 μm) narrowing gradually over its length (to 3 μm width). Anus ventral, at 87 U.

Remarks. *Chaetonotus retiformis* is similar to nine species in having the

arrowhead-shaped dorsal scales and long spines at the base of the furca: cf. *C. christianus* Schwank; *C. linguaeformis* Voigt; *C. maximus* Ehrenberg; *C. microchaetus* Preobrajenskaja; *C. multispinosus* Günspan; *C. pawlowskii* Kisielewski; *C. polyspinosus* Greuter; *C. pseudopolyspinosus* Kisielewski; and *C. ventrochaetus* Kisielewski. However, *C. retiformis* is easily distinguished from all but *C. linguaeformis and C. christianus* in having a three lobed head (Schwank, 1990).

Chaetonotus linguaeformis is recorded from Europe and Northern Asia except China (Voigt, 1902, 1904; Schwank, 1990). *Chaetonotus retiformis* differs from *C. linguaeformis* in the body length (170–190 μm vs. 310– 370?μm), head shape (round-shaped vs. tongue-shaped and obviously three lobed vs. barely three lobed), and the absence of eye-spot-like structures near the mouth ring: none vs. 2(Voigt, 1902, 1904; Schwank, 1990).

Chaetonotus christianus is recorded from Europe and Northern Asia, except China (Schwank, 1990). *Chaetonotus retiformis* differs from *C. christianus* (Schwank, 1990) in the number of lines of ventral scales (2–4 vs. 12), type of terminal plate (square-shaped with keel and spine vs. slender oval-shaped with keel but no spine) and body shape (ten-pin like vs. cylindrical).

***Chaetonotus machikanensis* n. sp.**

[New Japanese name: Machikane-itachimushi] (Figs. 4, 5)

Type locality. Japan, Honshu, Osaka Prefecture, Toyonaka, Machikane Pond (34° 80′N, 135° 45′E).

Type specimens. Holotype (OUM-GA-00003) and paratype (OUM-GA-00004)

deposited in Osaka University Museum (OUM). Other specimens in the first author's collection.

Etymology. After the type locality.

Diagnosis. A small-sized *Chaetonotus* of total length 95–124 μm including furca of 8.0–11.5 μm beginning at 87 U; PhIJ at 28 U; head slightly five-lobed, with cephalion and 2 pairs of sheaved sensory cilia. Eye-spots absent. Body enveloped by large scales, each with a long barbed spine, in 7 longitudinal alternating rows, with 15 in each. Three Y-shaped keeled scales located each side at base of furca. Ventral scales between two columns of ventral cilia, flattened and hexagonal shaped, in 2 or 3 longitudinal alternating rows. Two keeled scales present near the terminal plates. A pair of terminal plates, oval, keeled, and spined. Three small oval, keeled scales present each side of proximal part of furca.

Description. Based on an adult specimen, 99 μm in total length. Pharynx 28 μm in length from posterior edge of mouth to junction with intestine; pharyngeo-intestinal junction (PhIJ) at 28.5 U. Head slightly five-lobed. Diameter of mouth ring 6 μm and height 4 μm. Body size small, and tenpin-like shape, with distinct head, neck, trunk and caudal region. Widths of head / neck / trunk / caudal base are: 16.8 / 13.1 / 22.4 / 13.1 μm at 09 U / 28 U / 52 U / 84 U, respectively. Length of furca, 8 μm.

Sensory organs: Two pairs of 3 to 4 tufted cephalic sensory cilia, abut 13 μm length, on either side of head. Eye-spots and tactile bristles absent.

Cuticular armature: Body covered with alternating lines (7 longitudinal alternating rows, 15 in each) of spined, overlapping scales, except for cephalion and furca regions. Scales on neck region boomerang-shaped, width 5 μm, length 3.5 μm with

well-developed barbed spine 6 μm long. Scales of trunk region arrowhead-shaped, width 9 μm, length 8 μm with well-developed barbed spine 19.5 μm long. Dorsal terminal plates with 3 Y-shaped keeled scales located each side at base of furca, width 2 μm, length 5 μm. Ventral scales hexagonal, flattened, width 1 μm, length 2.5 μm; 2 scales keeled near pair of terminal plates. Terminal plates 3 μm wide, 8 μm long, with spine 3 μm in length. Three minute scales present each side of proximal part of furca.

Ventral ciliation: Two almost parallel bands of cilia extend from posterior edge of mouth (06 U) along most of trunk region, ending at 83 U.

Digestive tract: Mouth opening 6 μm wide, and 4 μm deep. Pharynx swollen at anterior and posterior ends; anterior swelling more obvious (6.5 μm in wide) than posterior (6 μm wide); central portion fairly constant width (5.5 μm). Intestine is straight, slightly wider at anterior (9 μm), narrowing gradually over its width to 2 μm. Anus is ventral at 83 U.

Remarks. *Chaetonotus machikanensis* is similar to *C. hystrix* Metschnikoff*, C. macrochaetus* Zelinka*,* and *C. persetosus* Zelinka, in having arrowhead-shape dorsal scales each with a long denticular spine.

Chaetonotus hystrix has been reported from Africa, Europe, North America, and Northern Asia except China (Schwank, 1990). *Chaetonotus machikanensis* is distinguished from *C. hystrix* in the furca length (8–11 μm vs. 14– 16 μm), the shape of ventral scales (flatten vs. keeled), and round-shaped scales at the base of furca: absent vs. present (Metschnikoff, 1865).

Chaetonotus macrochaetus has been recorded from Africa, Europe, Northern Asia (excluding China) and North and South America (Schwank, 1990). *Chaetonotus*

machikanensis differs from *C. macrochaetus* in the shape of the ventral scales, which are flattened vs. keeled (Zelinka, 1889).

Chaetonotus persetosus has been recorded in Europe and Northern Asia except China (Schwank, 1990). *Chaetonotus machikanensis* differs from *C. persetosus* in body length (95–124 μm vs. 70–100 μm), the edge shape of dorsal trunk region scales (long barb vs. wide face with short barb), the shape of scales in the neck region (boomerang-shaped vs. arrowhead-shaped), and the line pattern of flattened ventral scales (2–3 scales per horizontal row vs. 5 scales per horizontal row). Further, *C. machikanensis* has 2 keeled scales immediately rostral to the terminal plates, but *C. persetosus* has 8 rostral scales, 6 of which are keeled, with 2 non-keeled scales immediately adjacent to the terminal plates. In addition, *C. persetosus* has 1 keeled scale between the terminal plates (Zelinka, 1889).

***Chaetonotus* sp.**

[New Japanese name: kotsubu-itachimushi] (Figs. 6, 7)

Type locality. Japan, Honshu, Osaka Prefecture, Toyonaka, Machikane Pond (34° 80′N, 135° 45′E).

Type specimens. All specimens in the first author's collection.

Etymology. Japanese name in reference to its small body size.

Diagnosis. A small-sized *Chaetonotus* of total length 88-108 μm including furca of 9.5 μm beginning at 91 U; pharyngeo-intestinal junction (PhIJ) at 27 U; head lobed is not observed, with cephalion, hypostomion. Eye-spots absent. Body enveloped by large

circular-shaped, scales with a long thick branched spine. No information of ventral scales and head lobe.

Description. Based on an adult specimen, 105 μm in total length. Body small-sized tenpin-like shape with distinct head, neck, and trunk region. Widths of head / neck / trunk / caudal base are not observed. Length of distal furca 9.5 μm. Pharynx 28.0 μm in length, from posterior edge of mouth to junction with intestine; pharyngeo-intestinal junction (PhIJ) at 26 U. Head lobed was not observed, but with cephalion and hypostomion posterior of mouth was obserbed.

Sensory organs: Eye-spots absent.

Cuticular armature: Body coverd with spined scales, except for cephalion and furca regions. Dorsal trunk scale circular-shaped, diameter 9 μm. Spine on the scale relatively long and thick. Shape of all dorsal scales identical, but scales of posterior region are larger. Scales on neck region is 5 μm. Length of spines on neck scale 9.5 μm and on trunk scale 18 μm. Ventral scale data is needed to describe this specises.

Ventral ciliation: Ventral ciliation was present, but the start and end point could not be observed.

Digestive tract: No information.

Remarks. *Chaetonotus* sp. is similar to *C. erinaceus* Daday, 1905 from Paraguay in having the large circler dorsal scales with long spines. However, *Chaetonotus* sp. is easily distinguished from *C. erinaceus* in the body length (88-108 μm vs.140-170 μm), the diameter of dorsal scales (3-10 μm vs. 15-25 μm) and the spine feature (length 7-10 μm vs. 15-60μm, and unbranched vs. branched).

New species form the rice paddy

Systematics Family CHAETONOTIDAE Gosse, 1864

Subfamily CHAETONOTINAE Kisielewski, 1991

Genus *Lepidodermella* Blake, 1933

***Lepidodermella acantholepida* n. sp. Suzuki and Furuya**

[New Japanese name: kagiurokoitachimushi] (Figs. 8, 9)

Type locality. Japan, Honshu, Shiga Prefecture, Otsu(35o 08' 5.40″ N, 135o 54' 51.18″ E).

Type specimens. Holotype, mounted in glycerol, is deposited in Lake Biwa Museum (LBM1360000013). All specimens were collected by T.G. Suzuki.

Etymology. Latin adjective, describing the characteristic spined scale of the dorsal end plate.

Diagnosis. Medium-sized *Lepidodermella* of total length 140.0–147.4 μm including furca of 15.2–19.9 μm beginning at U86.0; pharyngeo-intestinal junction (PhIJ) at U29.7; head slightly five-lobed, with cephalion, hypostomion, and two pairs of sensory ciliary bundles. Eye-spots absent. Body enveloped by flat, rounded, scales. Two dorsal terminal plates with a claw-like spine. Two types of ventral scale between two columns of ventral cilia: rectangular anteriorly around the PhIJ at U31 and oval posteriorly. Rectangular scales in 1 longitudinal row. Oval scales in 3 longitudinal alternating rows. A pair of flat, oval terminal plates cover posterior end of ventral scales.

Description. Based on an adultspecimen (holotype), 144.6 μm in total length (Fig. 8). Body medium-sized, tenpin-shaped with distinct head, neck, and trunk region. Widths of head / neck / trunk / caudal base are 22.9 / 16.0 / 32.7 / 17.4 μm at U10 / U29

/ U58 / U84, respectively. Length of distal furca 17.2 μm. Pharynx 36.9 μm in length, from posterior edge of mouth to junction with intestine; PhIJ at U 29.7. Head with 5 shallow lobes and well developed cephalion. Ventral hypostomion located at posterior of mouth. Hypostomion divided into right and left sides, each of length 5.6 μm (Fig. 9).

Sensory organs: Two pairs of cephalic ciliary tufts isolated on either side of head, each consisting of seven or more sensory cilia: anterior pair 11 μm in length, posterior 20 μm. Eye-spots absent. Tactile bristles 10 μm in length at U25.0 on neck and U84.5 caudally.

Cuticular armature: Body covered dorsally with 28–32 columns of flat scales in 5–7 rows, except for regions of cephalion and furca. Dorsal scales flat, round, without spine, mean diameter 5.8 μm . Scales overlap, dorsally shape and size identical except for a pair of dorsal terminal plates which are rhombic, 6.7 μm in length, 5.1 μm in width, with a thick, simple spine of length 5.8 μm. Two types of ventral scale covering region between two columns of ventral cilia: one column of 17 rectangular scales of 7.1 μm in width, 2.3μm in length, located from 13.4U to 33.7U; 3 columns of 18 flat, oval scales, each 3.8 μm in length, 5.4 μm in width. A pair of oval terminal plates covering posterior end of ventral scales, their width 3.0 μm, length 9.4 μm; larger than other ventral scales.

Ventral ciliation: Densely packed field of cilia at posterior edge of hypostomion (07 U) extending posteriorly as two longitudinal bands, ending at U 88.

Digestive tract: Mouth opening with maximum and minimum diameters 8.2 μm and 5.2 μm. Pharynx cylindrical, shape width 11μm. Intestine straight, slightly wider anteriorly (9.7 μm), narrowing gradually over its length to 7.0 μm. Anus ventral, at U 83.4.

Remarks. *Lepidodermella acantholepida* **n. sp.** is similar to *L. squamata* (Dujardin) in the shape of body and scales. *Lepidodermella squamata* has a wide geographical distribution in Europe, Russia, Asia, America, and Africa (Schwank & Bartsch 1990). Amato and Weiss (1982) reported many variations in the shape and arrangement of scales in *L. squamata*, and Kånneby, Todaro and Jondelius (2012) suggest that the currently recognized *L. squamata* is probably composed of several cryptic species complexes. However, *L. acantholepida* **n. sp.** is easily distinguished from *L. squamata* by its hypostomion (linear pair vs. curved pair) and its pair of spined scales, which are absent in *L. squamata* (Schwank & Bartsch 1990).

Lepidodermella acantholepida **n. sp.** is similar to six species in dorsal scale shape and the presence of spines: cf. *L. amazonica* Kisielewski*, L. broa* Kisielewski*, L. intermedia* Kånneby*, L. minor chaetifer* Kisielewski, and *L. spinifera* Tretyakova. *Lepidodermella amazonica, L. broa,* and *L. minor chaetifer* are recorded from Brazil (Kisielewski 1991). *Lepidodermella acantholepida* **n. sp.** differs from *L. amazonica* in body length (140.0–147.4 μm vs. 199–200 μm), scale shape (oval vs. with antero-lateral horns), tactile bristles on the neck (a pair vs. absent), and spine at dorsal terminal plate (claw-like spine vs. no spine; Kisielewski 1991). *Lepidodermella acantholepida* **n. sp.** differs from *L. broa* in the ventral scales between the ciliary bands (rectangular and oval vs. absent) and spine at dorsal terminal plate (claw like spine vs. no spine; Kisielewski 1991). *Lepidodermella acantholepida* **n. sp.** differs from *L. minor chaetifer* in the number of spined scale (2 vs. 6), ventral terminal plate (flat vs. keeled spined), and ventral scales between ciliary bands (rectangular and oval vs. absent; Kisielewski 1991).

Lepidodermella intermedia was described from Mount Njulla, Swedish Lapland.

Lepidodermella acantholepida **n. sp.** differs from *L. intermedia* in body length (140.0–147.4 μm vs. 114 μm), the shape of the dorsal spined scale (large rhombic vs. small rounded) and the keeled scale (absent vs. dorsal plate near the cilia and terminal plate; Kånneby *et. al.* 2011).

Lepidodermella spinifera is recorded from Europe and Northern Asia excluding China (Tretyakova 1991). *Lepidodermella acantholepida* **n. sp.** differs from *L. spinifera* in scale shape (round vs. in the shape of a gourd or distended rectangle), terminal plate (oval vs. square) and spined scale (dorsal terminal plate vs. ventral scale; Tretyakova 1991).

Family DICHAETURIDAE Remane, 1927 Genus *Dichaetura*

[New Japanese name: Togeoitachimushi]

***Dichaetura filispina* n. sp. Suzuki and Furuya**

[New Japanese name: Ketoge-togeoitachimushi] (Figs. 10, 11)

Type locality. Japan, Honshu, Shiga Prefecture, Otsu (35o07'54.72" N, 135o53'50.16" E). 21, June, 2011, Suzuki.

Type specimens. Holotype deposited in Lake Biwa Museum (LBM1360000014). All specimens were collected by T.G. Suzuki. Five paratypes in the first author's collection

Etymology. Species name from the Greek *fili*, thin, and Latin *spina*, spine, describing the characteristic its thin spines on the base of furca; noun in apposition,.

Diagnosis. A medium-sized gastrotrich of total length 168–185 μm including furca of 20–23 μm beginning at

U89.8 ; PhIJ at U25.0. Head un-lobed, of simple rounded shape, without cephalion or hypostomion; one pair of sensory cilia; eye spots absent. Dorsal scales only caudally of basal constriction, each with a thin spine of length 11.0–15.9 μm. A thick caudal spine, length 7.2–11.0 μm, at base of furca. A small thick spine, length 5.0–6.2 μm, on median side of each limb of furca, about 39% of distance from furca base. Adhesive tube only in main furca, not in spine. No ventral scales in the area between the two columns of ventral cilia to terminal plates. Juvenile length about 125 μm. Basal constriction and spined furca are significant characteristics of this genus.

Description. Based on an adult specimen (holotype), 185.3 μm in total length. Body medium-sized, cylindrical shape with constriction at caudal region and no distinct neck region. Widths of head / neck / trunk / basal constriction /caudal base are 26.4 / 25.2 / 34.0 / 21.3 / 14.9 μm at U12.8 / U26.9 / U62.4 / U80.0 / U85.7, respectively. Length of distal furca 20.6 μm; spine on furca 8.1μm. Pharynx 45.0 μm in length, from posterior edge of mouth to junction with intestine; PhIJ at U25.0. Head of simple rounded shape, without cephalion or hypostomion.

Sensory organs: Three or more isolated cephalic sensory cilia on either side of head, length 10.0–15.2 μm. Eye-spots absent. Tactile bristles absent from both neck and caudal regions.

Cuticular armature: Scales only on dorsal region caudal of basal constriction, composed of 11 small round scales, each with a thin spine, arranged in 5 rows: 3 rows of 3 scales and a single scale anteriorly and posteriorly. Dorsal scales round, diameter 2 μm, spine length 11.1–15.9 μm. Ventral scales absent . Each limb of furca with a thick curved spine, length 6.2 μm, between which is a straight thick spine, length 9.8 μm.

Ventral ciliation: Densely packed field of cilia posterior of mouth ring (U4.8) splits into two parallel bands, one each side over most of trunk region, ending at U88. Each band widens then narrows again just posterior of head region, forming a characteristic triangular shape anteriorly.

Digestive tract: Mouth opening diameter 8.4 μm, 7.2 μm in depth. Pharynx is an irregular cylindrical shape (6.1–9.6 μm). Intestine straight, slightly wider anteriorly (11.1 μm) narrowing gradually over its length (to 4.7 μm width). Anus ventral, at U87.7.

Remarks. *Dichaetura filispina* **n. sp.** is similar to two species, *D. capricornia* and *D. piscator*, both from Europe (Metschnikoff 1865; Murray 1913). However, it is easily distinguished from these species in having independent sensory cilia and a branched furca, each limb of which has an adhesive tube. In this respect, *D. filispina* **n. sp.** can be distinguished from all other species (cf. Rudescu 1967; Schwank & Bartsch 1990). Metschnikoff (1865) described *D. capricornia* based on only one specimen, and subsequently Martin (1981) redescribed this species. *Dichaetura filispina* **n. sp.** is similar to *D. capricornia* in possessing spines on the inside of each limb of furca and in angle of furca. However, *D. filispina* **n. sp.** differs from *D. capricornia* in body length (168–185 μm vs. 102–150 μm), head sculpture (regular vs. three shallow lobes), the widest region of the body (trunk vs. head), and ventral scales (none vs. small spined scales).

Result and discussion

Chaetonotid fauna in Machikane Pond.

The freshwater Chaetonotida of the world consist of 22 genera, including 9 common genera: *Aspidiophorus, Chaetonotus, Dasydytes, Heterolepidoderma, Ichthydium, Lepidodermella,* and *Polymerurus*. *Chaetonotus* accounts for nearly half the freshwater species (163 species, 52.9%). Machikane Pond contains six species including four of the common genera: *Aspidiophorus* (1 species)*, Chaetonotus* (3 species including the 2 newly described species)*, Lepidodermella* (1 species)*,* and *Polymerurus* (1 species). In addition, further undescribed *Chaetonotus* species have been obtained from this pond. These chaetonotids are usually obtained from the muddy sediment and are associated with waterweed such as *Ceratophyllum*. Of the other three common genera, recorded from various localities across the world (Table 4), only *Dasydytes* is apparently absent from Japan.

There is a larger pond in the university campus, Nakayama Pond, about 200 m away from Machikane Pond. Its area is about 7000 m2, depth to 1.5 m, surrounded by a mixture of pine and evergreen broadleaf forest, with a partly artificially protected border. As with Machikane Pond, rainwater is the source of water, with no other flow into the pond; water is drained irregularly. The fauna of Nakayama Pond is more diverse than that of Machikane Pond because of its larger size and less artificial cultivation. Chaetonotids have also been examined in this pond, though not extensively, and 4 species have been found, belonging to 2 genera (unpublished observations): *Chaetonotus* (2 previously described species) and *Lepidodermella* (2 species). *Chaetonotus* species also predominate in this pond, but they are different from those in Machikane Pond. *Lepidodermella squamata* and *Chaetonotus cordiformis* Greuter, 1917 were found in both Machikane and Nakayama Ponds. Notably, *L. squamata* is a

common cosmopolitan chaetonotid recorded in Europe, Russia, Asia, America, and Africa (Schwank, 1990). In Japan, it has been identified in ponds in Hiroshima (Saito, 1937) and Lake Biwa (first author's unpublished data).

Ecology.

Freshwater gastrotrichs usually reproduce asexually and lay resting eggs that are able to survive long dry periods (Brunson, 1949). In Japan, rice paddies and nearby small ponds are dry during the winter season, but gastrotrichs appear in the wet season (Yamazaki et al., 2003, 2004), apparently from these resting eggs. This feature is presumably advantageous for extending the distribution of gastrotrichs, since resistance to drying enable resting eggs to be carried from one pond to another by migratory birds. For instance, *Lepidodermella squamata* probably has expanded its distribution in such a way.

However, not all gastrotrich species are cosmopolitan or have broad distributions. Many possibly endemic species are known (Sudzuki 1971a, b; Schwank, 1990; Kisielewski, 1991). Several species have been cultured to observe their feeding habitats. They have different niches and have various feeding habits depending on the species (first author's unpublished data). Small species (around 100 μm in body length) feed on bacteria and detritus. Middle- and large- sized species (over 150 μm) feed on bacteria and small particles of detritus at the juvenile stage, then algae and diatoms in the adult stage. However, most species were difficult to culture due to unknown or changing feeding habits but *L. squamata* is exceptionally easy to culture. It is relatively large (110–200 μm body length) but does not change its feeding habits as it grows. It displays

a wider range of feeding habits than the other species and can grow in an organically polluted environment, The wide range of feeding habit and high tolerance to organic pollution in *L. squamata* may contribute to its wide distribution.

Systematics.

More than 30 % of Japanese gastrotrich species have yet to be reported from non-Japanese locations. However, it is still unclear whether they are actually endemic species, because the gastrotrich fauna has not been sufficiently studied. Some species are distributed in two distantly remote areas. For example, *Aspidiophorus microsquamatus* Saito, 1937 is found in Poland and Japan (Saito, 1937; Kisielewski 1981), but has not been recorded in the Russian areas in between. Saito (1937) and Kisielewski (1981) identified the species that they found in Hiroshima and Poland as *A. microsquamatus*. However, it is unclear whether or not these are really the same species. As the number of described species increased last century, more morphological characters have now been defined. The early descriptions lack information on several morphological characters that currently have significant taxonomical value, such as the surface structure of the ventral part of the body and detailed morphology of the dorsal and ventral scales (Schwank, 1990). Redescriptions of the early-described species are urgently needed in order to reach a better understanding of gastrotrich phylogeny.

Rice paddy species

Dichaetura filispina and *L. acantholepida* were found only in rice paddies containing water from the River Mano, upstream from Lake Biwa. These two species have not

been found in Lake Biwa itself or small ponds nearby, so they may well be species endemic to rice paddies. Japanese rice paddies are dry for most of the year, except in May and June, and gastrotrichs are known to produce resting eggs that can withstand dry conditions for more than 2 years at room temperature (Brunson 1949). Rice paddy endemic species, such as *D. filispina* and *L. acantholepida*, presumably have such resting eggs to withstand dry periods.

An unexpectedly large variety of gastrotrich species was found in rice paddies during the present study, including several prospective rice paddy endemics, pond and swamp species, and acido- and baso- philic species. The rice paddy that is artificial wetland environment controlled by humans affords periodic drastic changes, this condition may be favorable to the species diversity of gastrotrichs.

Culture method for 3 species of chaetonotids

Introduction

Studies of the molecular phylogeny and taxonomy of microorganisms are greatly restricted unless the organisms can be raised in a culture system. This is particularly true in the gastrotrich, which is small and composed of relatively small numbers of cells. It is difficult to obtain DNA samples from a single individual. Sufficient numbers of genetically uniform specimens are required for molecular studies, and for this reason we established the *in vitro* culture system for gastrotrichs.

The first cultivation method for freshwater gastrotrichs was reported by Packard (1936). *L. squamata* was reared in a 0.1% malted milk solution for 22 months. *L. concinnum* and *Chaetonotus* sp. were also raised in the same medium for several weeks.

Using the same culture method, *L. squamata* was co-cultured with *Paramecium* sp. (Brunson, 1949) and with *Petalomonas* sp. (Sacks, 1955). Amato and Weiss (1982) reared *L. squamata* for a short period using a solution provided by experimental animal dealers (Connecticut Valley Biological Supply Co. Inc., MA, USA).

The present study created new culture methods for some gastrotrich species (chaetonotids) to allow a long-term culture and established strains of *C. machikanensis*, *I. podura*, and *L. squamata*. These strains cannot be cultured in the same manner because these species have different feeding habits. Thus, different culture systems were established for each species. And these culture systems realized pure strain of 3 gastrotrichs.

Materials and methods

Source of organisms

Three genera of chaetonotids, *C. machikanensis*, *I. podura,* and *L. squamata*. *C. machikanensis* and *I. podura* were collected from water plants (*Egeria densa* and *Spirogyra* sp.) in the Machikane pond on the Osaka University Toyonaka campus (Toyonaka, Japan). *L. squamata* was collected from shunken plants washed ashore on the Karasuma Peninsula of Lake Biwa (Kusatsu, Japan).

Chaetonotids were gently washed from the water plants. Specimens were carefully sorted using a pipette under a stereomicroscope (Olympus SZX16) to prevent contamination by other organisms. Each was transferred to a culture vessel.

Gastrotrich strains

Strains were created in three species of chaetonotids, *C. machikanensis*, *I. podura,* and *L. squamata*. Chaetonotids reproduce usually by parthenogenesis, so each strain was established from a single female individual.

Culture conditions for a small culture (Fig. 12)

Tap water was kept overnight and sterilized after filtering (using a pore size of 0.22 μm) (Millipore Inc. MA, USA). An ampicillin solution was prepared at 5 mg/ml in the filtered water. The filtered water and the ampicillin solution were then mixed in a 1:1 ratio. A drop of the mixed solution was put on a sterilized watch glass (70 mm in diameter). A chaetonotid was put in the drop and cultured at 20°C. The watch glass was kept in a plastic petri dish (90 mm × 20 mm) and surrounded with water to prevent evaporation. It was not necessary to feed chaetonotids during the culture period.

Culture conditions for a mass culture

1. Culture method for *I. podura* (Fig. 13 A, B')

Tap water was allowed to stand overnight and was used for stock water. A glass petri dish (85.5 mm × 20 mm) was autoclaved to be used as a culture dish. The bacteria were co-cultured using boiled wheat seeds. These seeds were first boiled by being submerged in water in separate glass petri dish (41.5 mm × 18 mm) and then incubated for 1 min in a. microwave oven (500W) for 1 min. The boiled wheat seeds were then placed in the culture dish, and a single chaetonotid was added and cultured at 20°C. *I. podura* usually reproduces at about pH 7–8 in the natural environment, so the culture was maintained at pH 7.8. One more wheat seed was added to the dish when the population reached 1,000

individuals. Half of the medium was changed when it became excessively cloudy, or every month. Boiled wheat seeds were changed every 2 months. *C. machikanensis* and *L. squamata* were not cultured successfully using this method.

2. Culture method for *C. machikanensis* and *L. squamata* (Fig. 13 A–D)

Tap water was allowed to stand overnight and was used for stock water. A glass petri dish (85.5 mm × 20 mm) was sterilized in a microwave oven (500W) for 1 min. An adequate amount of water to totally submerge the wheat seed and only one boiled wheat seed were put in the culture dish and maintained at 20°C for 1 week. When bacterial biofilms formed on the bottom of the dish, the wheat seed was removed and the water was replaced. A chaetonotid was then put in the dish and cultured at 20°C. A boiled wheat seed was added when the population reached 100 around 1 week later. *C. machikanensis* and *L. squamata* reproduce at about pH 7–8 in the natural environment, so they were maintained at pH 7.8 under culture conditions. Subsequent procedures were performed using the identical method to that used for *I. podura*. The change of the medium and boiled wheat seed was the same as in the method for *I. podura*.

Subculture method

Subculture was performed by transferring some individuals to new dishes. The subculture was started with five individuals. These increased to 3000 individuals around 2–3 weeks later. In the case of *I. podura*, bacterial biofilms were also transferred with the chaetonotids.

Observations

Chaetonotids were counted using a hand tally counter under a stereomicroscope (OLYMPUS SZX16). The culture conditions of the chaetonotids were checked every 2 days in the mass culture, and every 24 h in the small culture. Frequency of egg-laying per adult and lifespan were examined. After eggs were laid, they were removed to other dishes to keep a single adult in the dish.

Result

This study revealed that chaetonotid species *C. machikanensis*, *I. podura*, and *L. squamata* could not be cultured using a common method for other small organisms like rotifers. New methods enable me to establish strains of the three species, they have been maintained for more than 4 and half years.

Population growth

In the mass culture, the population of chaetonotids reached a peak on the 10th day after culturing began, and then fluctuated every 7–10 days in each species. The number of chaetonotids reached nearly 10,000 at their maximum. After 1 month the population fluctuated between 2,000 and 4,000 (Fig. 14).

Most individuals in these three species of chaetonotids were crawling on the bottom of the culture dish. This was especially true for *C. machikanensis*, which made changing the medium easy. But some of *L. squamata* and *I. podura* individuals were either crawling under the surface of the medium or swimming within the medium, respectively. Such individuals may be lost in changing the medium, but this number is kept minimal

because of the low frequency of medium change in our method.

Egg laying

In *I. podura*, eggs hatched within 24 h and the hatched juveniles matured in 24 h. Chaetonotids usually lay one, or rarely two eggs. The egg-laying cycle is approximately 24 h. After the first egg is laid, the second is laid within 24 h. After the third is laid, further egg laying is occasionally delayed. When chaetonotids are raised without the water being changed and with spoiled wheat seeds for 3 months, they produce resting eggs on the bottom of the culture dishes (Figs. 15-17).

The eggs experienced high population density took 4-15 days to hatch in the new culture dish. However, in this case, the juveniles did not mature, even in the new culture dish. Once culture conditions deteriorated, adults lay fewer eggs, and these took longer time to hatch than normal eggs. No hatching of resting eggs was detected in the culture system both after and before dried out.

Normal eggs and resting eggs

In *C. machikanensis* the normal egg within a female is oval and contained within a thin, transparent shell 36.9 ± 1.4 μm × 25.7 ± 1.0 μm (mean length ± S. D. (Standard Deviation) × mean width ± S. D.; n = 21) (Fig. 15C). The resting egg is oval; 36.7 ± 2.6 μm × 24.8 ± 1.4 μm (n = 27). The egg mass is spherical, 21.2 ± 1.5 μm (n = 27) in diameter (Fig. 15D).

In *L. squamata*, the normal egg within a female is oval and contained within a thin

transparent shell 45.7 ± 1.2 μm × 28.1 ± 1.1 μm (n = 21) (Fig. 16C). The resting egg is oval, contained in the shell; 46.0 ± 1.9 μm × 27.3 ± 1.7 μm (n = 21). There is no space between the shell and the egg (Fig. 16D). There are many protrusions in the external surface of shells in both normal and resting eggs. The protrusions are stuck firmly to bacterial films.

For *I. podura* eggs, the normal egg within a female is oval and contained within a thin transparent shell 43.5 ± 3.2 μm × 22.9 ± 1.0 μm (n = 20) (Fig. 17C). The resting egg is oval and contained in the shell; 42.0 ± 4.1 μm × 24.8 ± 1.7 μm (n = 22). The egg is oval; 29.2 ± 2.7 μm × 22.4 ± 12.3 μm (n = 22) (Fig. 17D).

Small culture

A small *I. podura* culture was initiated using an animal from the stock culture once it had reached logarithmic growth phase. We observed variations in the frequency of egg laying and the time required for hatching. Isolated individuals from logarithmically growing populations (after about 10 to 14 days) frequently laid eggs. Hatching occurred within 24 h, and juveniles grew fast, becoming adults and laying eggs within 24 h. Egg laying occurred at most four times per individual.

After the logarithmic growth phase, isolated individuals produced eggs less frequently. We identified another type of normal egg, whose hatching was delayed by at least 48 h. This egg was similar to normal eggs that had a thin egg shell and developing embryo. The juvenile grew slowly, and took 48 h to mature and produce its first egg.

The small culture system was useful for estimation of the organisms' lifespan, hatching, and rate of egg laying. However, this method was unsuitable for small species,

especially for *Chaetonotus* that has a long dorsal spine. The spines may reach water surfaces easily and the surface traps the organisms because of its long spines. Because of their small size, this species has limited locomotive ability and cannot escape from the water surface.

Discussion

In this chaetonotid culture system, we could not observe a stable phase after the logarithmic growth phase. At high population density, chaetonotids produced another type of normal egg, morphologically similar to normal eggs, but distinguished from them by their delayed hatching. While this makes them similar to resting eggs, which require a long time to hatch, they differed by having a thin shell and developing embryo. Resting eggs have a thick shell and development is arrested at the one-cell stage (Brunson, 1949).

High chaetonotid population density led to less frequent egg laying and a delay in hatching. Such an effect is possibly an adjustment mechanism to prevent overgrowth and to maintain the proper population size after the logarithmic growth phase.

Advantages of the new culture system

Previous culture methods for gastrotrichs were designed originally for observing their development and behavior (Packerd, 1936; Brunson, 1949; Sacks, 1955; Amato and Weiss, 1982). Therefore, these methods were more suitable for short-term culture than long-term culture. In these methods the liquid or small-particle feeds were directly added to the culture media. In culture systems using malted milk, the medium has to be

changed about every 5 days (Brunson, 1949). Our new culture system using boiled wheat seeds prolongs medium change, which has to be done only once per month. Reducing the number of medium change is also advantageous in terms of limiting unnecessary loss of organisms. Most chaetonotids are crawling under the bottom of the dish. However, *I. podura* and *L. squamata* may occasionally be found swimming or crawling under the surface of the medium. Because our method requires less frequent medium changes, it reduces the loss of these organisms.

Chaetonotid feeding habits

Our culture systems are based on the bacterial feeding habit of small-sized chaetonotids. It is not clear whether these systems are suitable for culturing middle- to large-sized chaetonotids (over 200 μm). Although feeding habits of these larger chaetonotid species are not known, green algae and diatoms are found in their intestines. Culturing *C. retiformis* using the boiled-wheat method was not successful, but some adults survived for 1 week and laid eggs with the co-cultured green alga *Gloeocystis* sp. as the feed (data not shown). This suggests that the adult *C. retiformis* eats small algae but not bacteria. The hatched juveniles survived for 1 week; however, they could not grow to adult body size, remaining two-thirds the size of the adult and laid no eggs. Although the staple diet is not clear, this culture system may be insufficient for juvenile growth.

The mouth (pharyngeal) size is likely correlated to the feeding habit, because gastrotrichs suck their food through the pharynge. The gastrotrich pharynge is of the

suction-type, and is oriented as a Y-shaped lumen in chaetonotids and as an inverted Y-shaped lumen in macrodasyids (Ruppert, 1982, 1991). These types of pharynge have been found in various phyla, and are not correlated to lineages (Nielsen, 2013). Juvenile bodies of *C. retiformis* are about 150 μm in length; almost the same size as the bacterial-eating chaetonotids, c.f. *I. podura* and *L. squamata*. One possibility is that chaetonotids such as *C. retiformis* change their food depending on their size. Because the head size of juvenile gastrotrichs is almost the same as that of adults, their feeding habits may not depend on the size of the mouth ring. The suction force seems to differ depending on the shape of pharyngeal lumen. Juveniles of the genus *Lepidodasys* (macrodasyida) have a circular pharyngeal lumen (Ruppert, 1982; Ax, 2001), and its suction force is weaker than the Y-shaped lumen (Nielsen, 2013). In the chaetonotid juvenile the pharyngeal lumen may not develop fully, and the suction force of the juvenile may be weaker than that of the adult. Thus, the feeding habit is likely to change according to growth.

Biological study of gastrotrichs using the new culture system

Although gastrotrichs are easily found in ponds, swamps, lakes, and all water bodies, it is hard to collect large numbers of individual of a single species. There is high diversity of gastrotrichs in the environment, but the species assemblage often varies (Suzuki *et al.*, 2013). Therefore, biological studies on gastrotrichs are greatly restricted. Our system allows the researcher to collect sufficient numbers of specimens from a single individual using a culture system. Previous culture methods involving a co-culture system with a eukaryote (parameciums or euglenas) were not suitable for

molecular analysis, because of contaminating eukaryotic DNA or RNA. Although our culture method only extends to three small species, it enables the culture of specific gastrotrichs without the inclusion of other gastrotrichs or eukaryotic organisms. Our culture systems allow for the investigation of basic, developmental, and molecular biology of gastrotrichs to be carried out under controlled and reproducible conditions.

Summary

The present study has increased the number of described species of gastrotrich in Japan from 33 to 45 (3 families, 8 genera), although the survey area was limited part of Osaka and Shiga Prefectures. In addition to new species and described species, 32 unidentified species were found in the rice paddies. It was an unexpected phenomenon that the species diversity occurs in the artificial environment. This should be especially noted. Freshwater gastrotrich species adapt to the broad range of environment changes (pH, temperature, humidity) of the Japanese paddy fields. Furthermore, planktonic species (新潟新井高校 1960) and gastrotrichs inhabiting the hot spring (Okada and Kamimura 1940) are recorded, suggesting they have a considerable capacity for adaptation. Thus, it is considered that the Japan has high species diversity in freshwater gastrotrich species. Most of the gastrotrich species would remain undiscovered in Japan, and so many species await description.

The culture systems of three chaetonotid species were established. *L. squamata* and *I. podura* show a worldwide distribution (Suzuki and Furuya, 2011), thus these culture systems can be used all over the world. Early developmental processes were studied in *L. squamata* (Brunson, 1949). This species is good for model organism of freshwater

chaetonotids. Strains of three species have been maintained laboratory culture system. If we can make good use of the resting eggs, the dried eggs could be transported to anywhere in the world.

It is difficult to obtain the DNAs sufficient to analyze the genome from a single individual of gastrotrich species. The mass culture was established in the gastrotrich strain, thereby the genome analysis project of gastrotrichs has started. Thus more accurate analysis on phylogenetic position will be possible using the phylogenetic marker genes, such as the Innexin gene involving gap junction proteins. The protein has the taxa-specific indels (insertion and deletion) in amino acid sequence (Suzuki and Furuya 2010).

Acknowledgements

This study was supported by grants from the Research Institute of Marine Invertebrates Foundation, Fujiwara Natural History Foundation (research grant no. 17), Lake Biwa Museum Comprehensive Research Program 06-02, and Lake Biwa Museum Environment Research and Technology Development Fund D-0906. I express my thanks to Prof. Maria Balsamo, Urbino University, and Ms. Masako Maeda, Hashikake in Lake Biwa Museum, for their valuable advice and comments.

References

Amato, A.J. & Weiss, M.J. (1982) Developmental flexibility of a cell-constant organism, Lepidodermella squammata (Gastrotricha). *Transactions of the American Microscopical Society*, 101(3), 229–240.

Ax P, (2001). Das System der Metazoa III. Spektrum, Heidelberg

Balsamo, M., d'Hondt, J.L., Pierboni, L. & Grilli, P. (2009) Taxonomic and nomenclatural notes on freshwater Gastrotricha. *Zootaxa,* 2158, 1–19.

Brunson, R.B. (1949) The life history and ecology of two North American gastrotrichs. *Transactions of the American Micro- scopical Society,* 68, 1–20.

Cavalier-Smith T, (1998) A revised six-kingdom system of life, Biol. Rev. 73: 203-266.

Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE, Smith SA, Seaver E, Rouse GW, Obst M, Edgecombe GD, Sorensen MV, Haddock SHD, Schmidt-Rhaesa A, Okusu A, Kristensen RM, Wheeler WC, Martindale MQ, Giribet G (2008) Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452: 745-749

Ehlers U, Ahlrichs W, Lemburg C, Schmidt-Rhaesa A (1996) Phylogenetic systematization of the Nemathelminthes (Aschel minthes). Verh Dtsch Zool Ges 89:8

Hummon W.D. & Todaro M.A. (2010) Analytic taxonomy and notes on marine, brackish-water and estuarine Gastrotricha. *Zootaxa* 2392, 1–32.

Kånneby, T., Todaro, M.A. & Jondelius U. (2012) A phylogenetic approach to species delimitation in freshwater Gastrotricha from Sweden. *Hydrobiologia*, 683(1), 185–202.

Kawamura, T. (1918) *Japanese Freshwater Biology*. Tokyo, Syoukabou, pp 113–115.

Kiritani, K. (2010) A comprehensive list of organisms associated with paddy ecosystems in Japan. The revised edition. The Institute of Agriculture and National

Environments & Biodiversity Agriculture Support Center, Tokyo, 60 pp.

Kisielewski, J. (1981) Gastrotricha from raised and transitional peat bogs in Poland. Poland, *Monografie Fauny Polski,* 11, pp 142.

Kisielewski, J. (1981) Gastrotricha from raised and transitional peat bogs in Poland. Poland, *Monografie Fauny Polski,* 11, pp142.

Kisielewski, J. (1991) Inland-water Gastrotricha from Brazil. *Annales Zoologici,* 43, 1–168.

Lacoul, P., Freedman, B. & Clair, T. (2011) Effects of acidification on aquatic biota in Atlantic Canada. *Environmental Reviews*, 19(NA), 429–460.

Lorenzen, S, (1985) Phylogenetic aspects of pseudocoelomate evolution. Ed by S. Conway-Morris, JD. George, R Gibson, HM Platt, The Origin and Relationships of Lower Invertebrates. Claredon Press, Oxford. 210: 2-23

Manylov OG, Vladychenskaya NS, Milyutina IA, Kedrova OS, Korokhov NP, Dvoryanchikov GA, Aleshin VA, Petrov NB, (2004) Analysis of 18S rRNA gene sequences suggests significant molecular differences between Macrodasyida and Chaetonotida (Gastrotricha). Molecular Phylogenetics and Evolution 30: 850-854

Martin, L.V. (1981) Gastrotrichs found in surrey. *Microscopy* 34, 286–300.

Metschnikoff, E. (1865) Über einige wenig bekannte niedere Tierformen. *Zeitschrift für Wissenschaftliche Zoologie,* 15, 450–463.

Murray J. (1913) Gastrotricha. *Journal of the Quekett Microscopical Club*, series 2, Vol 12(73), 211–238.

Nielsen C. (2001) Animal evolution: interrelationships of the living phyla. Oxford University Press, Oxford, pp 324-331

Nielsen C (2013) The triradiate sucking pharynx in animal phylogeny. Invertebr Biol 132(1): 1-13

新潟県立新井高等学校.1960. 野尻湖の自然と環境. 349 pp. 築地書館,東京.

Okada, Y. & Uyemura, M. (1940–1941) Studies on the Thermophilous Animals in the Hot Springs of Japan XXVII. The Thermal Fauna of the Simple and Carbonic Springs in Oita Prefecture, Japan. *Japanese Journal of Limnology*, 10(1–2), 41–67.

Packard CE (1936) Observations on the Gastrotricha Indigenous to New Hampshire. Trans Am Microsc Soc 55(4): 422-427

Rudescu, L. (1967) Gastrotricha. Fauna Republicii Socialiste Romania, Vol. 2. *Academia Republicii Socialiste Romania*, Bucuresti, 289 pp.

Ruppert EE (1982) Comparative ultrastructure of the gastrotrich pharynx and the evolution of myoepithelial foreguts in Aschelminthes. Zoomorphology 99: 181–220

Ruppert, E.E. (1991) Gastrotricha. *In*: Harrison, F.W. & Ruppert, E.E. (Eds), *Microscopic Anatomy of Invertebrates, Vol. 4, Aschelminthes*. Wiley-Liss, New York. pp. 41–109.

Saito, I. (1937) Neue und bekannte Gastrotrichen der Umgebung von Hiroschima (Japan) *Journal of Science of Hiroshima University,* 5, 245–265.

Schwank, P. (1990). *Gastrotricha und Nemertini*. In: A. Brauer, Editors, Gustav Fisher Verlag, Germany, Stuttgart, pp 1–252.

Sacks M (1955) Observations on the embryology of an aquatic gastrotrich, *Lepidodermella squamata* (Dujardin, 1841) J Morphol 96(3): 473-495

Segers, H., & Sanoamuang, L. (2007) Note on a highly diverse rotifer assemblage (Rotifera: Monogononta) in a Laotian rice paddy and adjacent pond. *International*

Review of Hydrobiology, 92(6), 640–646.

Shirayama, Y., Kaku, T. & Higgins, R.P. (1993) Double-sided microscopic observation of meiofauna using an HS-slide. *Benthos Research,* 44, 41–44.

Sudzuki, M. (1971a) Die das Kapillarwasser des Lueckensystems bewohnenden Gastrotrichen Japans. I. *Zoological Magazine*, 80, 256–257.

Sudzuki, M. (1971b) The Gastrotricha of Japan which live in the capillary water of the interstitial system: II. Bulletin of the *Biogeographical Society of Japan*, 27, 37–41.

Suzuki G.T., Tsuneki K., & Furuya H. (2010) Phylogenetic analysis of dicyemid mesozoans (Phylum Dicyemida) from Innexin amino acid sequences - Dicyemids are not related to Platyhelminthes. Journal of Parasitology. The American Society of Parasitologists. 96 (3):614-625.

Sudzuki, M. (1992) Seasonal and local occurrences of the Rotifera in southwestern islands of Japan. With lists and notes on other microscopic leading animals. *Proceedings of the Japanese Society of Systematic Zoology*, 46, 29–70.

Suzuki, T.G. & Furuya, H. (2011) Two new species of *Chaetonotus* (Gastrotricha, Chaetonotida, Chaetonotidae) from Japan. *Zootaxa*, 3011, 27–37.

Suzuki TG, Maeda M, Furuya H. (2013). Two new Japanese species of Gastrotricha (Chaetonotida, Chaetonotidae, *Lepidodermella* and Dichaeturidae, *Dichaetura*), with Comments on the Diversity of Gastrotrichs in Rice Paddies. Zootaxa 3691(2): 229-239

Todaro, M.A. & Tongiorgi, P. (2013) Freshwater Gastrotricha. Available in: http://www.gastrotricha.unimore.it/ (accessed 29 December 2013)

Tretjakova, E. I. (1991) *Lepidodermella spinifera*, a new species of Gastrotricha with

intermediate generic features. Byulletin Moskovogo Obshchestva Ispytatelej Prirody, *Otdel Biologiceskij*. 96, 79–85.

Usui, Y., Mowjood, M.I.M. & Kasubuchi T. (2003) Absorption and emission of CO2 by ponded water of a paddy field. *Soil Science and Plant Nutrition*, 49(6), 853–857.

Voigt, M. (1902) Drei neue *Chaetonotus*-Arten aus Plöner Gewässern. *Zoologischer Anzeiger*, 25 (662), 116–118.

Voigt, M. (1904) *Die Rotatorien und Gastrotrichen der Umgebung von Plön*. Germany, Verlag von E. Nägele, pp 1–178.

Whitehurst, I.T. (1991) The *Gammarus:Asellus* Ratio as an index of organic pollution. *Water Research*, 25, 333–340.

Yamazaki, M., Hamada, Y., Kamimoto, N., Momii, T., Aiba, Y., Yasuda, N., Mizuno, S., Yoshida, S. & Kimira, Y. (2003) Changes in the community structure of aquatic organisms after midseason drainage in the floodwater of Japanese paddy fields. *Soil Science and Plant Nutrition*, 49, 125–135.

Yamazaki, M., Hamada, Y., Kamimoto, N., Momii, T. & Kimura, M. (2004) Habitats of aquatic organisms in a paddy field during flooding period. *Edaphologia*, 74, 1–10.

Yoshida, S. & Hara, T. (1977) Effects of air temperature and light on grain filling of an indica and a japonica rice (*Oryza sativa L.*) under controlled environmental conditions. *Soil Science and Plant Nutrition*, 23(1), 93–107.

Zelinka, C. (1889) Die Gastrotrichen. Eine monographische Darstellung ihrer Anatomie, Biologie und Systematik. *Zeitschrift für Wissenschaftliche Zoologie*, 49, 209–384.

Zrzavý J, Mihulka S, Kepka P, Bezdek A, Tietz D (1998). Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. Cladistics 14: 249-285.

Figures

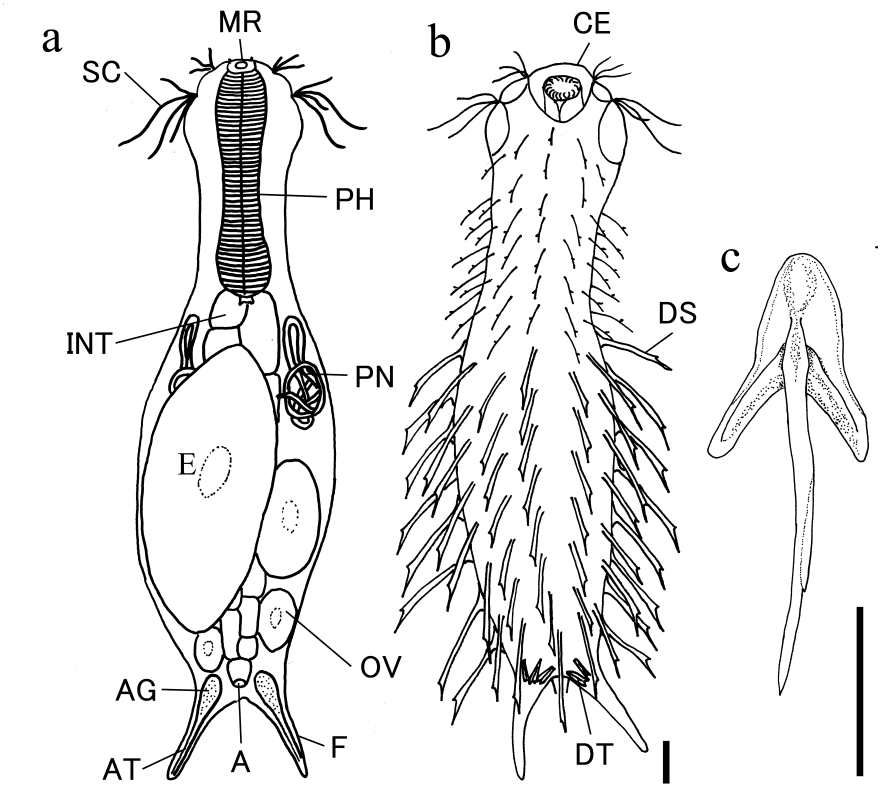

Fig. 1. Morphology of Chaetonotida. a, typical diagram of Chaetonotida; b, sketch of *C. machikanensis*; c, dorsal scale of *C. machikanensis*. Abbreviations: A, anus; AG, adhesive gonad; AT, adhesive tube; CE, cephalion; DS, dorsal scale; DT, dorsal terminal plate; F, furca; INT, intestine; MR, mouth ring; PH, pharynx; PN, protonephridium; SC, sensory cilium. Scale bars = 5 μm. Figs from Suzuki and Furuya 2011.

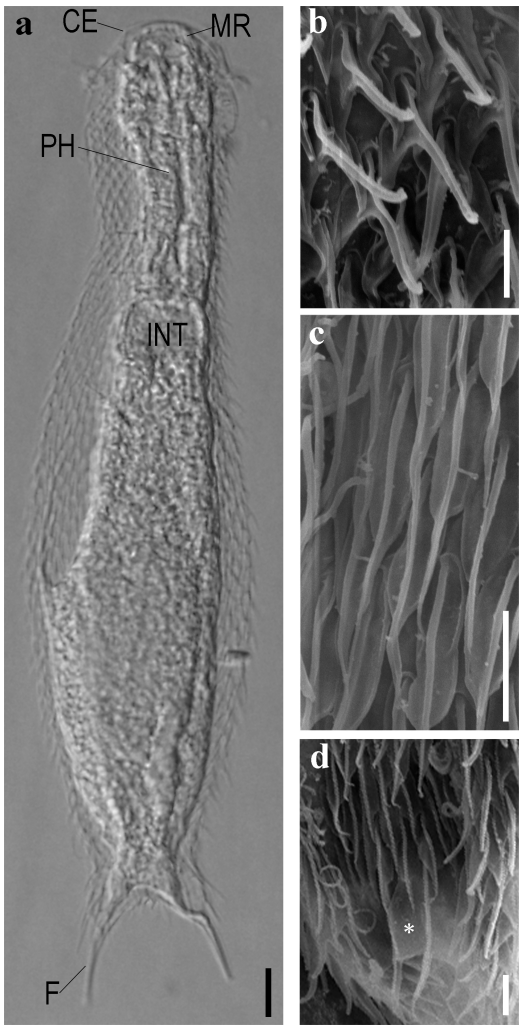

Fig. 2. *Chaetonotus retiformis* **n. sp.**, (Suzuki and Furuya 2011) light (a) and scanning electron (b–d) microphotographs of the holotype. a, Coronal view; b, dorsal scale; c, ventral scale; d, terminal plate (asterisk). Scale bars 10 μm in (a) and 2 μm in (b – d). Abbreviations: CE, cephalion; F, furca; INT, intestine, MR, mouth ring; PH, pharynx. Figs from Suzuki and Furuya 2011.

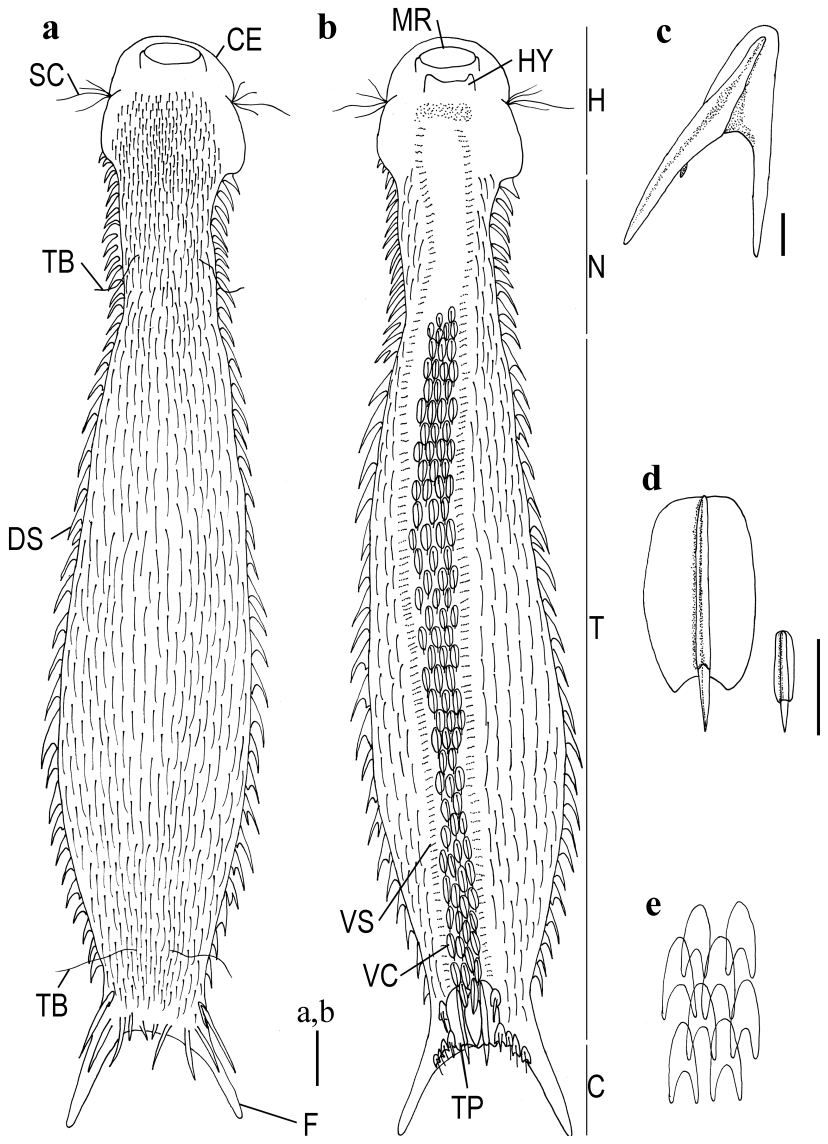

Fig. 3. *Chaetonotus retiformis* **n. sp.**, (Suzuki and Furuya 2011) line drawings of holotype specimen. a, dorsal view; b, ventral view; c, dorsal scale; d, terminal scale left and ventral scale right; e, arrangement of ventro-lateral scales. Scale bars = 10 μm in (a), (b), 1 μm in (c), 5 μm in (d). Abbreviations: C, caudal; CE, cephalion; DS, dorsal scale; F, furca; H, head; HY, hypostomion; MR, mouth ring; N, neck; SC, sensory cilium; T, trunk; TB, tactile bristle; TP, terminal plate; VC, ventral cilium; VS, ventral scale. Figs

from Suzuki and Furuya 2011.

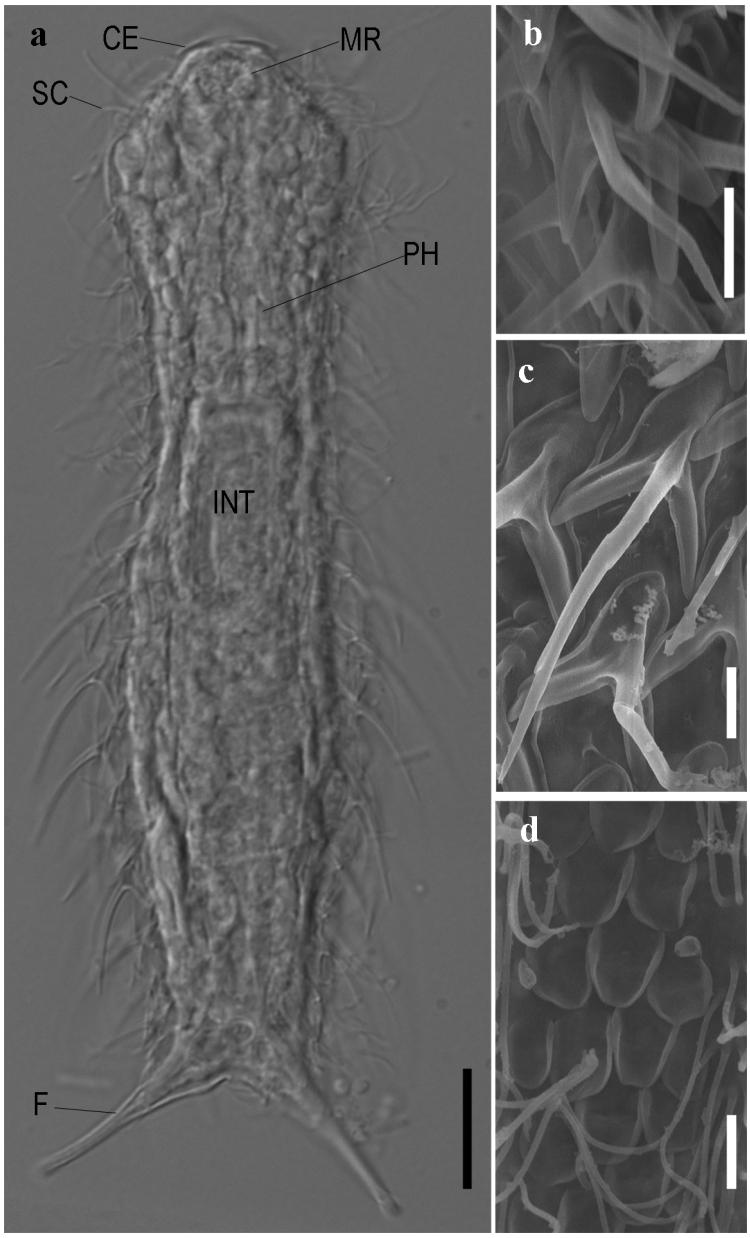

Fig. 4. *Chaetonotus machikanensis* **n. sp.**, (Suzuki and Furuya 2011) light (a) and scanning electron (b–d) microphotographs of holotype specimen. a, Coronal view. b, dorsal scale (neck). c, dorsal scale (trunk). d, ventral scales. Scale bars = 10 μm in (a) and 2 μm in (b–d). Abbreviations: CE, cephalion; F, furca; INT, intestine; MR, mouth ring; PH, pharynx; SC, sensory cilium. Figs from Suzuki and Furuya 2011.

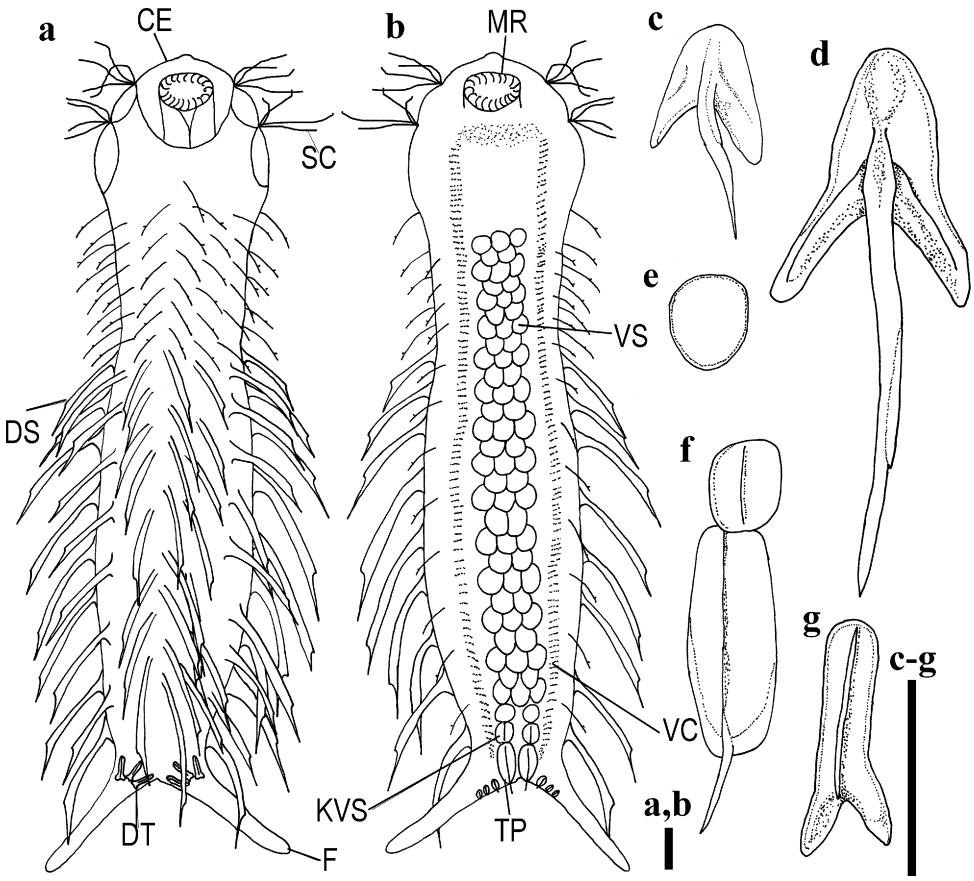

Fig. 5. *Chaetonotus machikanensis* **n. sp.**, (Suzuki and Furuya 2011) line drawings of holotype specimen. a, dorsal view; b, ventral view; c, dorsal scale (neck); d, dorsal scale (trunk). e, ventral scale; f, terminal plate and keeled ventral scale; g, dorsal terminal plate. Scale bars = 5 μm. Abbreviations: CE, cephalion; DS, dorsal scale; DT, dorsal terminal plate; F, furca; KVS, keeled ventral scale; MR, mouth ring; SC, sensory cilium; TP, terminal plate; VC, ventral cilium; VS, ventral scale. Figs from Suzuki and Furuya 2011.

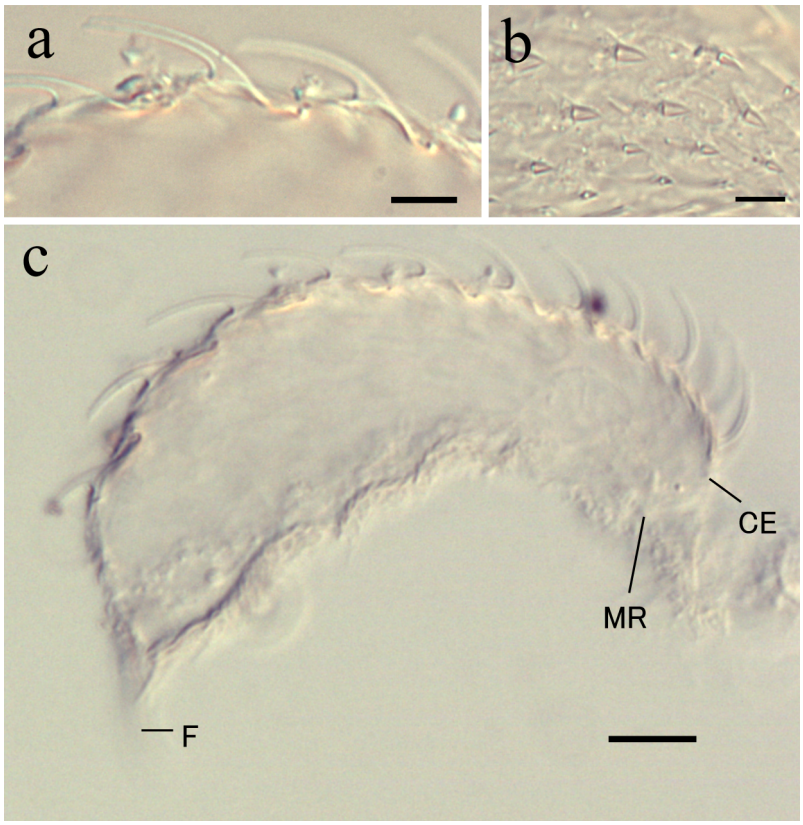

Fig. 6. *Chaetonotus* sp. a-b, dorsal scale and spine; c, side view of specimen. Scale bars = 10µm. Abbreviations: CE, cephalion; F, furca; MR, mouth ring.

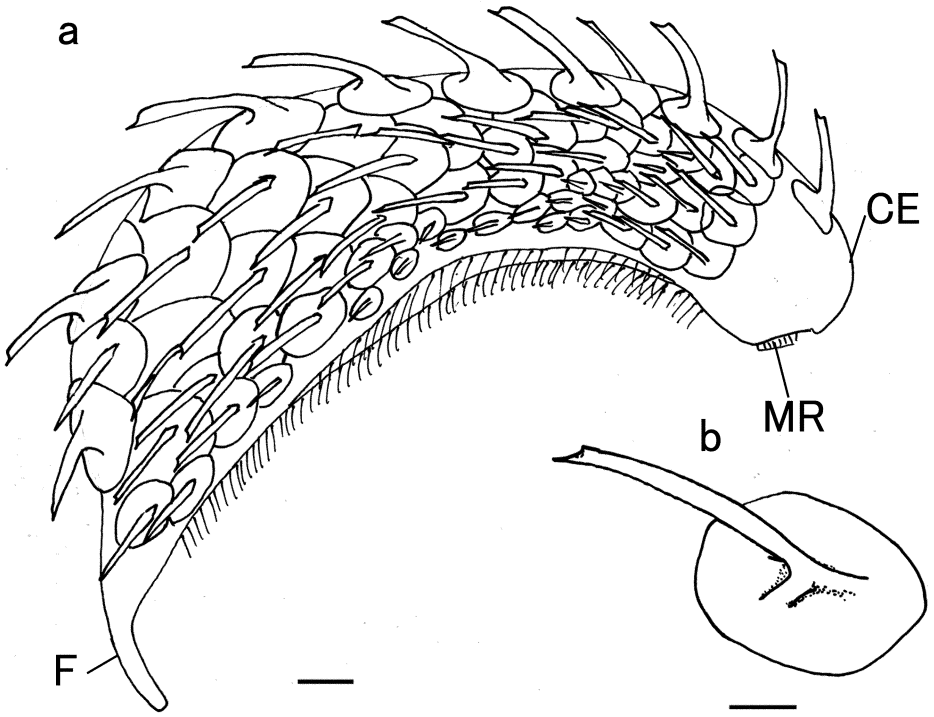

Fig. 7. *Chaetonotus* sp. line drawings of specimen. a, side view; b. scale and spine. scale bars = 5 μm. Abbreviations: CE, cephalion; F, furca; MR, mouth ring.

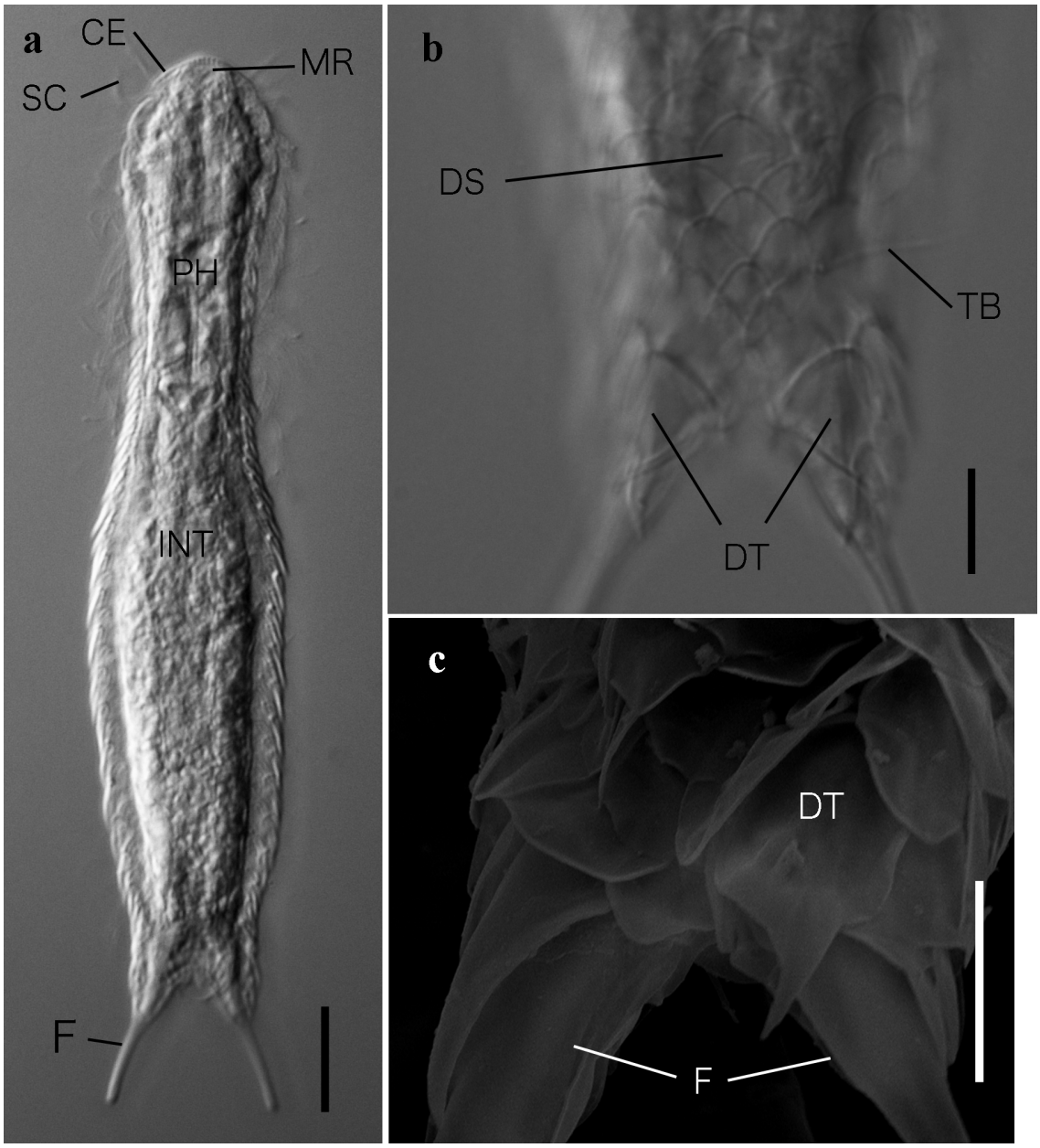

Fig. 8. *Lepidodermella acantholepida* **n. sp.**, (Suzuki *et. al.* 2013) light (a, b) and electron (c) micrographs of the holotype. a, coronal view; b, dorsal scale and dorsal terminal plate; c, dorsal terminal plate. Scale bars = 10 μm in (a) 5 μm in (b, c). Abbreviations: CE, cephalion; DS, dorsal scale; DT, dorsal terminal plate; F, furca; INT, intestine; MR, mouth ring; PH, pharynx; TB, tactile bristle. Figs from Suzuki et al.

2013.

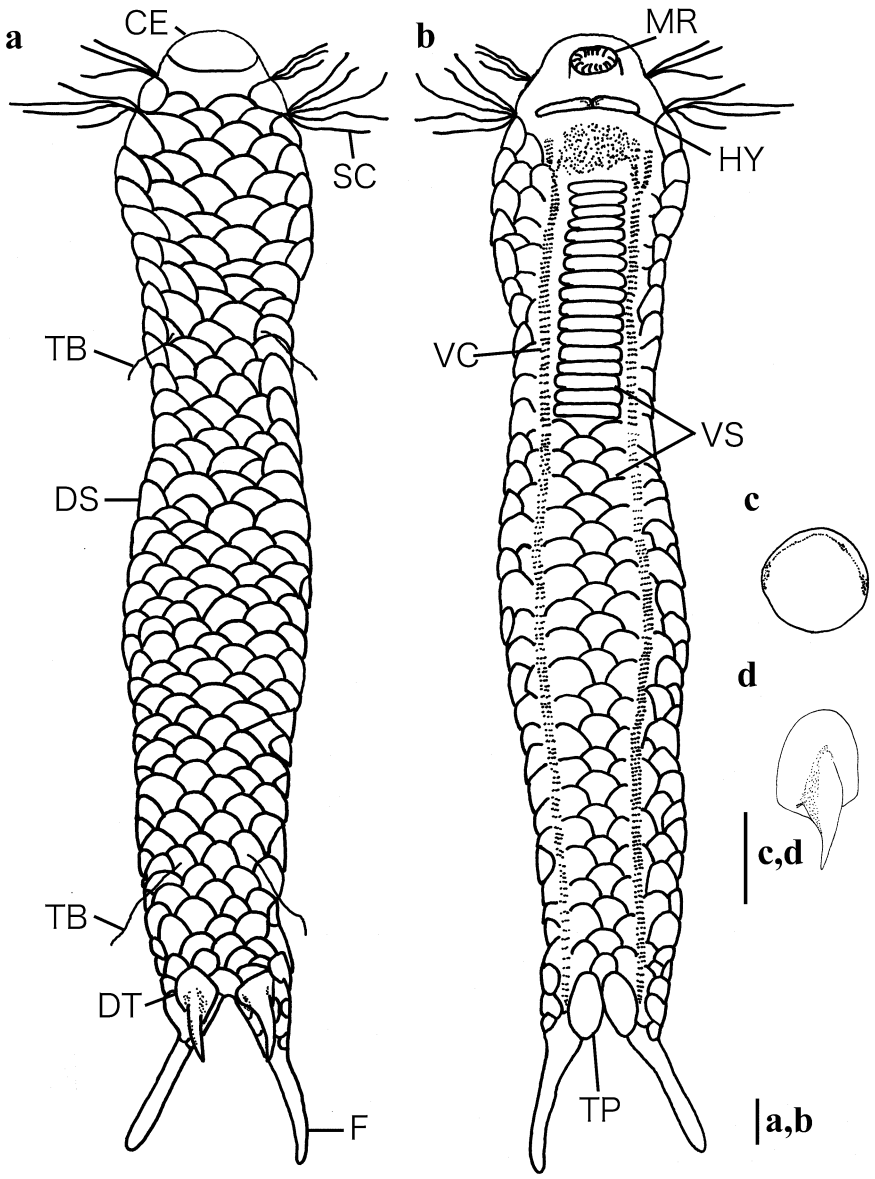

Fig. 9. *Lepidodermella acantholepida* **n. sp.**, (Suzuki *et. al.* 2013) line drawings of holotype specimen. a, dorsal view; b, ventral view; c, dorsal scale; d, dorsal terminal plate. Scale bars = 5 μm. Abbreviations: CE, cephalion; DS, dorsal scale; DT, dorsal terminal plate; F, furca; HY, hypostomion; MR, mouth ring; SC, sensory cilium; TB, tactile bristle; TP, terminal plate; VC, ventral cilium; VS, ventral scale. Figs from

Suzuki et al. 2013.

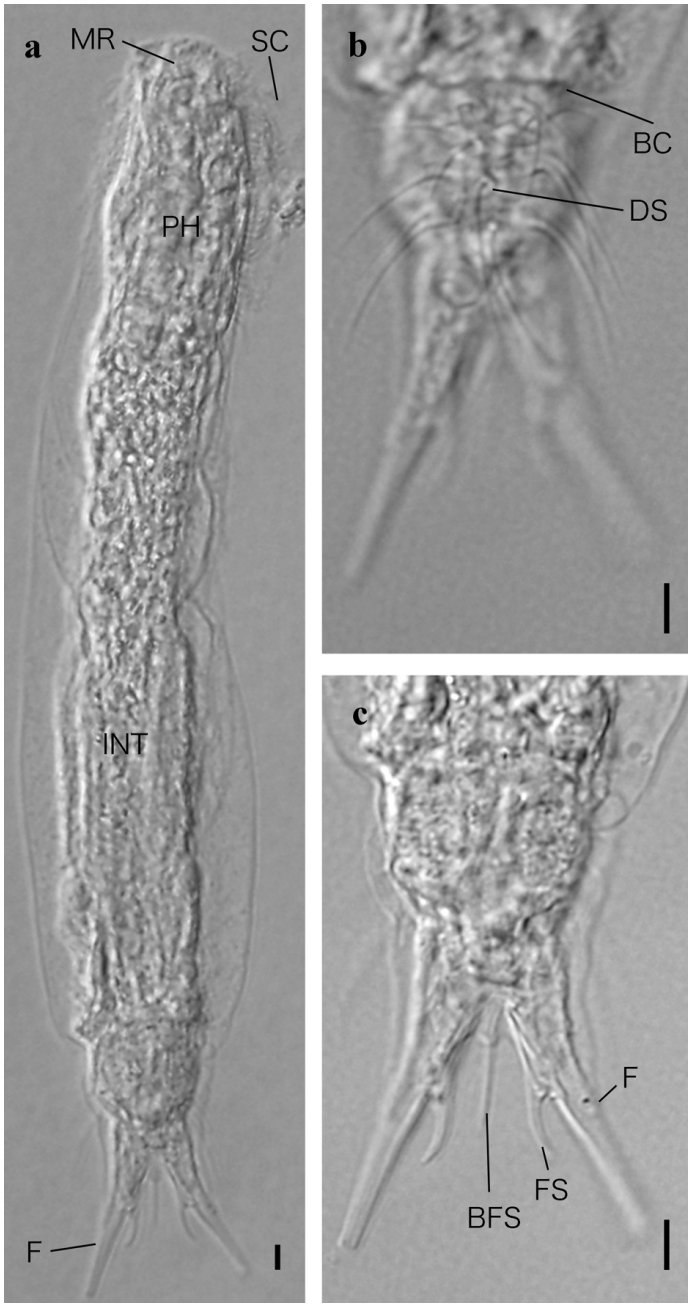

Fig. 10. *Dichaetura filispina* **n. sp.** (Suzuki *et. al.* 2013) light micrographs of the holotype. a, Coronal view; b, dorsal scale; c, furca spine and basal furca spine. Scale bars = 5 μm. Abbreviations: BC, basal constriction; BFS, basal furca spine; DS, dorsal scale; F, furca; FS, furca spine; INT, intestine; MR, mouth ring; PH, pharynx; SC, sensory cilium. Figs from Suzuki et al. 2013.

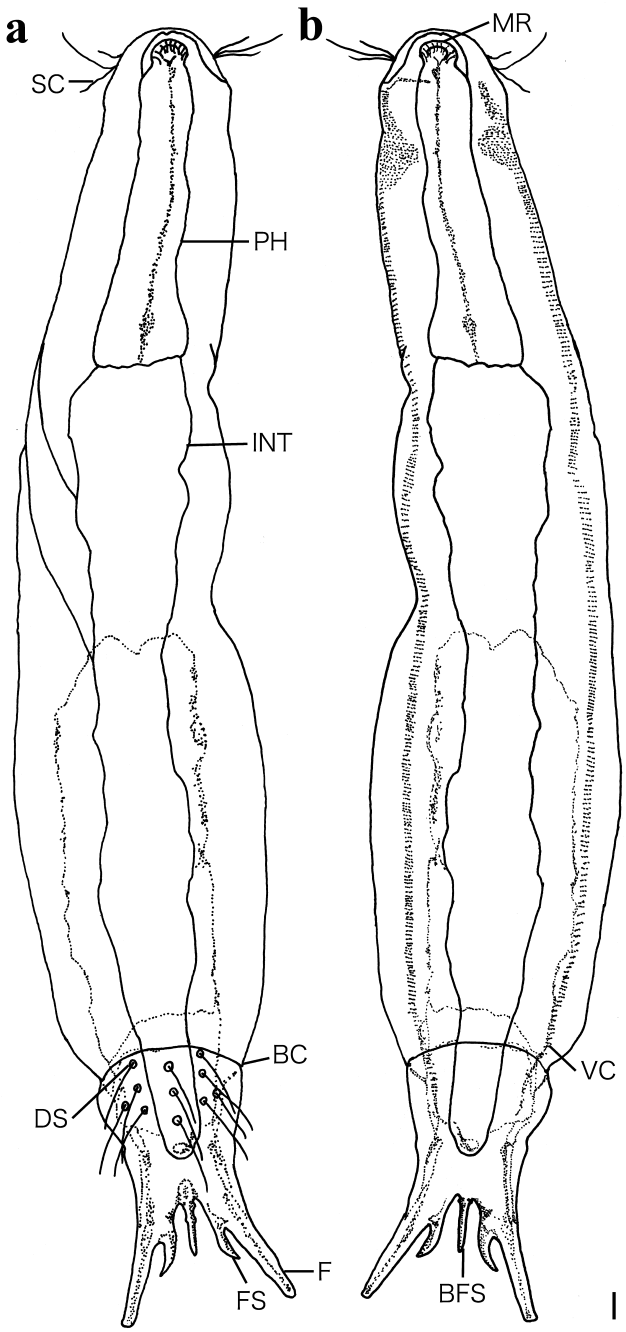

Fig. 11. *Dichaetura filispina* **n. sp.**, (Suzuki *et. al.* 2013) line drawings of holotype. a, dorsal view; b, ventral view. Scale bar = 5 μm. Abbreviations: BC, basal constriction; BFS, basal furca spine; DS, dorsal scale; F, furca; FS, furca spine; INT, intestine; MR, mouth ring; PH, pharynx; SC, sensory cilium; VC, column of ventral cilia. Figs from

Suzuki et al. 2013.

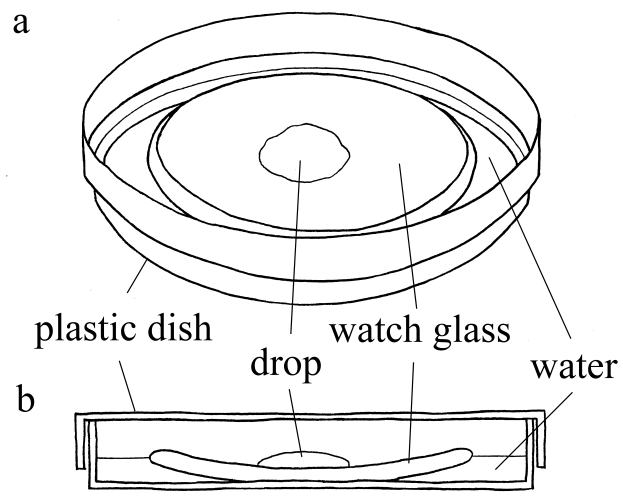

Fig. 12. Schematic diagram of the small culture system

Chaetonotids are cultured in a drop of water on the watch glass. a, overview; b, side view.

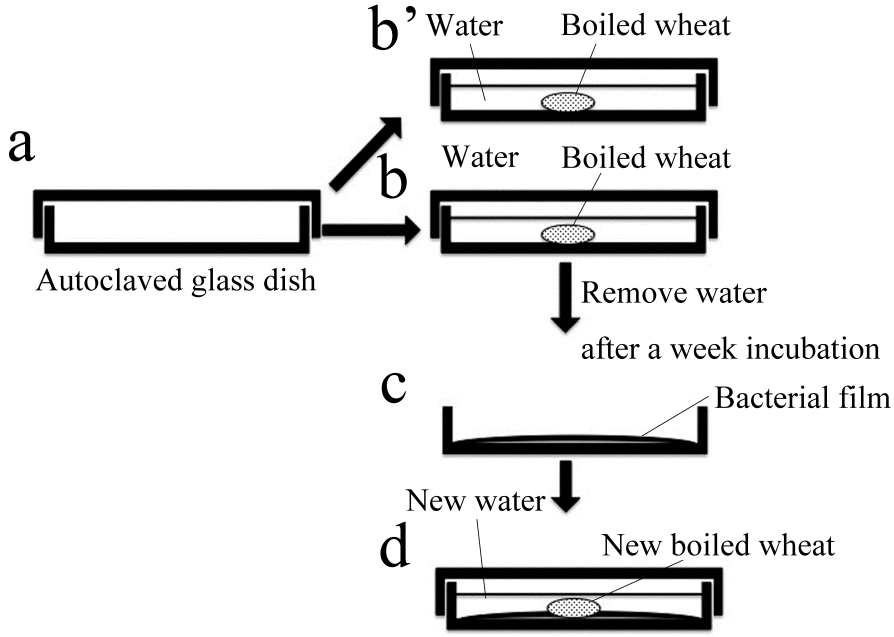

Fig. 13. Schematic diagram of the mass culture system. a, autoclaved glass dish; b', a glass dish of the culture system for *I. podura*; b-d, the culture system for *C. machikanensis* and *L. squamata*. After gastrotrichs are inoculated, each dish is covered and gastrotrichs are incubated under each condition.

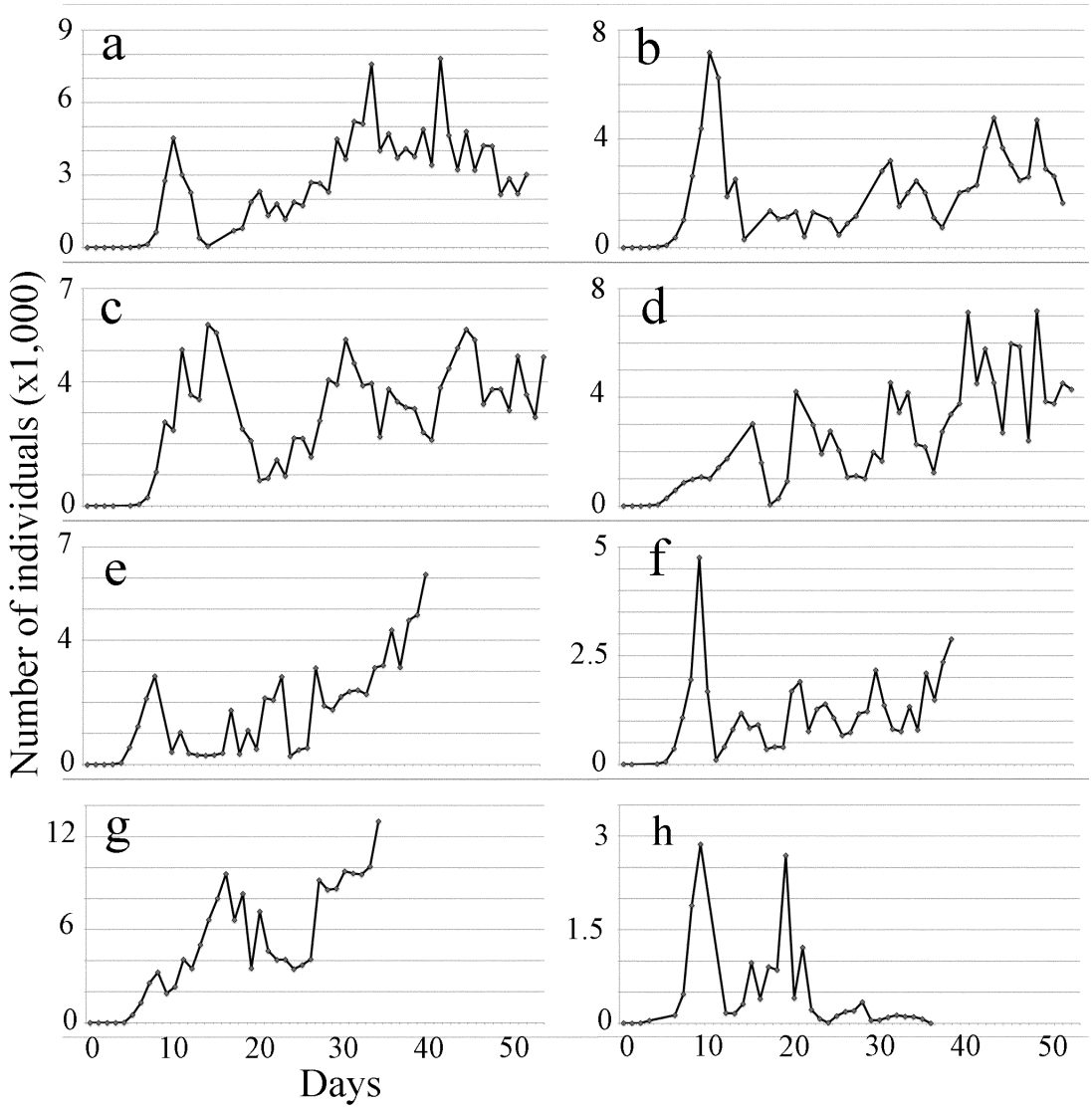

Fig. 14. Population growth of gastrotrichs in the mass culture. a-f, *Chaetonotus machikanensis*; g, *Lepidodermella squamata*; h, *Ichthydium podura*.

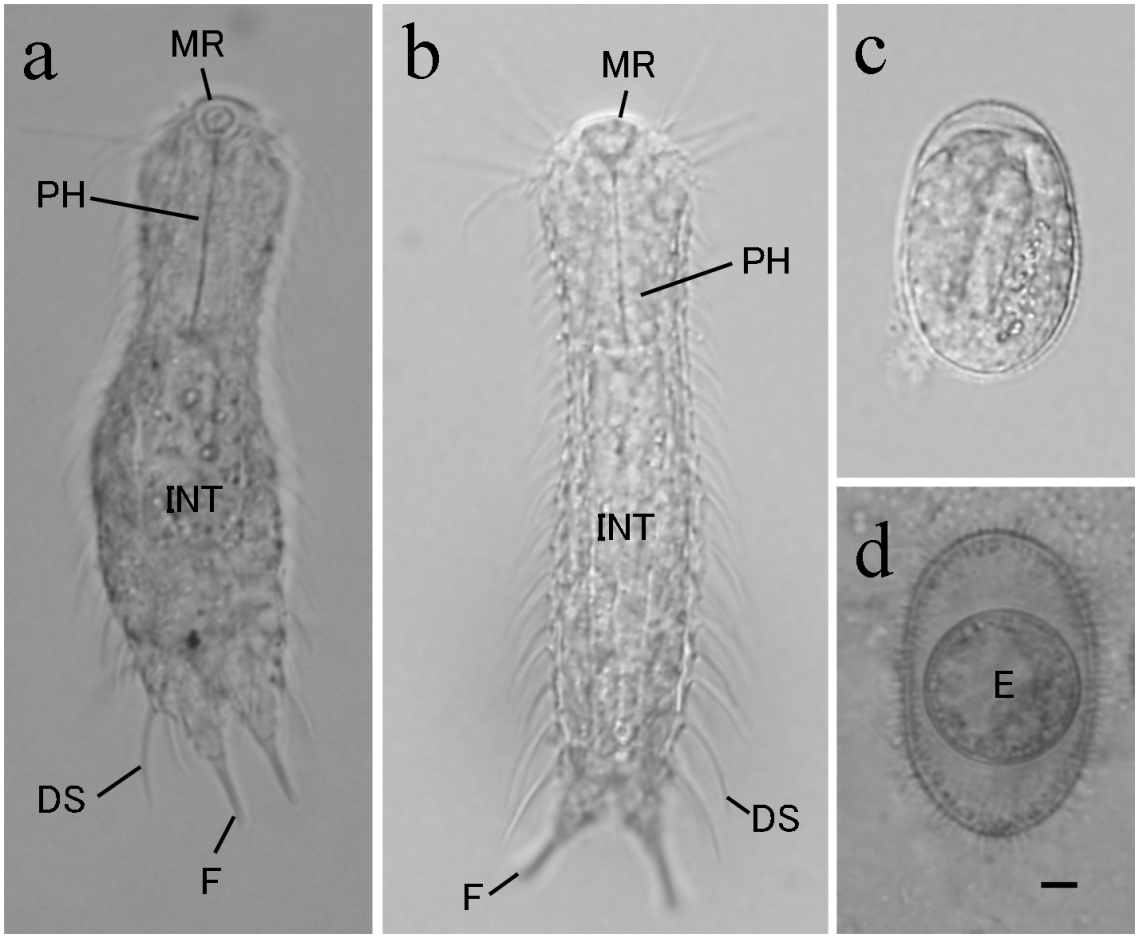

Fig. 15. Photograph of *C. machikanensis*. a, juvenile; b, adult; c, normal egg; d, resting egg. The embryo is formed in the egg shell (c), but not in (d), that is a spherial resting egg (d). The eggshell of resting eggs is thicker than that of the normal egg. Abbreviations: D, dorsal spine; E, egg; F, furca; INT, intestine; MR, mouth ring; PH, pharynx. Scale bar = 5μm.

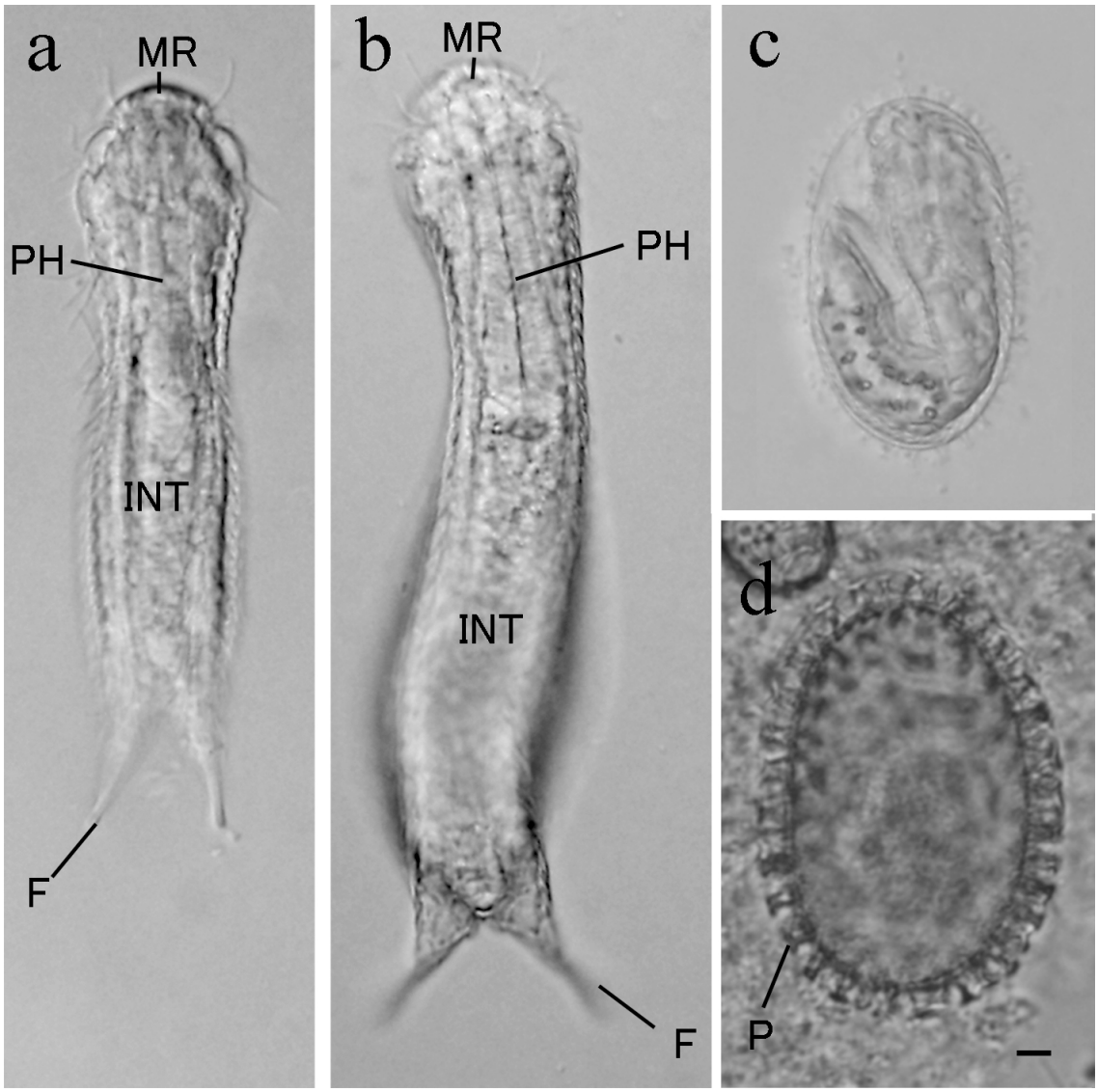

Fig. 16. Photograph of *L. squamata*. a. juvenile individual; b, adult individual; c, normal egg; d, resting egg. Embryo was formed in the eggshell (c), but not in the resting egg (d). The egg shell of resting eggs is thicker that of than the normal egg. Protrusions were found in the external surface of both resting and normal eggs. Abbreviations: F, furca; INT, intestine; MR, mouth ring; P, protrusion; PH, pharynx. Scale bar = 5μm.

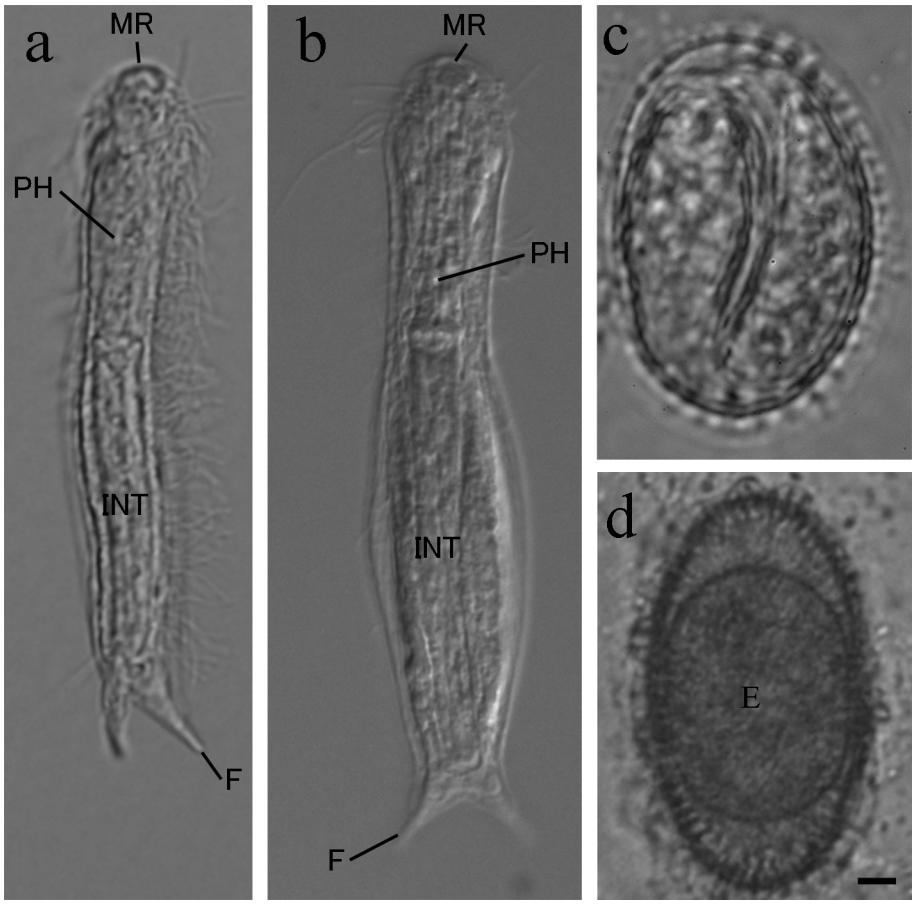

Fig. 17. Photograph of *I. podura*. a, juvenile individual; b, adult individual; c, normal egg; d, resting egg. Embryo was formed in the eggshell (c), but not in the resting egg (d), that the resting egg contains a oval egg (d). The egg shell of resting eggs is thicker that of than the normal egg. Abbreviations: E, egg; F, furca; INT, intestine; MR, mouth ring; PH, pharynx. Scale bar = 5μm.

Table 1 Identification of collection sites.

Site	Water sourse	Coordinates
1	Manogawa river	N35º07.878 E135º54.345
2	Manogawa river Pumped groundwater	N35º07.912 E135º53.836
3	Holding pond (Pumped from lake Biwa)	N35º08.090 E135º54.853

Table 2 Gastrotrich species recorded in present study.

Genus		Number of species	Remarks	Site
Dichaetura	*filispina*		n. sp.	3
Aspidiophorus	spp.	2		1,2,3
Chaetonotus	spp.	26		1,2,3
	*brevisetosus**			2
	*brevispinosus**			1
	*oculifer**		acidphilic	2,3
	*venustus**			2
Heterolepidoderma	spp.	3		3
	*macrops**		basophilic	1,3
	majus			3
	*obesum**			1,2
Ichthydium	sp.	1		2
Lepidodermella	*achanthofurca*		n. sp.	3
	squamata			1,2,3
Polymerurus	*nodicaudus*			1,2
	*nodifurca**			3

* First record from Japan

Table3 Chaetonotus species found in the rice paddies

Site	Species	Site	Species	Site	Species
1-a	*Chaetonotus* sp.1 *Chaetonotus* sp.2 *heterolepidoderma* sp.1	2-a	*Chaetonotus brevisetosus* *Chaetonotus venustus* *Chaetonotus* sp.1 *Chaetonotus* sp.2 *Chaetonotus* sp.3 *Chaetonotus* sp.6 *Chaetonotus* sp.7 *Chaetonotus* sp.9 *Chaetonotus* sp.10 *Chaetonotus* sp.11 *Chaetonotus* sp.12 *Chaetonotus* sp.17 *Heterolepidoderma obesum* *Heterolepidoderma sp.1* *Polymerurus nodicaudus* *Polymerurus nodifurca*	3-a	*Chaetonotus* sp.6 *Chaetonotus* sp.17 *Chaetonotus* sp.18 *Heterolepidoderma macropus* *Lepidodermalla achantholepida* *Polymerurus nodifurca*
1-b	*Chaetonotus* sp.1 *Chaetonotus* sp.2 *Chaetonotus* sp.3 *Chaetonotus* sp.5 *Heterolepidoderma obesum* *Lepidodermalla squamata*	2-b	*Aspidiophorus* sp.1 *Chaetonotus brevisetosus* *Chaetonotus* sp.3 *Chaetonotus* sp.6 *Chaetonotus* sp.13 *Chaetonotus* sp.14 *Ichthydium* sp.1 *Heterolepidoderma obesum* *Lepidodermella squamata*	3-b	*Aspidiophorus* sp.2 *Chaetonotus* sp.2 *Chaetonotus* sp.7 *Chaetonotus* sp.8 *Chaetonotus* sp.19 *Chaetonotus* sp.20 *Chaetonotus* sp.21 *Chaetonotus* sp.22 *Chaetonotus* sp.23 *Chaetonotus* sp.24 *Chaetonotus* sp.25 *Heterolepidoderma majus* *Heterolepidoderma* sp.2 *Lepidodermaella acantholepida* *Lepidodermalla squamata* *polymerurus nodifurca*
1-c	*Chaetonotus brevisetosus* *Chaetonotus brevispinosus* *Chaetonotus venustus* *Chaetonotus* sp.3 *Heterolepidoderma obesum* *Lepidodermalla squamata*	2-c	*Chaetonotus brevispinosus* *Chaetonotus venustus* *Chaetonotus* sp.1 *Chaetonotus* sp.15 *Heterolepidoderma obesum* *Polymerurus nodicaudus* *Polymerurus nodufurca*	3-c	*Chaetonotus* sp.5 *Chaetonotus* sp.26
1-d	*Chaetonotus* sp.1 *Chaetonotus* sp.2 *Heterolepidoderma macropus* *Heterolepidoderma obesum* *Heterolepidoderma* sp.2 *Heterolepidoderma* sp.3 *Lepidodermella squamata*	2-d	*Chaetonotus oclifer* *Chaetonotus* sp.3 *Chaetonotus* sp.16	3-d	*Chaetonotus oculifer* *Chaetonotus* sp.1 *Chaetonotus* sp.13

Table4 Gastrotrichs species described in Japan

Species	Place	Reference
Aspidiophorus heterodermus (Saito, 1937)	Japan (Hiroshima)	Saito, 1937
Aspidiophorus microsquamatus Saito, 1937	Japan (Hiroshima), Poland	Saito, 1937
Aspidiophorus nipponensis Schwank, 1990	Japan (Hiroshima)	Saito, 1937
Aspidiophorus paradoxus (Voigt, 1902)	Germany (Berlin, Holstein), Poland, France (western and central), England, Romania, Italy, North America (Ontario, Illinois), Japan (Yamanashi)	Sudzuki, 1971b
Aspidiophorus semirotundus Saito, 1937	Japan (Hiroshima)	Saito, 1937
Aspidiophorus sp.	Japan (Osaka)	This study
Chaetonotus bisacer Greuter, 1917	Germany (Berlin), Poland, Switzerland, Italy (Tuscany), England, Romania, Canada (Ontario), Argentina, Japan (Osaka, Hiroshima), U.S.A. (Indiana)	Saito, 1937, This study
Chaetonotus carpaticus Rudescu, 1967	Romania, Japan (Okinawa)	Sudzuki, 1992
Chaetonotus cordiformis Greuter, 1917	France (central), Poland, Romania, Japan (Osaka, Hiroshima)	Saito, 1937, This study
Chaetonotus crinitus Sudzuki, 1971	Japan (Yamanashi)	Sudzuki, 1971b
Chaetonotus disjunctus Greuter, 1917	Italy, Switzerland, Poland, Romania, Russia (Moscow), Germany (Berlin), Japan (Hiroshima), France (central)	Saito, 1937
Chaetonotus fujisanensis Sudzuki, 1971	Japan (Yamanashi)	Sudzuki, 1971b
Chaetonotus hystrix Metschnikoff, 1865	Hungary, Russia (central), Romania, Bulgaria, England, Italy, North America, Japan (Hiroshima)	Saito, 1937
Chaetonotus maximus Ehrenberg, 1831	France (western), Russia (central), Spain, Italy, Greece, England, Japan (Hiroshima), New Guinea, Algeria, North America	Saito, 1937
Chaetonotus machikanensis n. sp.	Japan (Osaka)	This study
Chaetonotus multispinosus Grünspan, 1908	France (central and westen), Austria, Hungary, Romania, Russia (Moscow), Italy, Japan (Hiroshima)	Saito, 1937
Chaetonotus persetosus Zerinka, 1889	Russia (central), Romania, Bulgaria, England, Japan (Hiroshima)	Saito, 1937
Chaetonotus retiformis n. sp.	Japan (Osaka)	This study
Chaetonotus scutatus Saito, 1937	Japan (Hiroshima)	Saito, 1937
Chaetonotus similis Zerinka, 1889	Hungary, Russia (central), Romania, Spain, Italy, England, Japan (Hiroshima), India, North America	Saito, 1937
Chaetonotus succinctus Voigt, 1902	Russia (central), Norway, France (central), Romania, Italy (Sardinia), Bulgaria, Japan (Hiroshima), U.S.A. (Virginia)	Saito, 1937
Chaetonotus zelinkai Grünspan, 1908	France, Russia (central), Romania, England, Italy, Finland, Norway, Japan (Hiroshima), North America	Saito, 1937
Heterolepidoderma gracile Remane, 1927	Germany (Holstein), Poland, Romania, France (central part), North America (Illinois, Ontario), Japan (Hiroshima)	Saito, 1937
Heterolepidoderma majus Remane, 1927	Germany (Holstein, Osthessen), Poland, France (central), Japan (Hiroshima)	Saito, 1937
Heterolepidoderma obliquum Saito, 1937	Japan (Hiroshima,)	Saito, 1937
Heterolepidoderma ocellatum (Metschnikoff, 1865)	France (western), Russia (central), Italy, England, North America (Illinois, Ontario), Japan (Hiroshima, Yamanashi)	Saito, 1937, Sudzuki, 1971b
Ichthydium forficula Remane, 1927	Germany (Holstein), Romania, Bulgaria, Japan (Hiroshima)	Saito, 1937
Ichthydium macrocapitatum Sudzuki, 1971	Japan (Yamanashi)	Sudzuki, 1971b
Ichthydium maximum Greuter, 1917	Switzerland, Poland, Romania, Japan (Hiroshima)	Saito, 1937

Ichthydium podura (Müller, 1773)	Russia (central), England, Denmark, Norway, Italy, Hungary, Romania, Bulgaria, Sudan, Madagascar, India, Japan (Hiroshima), North America	Saito, 1937
Lepidodermella aspidioformis Sudzuki, 1971	Japan (Yamanashi)	Sudzuki,1971b
Lepidodermella serrata Sudzuki, 1971	Japan (Yamanashi)	Sudzuki, 1971b
Lepidodermella squamata Dujardin, 1841	France (western), Russia (central), Hungary, Romania, Bulgaria, Spain (Ibiza), Tibet, Japan (Osaka, Hiroshima), India, Uruguay, North America, Eastern Africa	Saito, 1937, This study
Lepidodermella sp.	Japan (Osaka)	This study
Polymerurus nodicaudus (Voigt, 1901)	Germany (Holstein), Russia (central), Romania, Hungary, England, Italy, North America (Ontario, Indiana, Virginia), Japan (Osaka, Hiroshima, Nagano), India	Kawamura, 1918, Saito, 1937, This study
Polymerurus serraticaudus Voigt, 1901	Germany (Holstein), Russia (central), Hungary, Romania, Italy (Sardinia), Japan (Okinawa)	Sudzuki, 1992
Proichthydioides remanei Sudzuki, 1971	Japan (Nagano)	Sudzuki, 1971a