Embryological Studies of the Springtail *Tomocerus cuspidatus* Börner, 1909 (Hexapoda: Collembola, Tomoceridae)

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Shigekazu TOMIZUKA

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ABSTRACT

The embryonic and postembryonic developments of the collembolan *Tomocerus cuspidatus* Börner, 1909 were described in detail using the critical techniques such as electron and fluorescence microscopic observations and 3D reconstruction. The results obtained were discussed and compared with the previous embryological studies on other collembolans and hexapods, to reconstruct the groundplan of Collembola and evolutionary changes of groundplans of hexapods, referring to a robust phylogeny inferred from an international huge phylogenomic project “1000 Insect Transcriptome Evolution (1KITE)” (1KITE Phylogeny).

The embryonic development of Collembola is characterized by: 1) the holoblastic cleavage; 2) the formation of a primary dorsal organ by direct differentiation from blastoderm; 3) the simple blastokinesis; 4) the abdominal construction of six segments; 5) the embryonic membrane represented only by the serosa, which retains a potential to differentiate into the body elements; 6) the entognathy characterized by the mouth fold formed with the fusion and ventral extension of the intercalary, mandibular, maxillary, and labial terga and the construction of the postgnathal region by the labial tergal element of mouth folds extended from the both sides. Based on the critical out-group comparisons, the features Nos. 1-3 are regarded as being plesiomorphies of Hexapoda, and the feature No. 4 as autapomorphy of Collembola.

In contrast to the previous understanding, the serosa of Collembola was revealed to retain a potential to differentiate into the body element (feature No. 5). The trait “loss of ability in serosa for differentiating into body elements”,

which was previously bestowed to Collembola and Cercophora, is exclusively assigned to Cercophora as another comparative embryological autapomorphy of the group. The entognathy of Collembola (feature No. 6) was revealed to have close resemblance to that of Protura, and the monophyly of the assemblage of Collembola and Protura, *i.e.*, Ellipura is strongly suggested. On the other hand, the entognathy of Diplura shows a marked contrast with that of Ellipura, and the monophyly of Entognatha was revealed to be not always substantiated. The present comparative embryological analyses propose the hexapod basal splitting formulated as “Hexapoda = Ellipura (= Collembola + Protura) + Cercophora (= Diplura + Ectognatha)”. The hexapod basal splitting deduced from these comparative embryological analyses was entirely comparable with the 1KITE Phylogeny, reconstructing the groundplan of each group and its evolutionary changes.

1. INTRODUCTION

Insects are the most speciose organisms on the Earth, accounting for three quarters of all animal species, and the reconstruction of their evolution and groundplan is unarguably the most interesting subject. As a most reliable phylogeny of Hexapoda (Insecta *s. lat.*), has been widely accepted more than a half of century the "Entognatha-Ectognatha System" by Hennig (1953, 1969), which is formulated as "Entognatha (= Ellipura (= Protura + Collembola) + Diplura) + Ectognatha (= Monocondylia (= Archaeognatha) + Dicondylia (= Aptera (= Zygentoma) + Pterygota))".

However, recently Hennig's "Entognatha-Ectognatha System", especially concerning the status of Entognatha, has been challenged from various disciplines. Koch (1997, 2000) disputed the monophyly of Entognatha, pointing out a marked difference in the groundplan of entognathy between Diplura and Ellipura. Kukalová-Peck (1987) noticed that the entognathy had not been well established in a Carboniferous dipluran *Testajapyx thomasi* and deduced possible convergences in several times among Entognatha. Based on the critical comparisons of entognathy formations between three entognathan orders and the evolutionary review on the functional specialization between the embryo proper and embryonic membranes throughout Hexapoda, Machida and his colleagues (*e.g.*, Machida and Ando, 1998; Machida *et al.*, 2002; Machida, 2006, 2009) refuted not only the monophyly of Entognatha but also that of Ellipura, and simultaneously suggested a close affinity between Diplura and Ectognatha and the monophyly of Cercophora (= Diplura + Ectognatha). Recent molecular phylogenetics has provided several reconstructions of hexapod basal

splits as well. Molecular phylogenetic analyses on the 18S and/or 28S rRNAs such as Luan *et al*. (2005), Gao *et al*. (2008), and Dell'Ampio *et al*. (2009) postulated a close affinity between Protura and Diplura, discounting the monophyly of Ellipura, and proposed a taxon "Nonoculata" for the assemblage of Protura and Diplura. Sasaki *et al*. (2013) gave Protura a status of sister group to the remaining all major lineages of Hexapoda, based on three nuclear genes encoding the catalytic subunit of DNA polymerase delta and two subunits of RNA polymerase II. On the other hand, EST analysis of von Reumont *et al*. (2012), which aimed at testing a sister group relationship between Hexapoda and a primitive crustacean Remipedia, favored conventional Entognatha-Ectognatha system, supporting the monophylies not only of Ellipura but of Entognatha. Thus, the basal splitting of Hexapoda has been far from consensus and remains highly controversial.

In such background, an international huge project "1000 (1K) Insect Transcriptome Evolution (1KITE)" was launched in 2011, aiming at inferring a robust phylogeny of Hexapoda, based on transcriptomes of more than 1,000 species encompassing all major insect orders and lineages. As the first contribution from the 1KITE, Misof *et al*. (2014) inferred the phylogeny of insects from 1,478 protein-coding genes of 103 species, analyzing the nucleotide and amino acid sequences, and published statistically robust and congruent results resolving previously controversial phylogenetic relationships. In their phylogeny, the basal splitting of Hexapoda was reconstructed as: "Ellipura (= Protura + Collembola) + Cercophora (= Diplura + Ectognatha (= Archaeognatha + Dicondylia (= Zygentoma + Pterygota)))". Although still ambiguous branchings, for example, the positioning of Zoraptera and monophyly of

Polyneoptera are present, the tree inferred from the 1KITE is reliable enough to develop the discussion on the evolution of Hexapoda and is a scaffold robust enough for future comparative embryological analyses to rely on.

Whereas Ectognatha are defined with many autapomorphies, *e.g.*, the flagellate antennae, subdivision of tarsus, and fusion of posterior tentoria, three constituents of Entognatha, which only share plesiomorphies with each other, exhibit features too specialized in each lineage to reciprocally homologize the structures and body plans between them. This makes it difficult to illustrate the evolution of hexapod basal clades including three entognathan orders and to reconstruct the successive changes of their groundplans. Hereat, the comparative embryological approach is one of the most useful methods in this purpose, being able to follow the developmental processes of each structure and body plan (cf. Machida, 2000; Uchifune and Machida, 2005). The comparative embryology can refer to and discuss in the context of evolution the phenomena occurring and structures appearing only during the embryonic period as well (cf. Scholtz, 2004).

As for the embryology of Entognatha, detailed information has been accumulated for Protura and Diplura during these couple of decades (Ikeda and Machida, 1998, 2001; Fukui and Machida, 2006, 2009; Sekiya and Machida, 2009, 2011). On the other hand, although relatively a large number of studies have been done on Collembola (*e.g.*, Uzel, 1898; Claypole, 1898; Folsom, 1900; Hoffmann, 1911; Philiptschenko, 1912; Tiegs, 1942a; Bruckmoser, 1965; Jura, 1965, 1967; Uemiya and Ando, 1987a, b, 1991), our knowledge on their embryology remains old-fashioned, and the shortage of resolution in the collembolan embryology has largely prevented the comparative embryological

arguments among Entognatha. Accordingly, the embryological revaluation of Collembola is given a priority in the reconstruction of evolutionary changes of groundplan of Hexapoda.

The embryological studies on Collembola should be critically focused on the formation of entognathy and developmental potential of embryonic membranes. As addressed, Misof *et al.* (2014), as the first contribution from the project "1000 (1K) Insect Transcriptome Evolution (1KITE)" project, proposed a very robust hexapod phylogeny which formulated as: "Ellipura (= Protura + Collembola) + Cercophora (= Diplura + Ectognatha)". On the other hand, the comparative embryological analyses by Machida and Ando (1998), Machida *et al.* (2002) and Machida (2006, 2009) reconstructed the hexapod basal splitting as "Protura + (Collembola + Cercophora (= Diplura + Ectognatha))", and rejected not only Entognatha but also Ellipura. The most crucial in their argument is the loss of ability for the serosa to differentiate into the body elements, which is to be shared with Cercophora as their synapomorphy. However, their conclusion was based only on a brief description of a collembolan, *Isotoma cinerea* made by Philiptschenko (1912), which is only one article available for this topic concerned. The second is that they concluded the collembolan entognathy to be formed in the way different from those of either Protura or Diplura. The conclusion was based on Uemiya and Ando's (1987a) embryological study on *Tomocerus ishibashii*, which is the newest and the most detailed in the description of entognathy formation in the embryological studies hitherto done for Collembola, but different understandings actually can be found in several previous articles (cf. Claypole, 1898; Folsom, 1900; Hoffmann, 1911; Philiptschenko, 1912; Bruckmoser, 1965). Therefore, it is crucial to re-examine

the embryonic membranes and their developmental potentials in Collembola, and to totally understand the formation of entognathy in Collembola, employing the critical techniques as used in the studies in Protura and Diplura (Ikeda and Machida, 1998, 2001; Fukui and Machida, 2006, 2009; Sekiya and Machida, 2009, 2011), *e.g*., the scanning and transmission electron microscopies and fluorescence one.

The aim of the present study is: 1) to re-examine and describe the development of Collembola in detail, using a largest-sized Japanese arthropleonan collembolan *Tomocerus cuspidatus* Börner, 1909 as materials, with special reference to i) the formation of entognathy, and ii) the embryonic membranes or the serosa and its developmental potentials; 2) to reconstruct the groundplan of Collembola (as for the groundplan, see Ax (1984), Miki (1989), Brusca and Brusca (1990), Valentine and Hamilton (1997) and Kuratani (2004)); and 3) to illustrate the basal splitting of Hexapoda and evolutionary changes of their groundplans, mapping on the robust tree of Hexapoda inferred from 1KITE (Misof *et al*., 2014) the embryological information newly revealed in the present study together with the previous information on the embryogeneses of hexapod basal lineages.

2. MATERIALS AND METHODS

2. 1. Materials

Tomocerus cuspidatus (Fig. 1) has a univoltine lifecycle. It overwinters under bark or in soil and has a synchronized short breeding period. Eggs are laid in the litter layer in April (Hisamatsu and Matsunaga, 1994). Hibernating adults were collected in a larch forest in Sugadaira, Nagano Prefecture, Japan. About 50 insects were kept in a plastic case with a soil bottom kept at 5°C and were fed on green algae grown on larch bark (Fig. 2). Fourteen to 18 days later, each female laid about 60 eggs on the soil surface. These eggs were incubated under humid conditions (~ 100%) at 5°C.

2. 2. Cleaning and fixing of eggs

Eggs were cleaned with a soft brush on wet tissue paper and soaked in a commercial bleach (Kitchen bleach, Mitsuei Chemical, Tokyo, Japan) for approximately 30 sec. The eggs were then rinsed in a modified Ephrussi-Beadle solution (0.38% NaCl, 0.02% KCl, 0.01% $CaCl_2$). The eggs were fixed with Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde 0.1 M HCl-sodium cacodylate buffer solution, pH 7.2 [SCB]) at 4°C or F. A. A. (acetic acid 1 parts, formalin 6 parts, alcohol 16 parts, water 7 parts) at room temperature for 10 h. The chorion and blastoderm cuticles were removed with fine forceps and needles during or after fixation.

2. 3. Observations of external morphology

The fixed eggs and embryos were stained with DAPI solution (4',

6-diamidino-2-phenylindole dihydrochloride, 10 µg/ml with SCB) for 50 min. Specimens stained with DAPI were observed with a fluorescence stereomicroscope (MZ FL III + FULCOMBI, Leica, Heerbrugg, Switzerland) under UV excitation at 360 nm. The series of images of DAPI stained embryos were captured at different depth of field with a digital camera (E-620, Olympus or DS-Fi2, Nikon, Tokyo, Japan), and they were integrated through the public domain image processing package ImageJ (http://rsb.info.nih.gov/ij/) to produce a single image with all sections of the specimen in focus.

The fixed eggs, embryos and larvae were post-fixed with 1% OsO_4 for 20 min. They were dehydrated through a graded ethanol series and dried with a CO_2 critical point dryer (Samdri-PVT-3D, tousimis, Rockville, USA). The dried specimens were stuck to a sharpened tip of a 0.1 mm flexible cupper wire which enables the observations from all directions. Then they were coated with gold in an ion sputter (Ion Sputter JFC-1100, JEOL, Tokyo, Japan) and observed under a scanning electron microscope (SM-300, TOPCON, Tokyo, Japan) at 15 kV. A part of specimens without coated were observed under a low-vacuum SEM (SEM-300 Wet-4, TOPCON, Tokyo, Japan).

2. 4. Histological observations

Fixed embryos were dehydrated in a graded ethanol series and embedded in a mixture of a methacrylate resin (Technovit 7100, Külzer, Wehrheim, Germany) and styrene. Semithin sectioning was performed at a thickness of 2 µm using a semithin microtome (H-1500, Bio-Rad, Hercules, USA) equipped with a tungsten carbide knife (SHK20SB, Meiwafosis, Tokyo, Japan). Methacrylate sections were stained with Mayer’s acid haemalum, eosin gelblich,

and fast green FCF (Machida *et al*., 1994a, b). Semithin sections were observed with a biological microscope (Optiphot-2, Nikon, Tokyo, Japan).

2. 5. 3D reconstructions

The embryos in early and late stages of dorsal closure and first instar larva were reconstructed three-dimensionally from complete series of semithin sections. All serial sections of the embryo were captured, and the serial pictures were automatically aligned to each other through ImageJ. Subsequent segmentation and rendering were accomplished with Reconstruct (http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm) and Blender (http://www.blender.org/) (Fiala, 2005; Blanke *et al*., 2012).

2. 6. TEM observations

The embryos fixed with Karnovsky's fixative were postfixed with 1% OsO_4 for 2 h. They were embedded in Araldite CY212 (TAAB Laboratories Equipment Limited, Berkshire, England) and processed with an ultramicrotome (MT-XL, RMC, Tucson, USA) equipped with a diamond knife (Histoknife Wet 8 mm, Drukker, Cuijk, Netherlands) into sections 80 nm thick, which were stained with uranyl acetate and lead citrate and observed under a TEM (HT-7700, Hitachi, Tokyo, Japan) at 80 kV.

3. RESULTS

3. 1. Eggs

Just after oviposition, eggs are spherical, about 200 µm in diameter, and yellowish in color (Fig. 3A). About 17 days after oviposition at 5°C, the chorion splits in two halves, and the first blastodermic cuticle, which has already been formed under the chorion, makes its appearance on the surface (Fig. 3B). At this time, the eggs are spheroidal with a long diameter of 250-280 µm and short diameter of 200 µm. The blastoderm cuticle has four large curved spines and many small projections on its surface.

The polarity of eggs in which the embryo has not yet developed cannot be designated. In this paper, the orientation of individual eggs is designated as follows: the dorsal and ventral sides of eggs are respectively where the primary dorsal organ (PDO) develops and its opposite; the anterior and posterior sides of eggs are respectively where the cephalic region of the embryo develops and its opposite.

3. 2. Embryonic development

The egg period of *Tomocerus cuspidatus* is approximately 80 days at 5°C (Fig. 4A-L). The embryonic development of *T. cuspidatus* is described, being divided into 11 stages (Table 1).

Stage 1

The cleavage of *Tomocerus cuspidatus* is initially holoblastic.

The first cytoplasmic division occurs at the 4-cell stage (Fig. 4A). The same as in other collembolans, for example, *Tetrodontophora bielanensis*, with its progression, the cleavage transitions from the holoblastic to the superficial type (cf. Jura, 1965, 1972). Then, the yolky part of the blastomeres is isolated centripetally and a large yolk mass forms in the center of the egg. Some nuclei migrate from the blastomeres inside the yolk mass and proliferate there. These nuclei represent the yolk cells, scattered in the yolk mass, and primordial germ cells that form a large cellular mass of about 80 cells at the center of the yolk (cf. Fig. 5A).

Stage 2

The blastomeres devoid of yolk at the egg surface continue to divide. They become columnar and finally a superficial unicellular-layered blastoderm (Figs. 4B, 5A). The cells of the newly formed blastoderm undergo tangential division (Fig. 5C, arrow) and form the inner cellular layer below the entire blastoderm (Fig. 5A, C, white arrowheads); the outer and inner cellular layers, respectively, represent the ectoderm and the mesoderm. The nuclei of the mesoderm are spherical and slightly smaller than those of the ectoderm. The mesodermal cells themselves proliferate by radial division (Fig. 5A, B, black arrowheads).

Stage 3

Blastoderm cells at the center of the dorsal side of the egg become taller to form the primary dorsal organ (Figs. 4C, 6). The cells of the primary dorsal organ are clearly distinguished from other blastoderm cells in their greater

height and the larger size of their nuclei. The mesodermal cells, which were found beneath the entire blastoderm (Fig. 6, white arrowheads), have disappeared at the region of the primary dorsal organ (Fig. 6). Thus, in the eggs at this stage, two regions are distinguished, namely, i) the primary dorsal organ and ii) the blastoderm lined with the mesoderm.

Stage 4

As is well known in other collembolans, both the primary dorsal organ and the blastoderm cells secrete the first blastoderm cuticle, and the latter secretes the second blastoderm cuticle as well (cf. Philiptschenko, 1912; Uemiya and Ando, 1987b). During the secretion of the first blastoderm cuticle, numerous protrusions and wrinkles of blastoderm form (Figs. 4D, 7A, A'). As in *Tomocerus ishibashii* (Uemiya and Ando, 1987b), these protrusions and wrinkles function as molds for characteristic structures on the first blastoderm cuticle, that is, two pairs of large cuticular spines and numerous cuticular projections (Fig. 7A', cf. Fig. 3B). After the first blastoderm cuticle is finished, the surface of the blastoderm becomes smoothed out again (Fig. 7B), and the secretion of the second blastoderm cuticle commences. The primary dorsal organ become radial arranged, and filamentous outgrowths of primary dorsal organ or tendrils extend between the first and second blastoderm cuticle (Fig. 7B, B').

Around the time of commencement of the secretion of the second blastoderm cuticle, the mesoderm, which has been distributed beneath the entire blastoderm, starts to migrate towards the presumptive

embryonic area, and becomes multi-layered there (Fig. 8A, white arrowheads). This implies the differentiation of the embryonic and extra-embryonic areas: the area onto which the mesoderm has migrated is the embryonic area, being called the “embryo”, while the area from which the mesoderm has receded is the extra-embryonic area, representing the serosa (Fig. 8A). The embryonic area (embryo) and the extra-embryonic one (serosa) that have just differentiated, the latter of which is yet to be attenuated, are not well distinguished in terms of thickness but only in terms of the presence/absence of mesoderm (Fig. 8A). The concentration of mesoderm is more progressive in the anterior region of the egg. Although the embryo has already differentiated in its cephalic region at this stage, the thoracic and abdominal regions have yet to be differentiated; namely, the area other than the cephalic region of the embryo and the dorsal organ retains the features of the blastoderm, which is uniformly lined with the mesoderm (Fig. 8B, white arrowheads). Thus, in the egg at this stage, four regions are distinguished, namely, i) the primary dorsal organ, ii) the cephalic region as the differentiated embryonic area, iii) the serosa as the differentiated extra-embryonic area, and iv) the blastoderm as the undifferentiated primary ectoderm.

Stage 5

The differentiated cephalic region of the embryo condenses. Segmentation starts, and the appendages or the antennae, mandibles, maxillae, and labia are formed as well as an unpaired clypeolabrum (Fig. 4E). The intercalary appendages differentiate as a pair of low swellings (cf. Fig. 9). Owing to the condensation of the embryo, the serosa of anterior half of the egg

is on the contrary widened and becomes attenuated. Its cell density being lowered, the serosa appears dark in a DAPI-stained specimen, and the embryonic region and serosa become clearly defined (Fig. 4E). In the posterior half of the egg, however, the blastoderm is yet to differentiate into the embryonic and extra-embryonic areas (Fig. 4E, asterisk).

Stage 6

Owing to the increase of egg volume, the chorion ruptures, and the first blastoderm cuticle appears on the egg surface. The increase of egg volume causes the newly developed structures of embryos to stretch out, making them obscure (Fig. 4F). The stomodaeal invagination is formed just posterior to the clypeolabrum (Fig. 9A). The migration of the mesoderm or the differentiation of the embryonic and extra-embryonic area also starts in the posterior half of the egg (Fig. 10A). The thoracic region of embryo condenses, and the segmentation and formation of appendages proceed from the first thoracic segment posteriorwards. The condensation of the thoracic region also leads to attenuation of the serosa around the thoracic region. The serosa apparently increases in terms of its extension compared with that at the previous stage (Fig. 4G).

The attenuation of the serosa is more progressive in the anterior region of the egg, because the condensation of the embryo is more progressive there. Figure 10 shows a transverse section through the labial and abdominal segments. The serosa in the labial segment is obviously flattened (Fig. 10A, B, white arrowheads), but that in the abdominal region is still less flattened (Fig. 10A, B, black arrowheads). The

DAPI-stained embryo looks broadened in the abdominal region because the less attenuated serosa is demonstrated similarly to the embryo (Figs. 4G, 9A).

The cephalic and thoracic appendages elongate. Later at this stage, the maxilla, labium and thoracic appendages divide into proximal a coxopodite and a distal telopodite. The telopodites of maxilla and labium are the future maxillary and labial palpi (Fig. 9A, B). In mandible, no elongation comparable to the telopodites of the maxillary, labial and thoracic appendages develops throughout the development (Figs 9A, B, 23A, B. 24A, B, 27A, B, 28A, C), and they are represented only by the coxopodite.

In differentiated segments, a pair of coelomic sacs are formed in the cavities of appendages as “appendicular coelom”, and the coelomic sacs of both sides are connected with the mesodermal sheet called the “median mesoderm” (Fig. 10A, C). Cells which are characterized by their larger size have differentiated in the ectoderm between appendages of both sides or a pair of ganglia, representing the neuroblasts (Fig. 10A, C).

Stage 7

The embryo starts to bend ventrally at the thoracic region, and blastokinesis commences (Figs. 4H, 11A-D). As a result of blastokinesis, the ventral surface of the gnathal segments and that of the abdominal ones come to face each other, and the embryo assumes a C-shape (Fig. 11D). The embryo keeps this posture until hutching. During blastokinesis, the primary dorsal organ reaches its largest size (Fig. 12A). The antennae become segmented into four parts (Fig. 11D’). With progressive blastokinesis, the condensation and segmentation of the abdominal region of embryo and the formation of

abdominal appendages proceed from anterior toward posterior (Fig. 11A-D). Appendages of the first, third and fourth abdominal segments develop into the ventral tube (Fig. 11A), tenaculum (Fig. 11B) and furca (Fig. 11C), respectively. Early in blastokinesis, the proctodaeal invagination is formed near the abdominal end (Fig. 11A). Late in blastokinesis, a pair of subanal lobes and a supraanal one form, surrounding the proctodaeum (Fig. 11D).

The serosa drastically increases in extension (Fig. 11A-D), and the serosal cells become strongly flattened (Fig. 12A, B, white arrowheads). At this stage, the serosa is the largest in extension and the lowest in cellular density. Mitoses are rarely found in the serosa (Fig. 12A, B, black arrowhead).

The developing terga of intercalary and mandibular, maxillary and labial terga become defined (Fig. 25A-B'). They unite together and ventrally extend to form mouth fold (Figs. 23A, 26A-B', 27A). The dorsal limit of the mouth fold can be defined as a line connecting the bases of antenna and foreleg (Fig. 27, dotted line). The mouth fold communicates with the clypeolabrum, passing through the region of intercalary tergum (Fig. 26B, B'). The clypeolabrum extend ventrally and develops over the stomodaeum (Figs. 23A, 27A, B). In the three gnathal appendages, a pair of endites develops, and soon differentiates into the medial molar and lateral incisor in mandible, into the medial lacinia and lateral galea in maxilla and into the medial glossa and lateral paraglossa in labium (Figs. 24A, 27B, C). A pair of superlingua is formed between the mandibles (Figs. 24A, 27B). The coxopodites of

maxilla and labium are divided into proximal subcoxa and distal coxa (Figs. 23A, 25A-B').

Stage 8

Blastokinesis has been completed (Fig. 4I). The telopodites of the thoracic appendages become three-segmented into the trochanter, femur and tibiotarsus (Fig. 13B). Lateral body walls on both sides start to extend dorsally and definitive dorsal closure begins (Figs. 4I, 13A, 19A).

Different from the serosal cells of Ectognatha, of which nuclei usually undergo polynucleation or polyploidy (Machida *et al*., 1990; Handel *et al*., 2000; Kobayashi *et al*., 2002; Johannsen, 1929; Lamer and Dorn, 2001), the serosal cells of *Tomocerus cuspidatus* do not suffer from such changes (Fig. 20A). Figure 22A to A" show an embryo at this stage under dorsal closure, which was reconstructed three-dimensionally from serial semithin sections. A total of 257 cells are counted in the serosa of this embryo. The serosal nuclei in the cephalic and abdominal regions are evenly scattered (Fig. 22A, A"), but those in thoracic segments are roughly arranged in two or three longitudinal rows in parallel with the embryonic margins (Fig. 22A'; cf. Fig. 19B). Two to five serosal cells are seen per transverse section (Fig. 20A).

The ventral elongation of clypeolabrum, which divides itself into the proximal clypeus and distal labrum, completes, simultaneously acquiring its definitive position (Figs 13A, 23B, 28A). The newly differentiated clypeus communicates continuously with the mouth fold (Figs. 23B, 28A). The mouth fold further extends ventrally, and almost covers mandible, maxilla and labium, exclusive of their apices (Figs. 23B, 28A). The mouth fold, mostly labial tergal

elements of the mouth fold, extends posteromedially as well (Fig. 28B, D, white arrowheads). Late in Stage 8, a swelling representing the lingua is formed in the maxillary segment, just posterior to the superlingual pair (Figs. 24B, 28C).The labial palpi start to degenerate (Fig. 28D). A pair of invagination of labial glands is formed at the medial bases of labial appendages (Figs. 24B, 28C, D, black arrowheads). The labial appendages of both sides start to approach each other (Figs. 24B, 28C).

Stage 9

Six paired ommatidia differentiate as black spots on each side of the head lobe (Figs. 4J, 14A, 23C). In the thoracic appendages, the coxopodites are divided into the proximal subcoxa, which are subdivided into two segments, and distal coxa, and the articulation of thoracic appendages completes (Fig. 14B).

With progressive definitive dorsal closure, body walls on both sides further extend dorsomedially (Figs. 4J, 14B, 19B). The epidermal cells of the body wall are cuboidal with spherical nuclei, but the dorsomedial epidermal cells are attenuated with their nuclei flattened (Figs. 20B, 21A). These dorsomedial cells overlap the medially neighboring cell, with their cell membranes running obliquely, the same as in the serosal cells overlapping each other (Fig. 21A, B). The serosal cells do not show any signs suggestive of degeneration, such as the condensation of chromatin and organelles, which is one of the key features of apoptosis (Allen *et al*., 1997; Barni *et al*., 1997) (Fig. 21B). Comparing Figure 20 A and 20B, we know that the lateralmost serosal

cells (Fig. 20B, arrow) come close to the dorsomedial epidermal cell of the body wall (Fig. 20B, arrowheads), although the distance between the serosal cells is basically unchanged.

The subcoxae of labial appendages of both sides fuse at the midline in this stage (Figs. 24C, 29A). The paired invaginations of labial glands, which arose in the medial bases of labial appendages, also fuse with each other. They thus form a common duct of the glands, which opens at the distal extremity of the fused labial subcoxae (Figs. 24C, 29A, B, black arrowhead). The labial tergal elements of the mouth folds of both sides extend medially on the postgnathal region (Figs. 29A, 30A, white arrowheads). The newly fused labial subcoxa is compressed into a form of longitudinal narrow plate with the mouth folds or the labial terga extended from laterals (Figs 24C, 29A, 30A). Late in this stage, labial tergal elements of mouth folds of both sides further extend medially and they finally fuse with each other, and confine the postgnathal region (Figs. 29B, 30B).

Stage 10

The third and fourth antennal segments elongate. The eye regions become deeply pigmented (Figs. 4K, 15). The dorsal closure further proceeds (Fig. 19C), and the serosal cells decrease markedly in number: only one to three cells are found in a section (Fig. 20C). Figure 22B to B’’ are 3D reconstructions of an embryo at this stage. This embryo has 106 serosal cells. The serosal cells have migrated dorsomedially and aligned roughly in a row along the dorsal midline of the embryo (Fig. 22B’, B’’). The serosal cells do not show any sign suggestive of apoptosis (Fig. 20C).

The formation of entognathy almost completes (Fig. 23D, 24D, 31A). The labial coxae of both sides to form a plate-like prementum (Figs. 23D, 24D, 31A, B). The postgnathal region of entognathy elongates, and a median longitudinal groove becomes conspicuous, forming the anterior part of “ventral groove” (cf. Fig. 33B) which attains the ventral tube in the first abdominal segment (Fig. 31B). Posterior tentorial pits are observed externally as a pair of invaginations (Figs 23D, 31A, 32B, white arrowheads) between the maxillary and labial terga (Fig. 31A). Anterior tentorial pits are located in the region of the cranium ventrolateral to the antennal bases (Figs. 31A, 32A, black arrowheads), although the pits are concealed at the base of the antenna.

Stage 11

The embryo acquires its definitive form, and the larval cuticle is secreted, on which the chaetotaxy appears (Figs. 4L, 16, 17, 19D). The third and fourth antennal segments are annulated (Figs. 4L, 16). The primary dorsal organ sinks into the lumen of the developing midgut (Figs. 17, 20D). Its degeneration has not completed by the hatching: even in full grown embryos as shown in Figure 17, the filamentous outgrowths sprung out of the primary dorsal organ can be seen in the first thoracic segment. The midgut epithelium is derived from the yolk cells (Fig. 20) as in the other collembolans (cf. Jura, 1972). The closing membrane between the foregut and midgut (Fig. 17) and that between the hindgut and midgut break down, and the communication of the guts is established. Due to the completion of the definitive dorsal closure, the

heart completes just beneath the terga newly completed (Figs. 17, 20D').

Dorsal closure is completed, with the first thoracic segments closing just before hatching. The dorsal epidermis that has just closed is still somewhat flattened (Fig. 20D), but soon gains thickness, becoming cuboidal (Fig. 20D'). In the dorsal epidermis as well as beneath it inside the body, any special structures suggestive of the degeneration of serosal cells are not found (Fig. 20D, D').

3. 3. Postembryonic development

First instar larva

Body length is about 800 µm, whitish in color, and devoid of scales (Fig. 18A). Antennae are brownish in the first, third and fourth segments, and paler in the second one (Fig. 18A). The fourth one among the four segments of antennae is the longest (Fig. 18A), while the third one is the longest in adults (cf. Fig. 1).

The tentorial pits cannot be designated externally under SEM (Fig. 33A), but they are clearly demonstrated in sections of the head (Figs 34, 35A, black and white arrowheads). Figure 36A to B' are 3D representation reconstructed from the serial sections of the head of a first instar larva. Figure 34 shows the anterior tentorial pit (black arrowhead) taking its position at the cranium ventrolateral to the antennal base. The anterior tentorium extends internally from its external pit and first divides into two branches: one branch extends in the direction toward the stomodaeum (Figs. 34, 36B, black arrows), and another extends along the gnathal pouch of mandible (Figs. 34, 36B, white arrows), which then bifurcates (Fig. 36A', B). Figure 35A and B show the posterior tentorium. The posterior tentorial pit is in the lateral region of the cranium,

inside which the proximal end of the maxilla is located (Fig. 35A, white arrowhead). The posterior tentorium extends internally along the blind end of the gnathal pouch, which enfolds the maxilla (Fig. 35B), and then, ventromedially runs (Fig. 36A', B).

Second and subsequent instar larvae

The second (Fig. 18B), third (Fig. 18C) and fourth (Fig. 18D) instar larvae are respectively about 1 mm, 1.2 mm and 1.4 mm in body length, respectively. Dark-colored, glossy scales appear on the body surface from the second instar (Fig. 18B-D). The third segment of antenna elongates relative to other segments with the progressive growth, and at the fourth instar it becomes apparently the longest among four antennal segments (Fig. 18D).

4. DISCUSSION

4. 1. Cleavage

Collembola have been reported to perform the holoblastic cleavage, as revealed in *Tomocerus cuspidatus* (Tomoceridae) in the present study (Fig. 4A) (*e.g.*, Neanuridae: Claypole, 1898; Isotomidae: Philiptschenko, 1912; Onychiuridae: Jura, 1965). The holoblastic cleavage can be regarded as being one of the features in the groundplan of Collembola.

It is well known that the myriapods, *e.g.*, classes Symphyla (Tiegs, 1940), Pauropoda (Tiegs, 1947) and Diplopoda (Dohle, 1964) perform the cleavage of holoblastic type. Cleavage in Chilopoda is seemingly intralecithal as Anderson (1973) pointed out, but Machida *et al*. (1990) concluded that the yolk divisions appearing in the earlier stages of chilopods development should represent the vestiges of total cleavage. Their interpretation is supported by recent developmental re-examination of geophilomorphan centipede, *Strigamia maritima* (Brena and Akam, 2012), which shows a typical holoblastic cleavage. The cleavage patterns of Crustacea are highly divergent and it is difficult to characterize their cleavage. Nothing is known about the embryology of Remipedia or Cephalocarida, which are often bestowed the sister group position to Hexapoda in recent molecular phylogenetic analysis (Regier *et al*., 2010; von Reumont *et al*., 2012; Misof *et al*., 2014). The analysis of the distribution of cleavage patterns based on the phylogenetic relationships of other crustaceans leads clearly to the inference that the stem species of Crustacea underwent total cleavage, and modification of cleavage have arisen during crustacean evolution (Anderson, 1973; Scholtz and Wolff, 2013).

The cleavage type in Mandibulata (= Myriapoda + Crustacea + Hexapoda) is basically holoblastic and in each lineage within Mandibulata the cleavage suffers from modifications: for example, the modification into superficial type in Diplura and dicondylian hexapods is well known (Diplura: Uzel, 1898; Zygentoma: Jura, 1972; Pterygota: cf. Johannsen and Butt, 1941; Anderson, 1973). Primitive hexapods retain the basic type of cleavage for Mandibulata as well (Protura: Fukui and Machida, 2006; Archaeognatha: Machida *et al*., 1990). The holoblastic cleavage in Collembola is therefore regarded as being plesiomorphic in Hexapoda.

4. 2. Primary dorsal organ

In *Tomocerus cuspidatus*, the primary dorsal organ is directly formed from the blastoderm before the stage of differentiation of embryo and serosa as well as in the other collembolans (*e.g.*, Neanuridae: Claypole, 1898; Folsom, 1900; Hypogastruridae: Tiegs, 1942a; Onychiuridae: Jura, 1965, 1967; Entomobryidae: Tiegs, 1942a; Isotomidae: Philiptschenko, 1912; Tomoceridae: Uemiya and Ando, 1987a; Sminthuridae: Tiegs, 1942a). The primary dorsal organ is also formed in Protura (Fukui and Machida, 2006), Diplura (Uzel, 1898; Tiegs 1942b; Ikeda and Machida, 1998, 2001; Sekiya and Machida, 2009), Crustacea (*e.g.*, Manton, 1928; Strömberg, 1972; Anderson, 1973; Meschenmoser, 1989; Wolff, 2009) and Myriapoda (Symphyla: Tiegs, 1940; Pauropoda: Tiegs, 1947). The primary dorsal organs hitherto reported in variable groups of arthropods share the major aspects such as: 1) the radial arrangement of cells (cf. Figs. 7B, 12A), 2) possession of tendrils sprung out from its center (cf. Fig. 12A), and 3) dorsal positioning (cf. Fig. 4C-K), and can be

homologized with each other. However, Diplura is different from all the others possessing the primary dorsal organs in the manner of formation of the organ. The primary dorsal organs are formed by the direct differentiation of the blastoderm in the above-mentioned arthropods exclusive of Diplura, in which, however, the primary dorsal organ is formed by the concentration of the serosa (Uzel, 1898; Ikeda and Machida, 2001; Sekiya and Machida, 2009). The manner of formation of the organ in Diplura is unarguably derived, and the formation by the direct differentiation from the blastoderm is the basic or plesiomorphic type in Arthropoda.

Nothing definite is known on the function of primary dorsal organ. However, the primary dorsal organs including those of Collembola are likely to serve as an anchor and a generator of driving force for the blastokinesis from the circumstantial evidence: i) the primary dorsal organ is fixed to the egg shells with filamentous outgrowths (cf. Fig. 7B'); ii) the primary dorsal organ is the most developed during the blastokinesis (cf. Fig. 12A).

4. 3. Blastokinesis

The blastokinesis of *Tomocerus cuspidatus* is a simple flexion of embryo (Figs. 4H, 11A-D), the same as in other collembolans (*e.g.*, Claypole, 1898; Folsom, 1900; Philiptschenko, 1912, Bruckmoser, 1965; Uemiya and Ando, 1987a). The blastokineses of similar type are performed in other entognathan Protura (Fukui and Machida, 2006) and Diplura (*e.g.*, Uzel, 1989; Ikeda and Machida, 1998, 2001; Sekiya and Machida, 2009), as well as in Myriapoda (*e.g.*, Symphyla: Tiegs, 1940; Pauropoda: Tiegs, 1947; Diplopoda: Dohle, 1964; Chilopoda: Heymons, 1901), Crustacea (*e.g.*, Shiino, 1950; Anderson, 1969,

1973; Zilch, 1974) and Chelicerata (Brauer, 1895; Reuter, 1909; Holm, 1952; Moritz, 1957; Yoshikura, 1961). The simple blastokineses as shown in these arthropods can be regarded as being plesiomorphic in Hexapoda, as Ikeda and Machida (1998) suggested. Ectognatha perform the blastokineses in which the formation and regression of amnioserosal fold are involved in and is often accompanied by a substantial reversal of the embryo's directions (cf. Johannsen and Butt, 1941).

4. 4. Formation of abdominal segments

The abdomen in *Tomocerus cuspidatus* is composed of only six segments, and the first, third and fourth abdominal segments bear ventral tube, tenaculum and furca, respectively (cf. Fig. 11A-D) as in other collembolans (Claypole, 1898; Folsom, 1900; Uemiya and Ando, 1987a). In Protura, the prelarva hatches out with nine abdominal segments, and the abdominal segments increase up to 12 during the postembryonic development (Fukui and Machida, 2006; Fukui, 2010). In Diplura, the abdomen is revealed to be composed of 10 abdominal segments throughout the embryonic and the postembryonic developments (Uzel, 1898; Ikeda and Machida, 1998). The abdomen of Ectognatha, which accounts for *ca*. 99% of Hexapoda, is composed of 11 segments (Matsuda, 1976). Thus, each of hexapod major lineages has an own number of abdominal segments specific to itself, and each of them may be safely asserted to be an autapomorphy of each lineage.

4. 5. Development and developmental potential of serosa

4. 5. 1. Differentiation of embryo and serosa

In *Tomocerus cuspidatus*, the mesoderm is segregated beneath the entire blastoderm that has just been completed (cf. Fig 5A). The mesoderm then concentrates towards the presumptive embryonic area, and the areas with and without mesoderm lining differentiate. The former and latter areas respectively represent the embryonic area or the embryo and the extra-embryonic area or the serosa (cf. Fig. 8A). The embryonic and serosal cells closely resemble each other, being distinguished only by the presence/absence of the mesoderm lining. Hereafter, owing to successively occurring phenomena, namely, the condensation of embryo, the increase of egg volume in association with the rupture of chorion, and the change of the embryo's posture caused by blastokinesis, the serosa markedly increases in terms of its extension and is attenuated (cf. Figs. 10B, 12A, B). The serosa and the embryonic ectoderm become distinguishable from each other in terms of their thickness.

In *Anurida maritima* (Neanuridae) (Claypole, 1898) and *Isotoma cinerea* (Isotomidae) (Philiptschenko, 1912), the embryo and serosa are differentiated in a similar manner to those in *Tomocerus cuspidatus*, as revealed in the present study. Therefore, one of the groundplans in collembolan embryogenesis may involve this manner of differentiation of the embryo and serosa; namely, the areas towards which the mesoderm concentrates and from which the mesoderm recedes, respectively, differentiate into the embryo and the serosa. Uemiya and Ando (1991) reported in *Tomocerus ishibashii* (Tomoceridae) that the serosa is derived from a small area around the primary dorsal organ, and that the mesoderm is segregated only in the presumptive embryonic area. However, I can find several cells that appear to be mesodermal beneath the "serosal area" in Uemiya and Ando's (1991) Figure 5, and their observation should be tested in

the same species as they examined. Jura (1965) reported that the regional differentiation of mesoderm was restricted beneath the embryonic area in *Tetrodontophora bielanensis* (Onychiuridae), but his observation on mesodermal segregation lacks detail.

In *Tomocerus cuspidatus*, the mitosis was observed in the serosa during the blastokinesis in which the serosal area drastically expands (Fig. 12B). Although it may be critically re-examine whether this is abnormal phenomenon or not, this finding should be paid much attention, since the serosal cells of most of Hexapoda, *i.e.*, Ectognatha, do not possess mitotic ability. For example, those of Archaeognatha never undergo proliferation (Machida *et al*., 1990, 1994a). The serosal cells of a coleopteran *Tribolium castaneum* are not stained with anti-phosphohistone H3 antibody, which is specific to the chromosomes in mitosis (Handel *et al*., 2005; van der Zee *et al*., 2005). In some representatives of Ectognatha, it was reported that the serosal cells are often more basophilic than somatic cells, showing polynucleation and/or polyploidy (Archaeognatha: Machida *et al*., 1990; Coleoptera: Handel *et al*., 2000; Kobayashi *et al*., 2002; Lepidoptera: Johannsen, 1929; Lamer and Dorn, 2001). The serosal cells of *T. cuspidatus* invariably show similar stainability to somatic cells, with their nuclei not showing an irregular shape, and suffer from neither polynucleation nor polyploidy (cf. Figs. 12B, 20A-C, 21A, B). The facts mentioned above may represent that in *T. cuspidatus* the serosal and somatic cells have not well been differentiated.

4. 5. 2. Dorsal closure and participation of serosa to it

In Ectognatha, the serosa sinks into the yolk to degenerate there, in

association with definitive dorsal closure, which is exclusively accomplished with the extension of embryonic body walls, usually forming a cellular mass called the secondary dorsal organ (cf. Johannsen and Butt, 1941: Barni *et al*., 1997). The serosal cells heading for degeneration or those of the secondary dorsal organ show features typical of apoptosis, such as the condensation of chromatin and organelles (cf. Barni *et al*., 1997). The serosal cells of Archaeognatha, which represents the basalmost clade of Ectognatha, also degenerate in association with definitive dorsal closure, without forming such a large cellular mass as the secondary dorsal organ, but a doughnut-shaped condensation only encircling the egg circumference, because the degeneration of the serosal cells occurs too rapidly to form a solid structure (Machida *et al*., 1994a). In Diplura, which is sometimes bestowed a sister group position to the Ectognatha (Machida, 2006; Misof *et al*., 2014), the serosal cells concentrate towards the dorsal side of the egg and form a disc-like structure called the primary dorsal organ before blastokinesis occurs. Then, the primary dorsal organ or the serosa of Diplura sinks into yolk in the final stage of definitive dorsal closure, which is exclusively fulfilled with the embryonic body walls, and degenerates there (Uzel, 1898; Tiegs, 1942b; Ikeda and Machida, 1998, 2001; Sekiya and Machida, 2009). Thus, in Diplura and Ectognatha, the serosa degenerates, usually forming a degenerative, special structure, without participating in definitive dorsal closure, and the latter is exclusively performed by dorsomedial extension of the embryonic body walls.

Philiptschenko (1912) stated, in his embryological study on *Isotoma cinerea*, “Diese Zellen (of serosa) spielen ziemlich lange Zeit hindurch die Rolle eines primären Integuments des Rückens und der Seiten, gehen aber nicht als

solche auf die erwachsene Form über, indem sie … durch das nach oben wachsende Ectoderm des Keimstreifens verdrängt werden" (p. 643) ("The serosal cells play a role in provisional dorsal closure (*i.e.*, the dorsal covering with embryonic membranes in the embryonic period) for a fairly long time, but without participating in definitive dorsal closure, they are replaced by the dorsally extending ectoderm of the embryo") although without any figures illustrating this. However, the formation of any degenerative structures such as the secondary dorsal organ has not been reported, nor has evidence supportive of the degeneration of serosal cells in the collembolans hitherto studied (*e.g.*, Neanuridae: Claypole, 1898; Folsom, 1900; Isotomidae: Philiptschenko, 1912; Tomoceridae; Uemiya and Ando, 1987a). As described in the present study, I could not obtain any evidence supportive of the degeneration of serosal cells. Furthermore, in the transmission electron microscopic observation, I could not find any degenerative figures of serosal cells throughout all the developmental stages, including the final step of dorsal closure (cf. Fig. 21A, B). Philiptschenko's (1912) description above should thus be re-examined.

In *Tomocerus cuspidatus*, after blastokinesis has been completed, the serosa is attenuated (cf. Figs. 12A, B), and the embryonic and serosal cells become distinguishable in terms of thickness: the former are cuboidal with a spherical nucleus, and the latter are attenuated with a flattened nucleus (cf. Figs. 20A-C, 21A, B). The serosal cells of *T. cuspidatus* decrease in number, with progressive definitive dorsal closure. Comparing an embryo in the early stage of dorsal closure shown in Figure 22A-A" with an embryo in the late stage of dorsal closure shown in Figure 22B-B", the serosal cells were lower in number by about half. We also have to pay attention to the following findings: in the late

stage of dorsal closure, the serosal cells are often observed to come close to the extending body walls of the embryo (cf. Fig. 20B), and the cells at the frontier of the extending body walls are always highly attenuated (cf. Fig. 21A, B). Taking these findings into consideration as well as the fact that the serosal cells should not degenerate, it may be safely asserted that, in *T. cuspidatus*, the serosal cells should be integrated into the developing body walls of the embryo and should form definitive dorsal closure together with the embryonic body walls, without degeneration. It may be noticeable that the cells at the frontier of the developing body wall overlap with neighboring cells with their cell membranes running obliquely, just like the serosal cells that overlap with each other (cf. Fig. 21A, B). An oblique cellular arrangement is often found in the serosal cells of other hexapods such as Archaeognatha (Machida *et al*., 1994a). It is also noteworthy that, in *T. cuspidatus*, the serosal cells have a potential to proliferate (see the above), and do not suffer from polyploidy and/or polynucleation (Figs. 12B, 20A-C, 21A, B), the same as in ectodermal somatic cells. Hereat, it can be concluded that the serosal cells of *T. cuspidatus* possess the ability to differentiate into the body wall or to participate in definitive dorsal closure, different from Diplura and Ectognatha, the serosal cells of which have lost such ability.

It was revealed that the serosa of Protura retains the ability to participate in definitive dorsal closure (Fukui and Machida, 2006; Machida, 2006). The serosal cells of Protura are arranged roughly in two or three longitudinal rows along the midline during the late stage of dorsal closure, the same as in *Tomocerus cuspidatus* (Fukui, 2010, in prep.). It is known that the dorsal extra-embryonic ectoderm in Crustacea and Myriapoda, which can be equivalent

to hexapod serosa, does not degenerate and participates in definitive dorsal closure (see Machida and Ando, 1998; Machida *et al*., 2002). The ability to differentiate into the definitive dorsal closure in Collembola and Protura may represent a plesiomorphic condition to Hexapoda.

4. 5. 3. Evolutionary changes of the embryonic membranes and their developmental potentials in Hexapoda

Machida and colleagues demonstrated anagenetical evolutionary transition in the functional specialization between the embryo proper and embryonic membranes, to provide a persuasive phylogenetic hypothesis in Hexapoda (*e.g.*, Machida and Ando, 1998; Machida *et al*., 2002; Machida, 2006, 2009). 1) In Protura, the same as in Crustacea and Myriapoda, the embryonic membranes are represented only by the serosa, which secretes blastoderm cuticle in cooperation with the embryo proper; the serosa retains the ability to differentiate into the body element and form definitive dorsal closure together with the embryo; the condition shown in Protura is the most ancestral or plesiomorphic in Hexapoda. 2) In hexapods excluding Protura, namely, Collembola, Diplura, and Ectognatha, the serosa lost the ability to differentiate into the body element, and this function became exclusively performed by the embryo; this condition is regarded as synapomorphy of Collembola, Diplura, and Ectognatha. 3) In Diplura and Ectognatha, a second embryonic membrane or the amnion was acquired, leading to the temporal specialization concerning the provisional dorsal closure with the serosa and amnion; the acquisition of amnion and temporal specialization concerning the provisional dorsal closure with the serosa and amnion can be regarded as synapomorphy of Diplura and

Ectognatha, suggesting the monophyletic Cercophora. 4) In Ectognatha, the embryo lost the ability to secrete the cuticular egg envelope (blastoderm cuticle); in association with this, the embryonic membrane (serosal or amnioserosal) fold was acquired, which ensured the secretion of the cuticular layer beneath the embryo, and the continuity of the cuticular egg envelope (serosal cuticle) was maintained; this condition is regarded as autapomorphy of Ectognatha. Summarizing the above, the phylogeny formulated as "Protura + (Collembola + Cercophora (= Diplura + Ectognatha))" was proposed. Here, the affinity of Collembola and Cercophora drawn from the second step mentioned above largely depends on Philiptschenko's (1912) description that the serosa of Collembola degenerates without participating in definitive dorsal closure (cf. Machida and Ando, 1998).

In the present study, it was concluded that the serosa of Collembola retains the ability for differentiating into the body element, like that of Protura. Hereat, the loss of this ability should be attributed exclusively to Cercophora. Consequently, the affinity of Collembola and Cercophora is ruled out. On the other hand, the status of Cercophora becomes more reinforced by the assignment of another autapomorphy "loss of ability for differentiating into the body element by the serosa." At this point of time, the phylogeny previously proposed by Machida and his colleagues should be reconstructed as "Protura + Collembola + Cercophora (= Diplura + Ectognatha)", because Protura and Collembola share only symplesiomorphies.

4. 6. Formation of entognathy

4. 6. 1. Tentoria and mouth folds

Folsom (1900) investigated the tentorial structures in *Anurida maritima*. He failed to find the tentorial invaginations and addressed that "…tentorium of *Anurida* is derived from proliferated ectodermal cells which are in no way, except in position, distinguishable from young ganglion cells (p. 140)". His understanding, however, is suspect. His tentorium of *A. maritima*, which has dorsal and ventral arms and assumes an H-shape, is the structure that is correctly identified as a complex of the mandibular transverse tendon and the dorsal and ventral suspensory muscles (compare Fig. 35 of Folsom (1900) with Fig. 37 of Manton (1964)). Furthermore, it is well known that collembolan tentoria of both sides do not fuse with each other and never assumes an H-shape (Manton, 1964; Koch, 2000).

The present study revealed that the anterior tentorium of *Tomocerus cuspidatus* extends internally and divides into two branches, *i.e.*, one branch extending towards the stomodaeum and the other extending along the gnathal pouch of mandible which then bifurcates into two directing posteriorly and ventrally (cf. Fig. 36A, B'). The posterior tentorium runs internally along the blind end of maxillary gnathal pouch and heads ventromedially (cf. Fig. 36 A' B). The tentorial structures revealed in *T. cuspidatus* agree with those in other collembolans (Manton, 1964; Goto, 1972; Koch, 2000). Comparing the head of the first instar larva reconstructed three dimensionally (Fig. 36A, B) and that of embryo in the later stages of entognathy formation (Fig. 31A), the ectodermal invaginations at the base of antenna and the postcephalic region found in the late embryo (Fig. 31A, black and white arrowheads) were revealed to exactly correspond to the anterior and posterior tentorial pits on the cranium of the first instar larva (Fig. 36A, B, black and white arrowheads), respectively. Hence,

these invaginations found in the late embryo's cephalic region can be identified as the tentorial invaginations or tentorial pits.

The mouth fold of *Tomocerus cuspidatus* is the ventral extension of the fused terga of intercalary, mandibular, maxillary and labial segments (cf. Figs. 26B, B', 37A). The mouth fold can be defined as the cranial structure extending ventrally, surpassing the appendicular bases and covering the sides of gnathal appendages. Early in entognathy formation, the dorsal limit of mouth fold can be defined as dotted line as shown in Figure 27A, which connecting the bases of antenna and foreleg. They do not change their position during entognathy formation (cf. Figs. 23A-D, 27A, 28A, 31A). Hence, the dorsal limit of mouth fold of the embryo in the later stage of entognathy formation should be also defined as the line connecting bases of antenna and foreleg (cf. Fig. 31A, dotted line). Figure 31 shows that the dorsal limit of the mouth fold (dotted line) can be approximated as the line connecting the anterior (black arrowhead) and posterior (white arrowhead) tentorial pits. In Ectognatha, anterior and posterior tentorial pits are connected by a lateral submarginal groove on the cranium or subgenal suture, which define a dorsal limit of a narrow marginal area on the side of the cranium or subgena (Snodgrass, 1935, Matsuda, 1965). Hence, the mouth fold in Collembola and subgena in Ectognatha are approximately homologous each other. Accordingly, the mouth fold of Collembola (possibly also the mouth folds of other two entognathan orders Protura and Diplura) may be regarded as the ventral extension of subgena of Ectognatha. Actually, the subgena of Archaeognatha which represents the most primitive clade of Ectognatha have a well-developed a ventral extension of subgena named the "pleural fold", which covers and supports the unstable monocondylic mandibular bases like the

entognathan mouth fold (see. Manton, 1964). Manton (1964) suggested that the ancestral forms of Hexapoda have a cephalic construction as found in the modern Archaeognatha. It is probable that on the one hand the mouth fold has been evolved from the pleural fold as found in Archaeognatha, this being regarded as a “preadaptation” leading to entognathy. On the other, in association with the stabilization of mandibles brought about from the dicondylization, the pleural fold has been degraded in the lineage leading to Dicondylia.

4. 6. 2. Formation of entognathy in Collembola and other two entognathan orders

Development and construction of entognathy in Collembola have been investigated and differently understood by authors (*e.g.*, Folsom, 1900; Hoffmann, 1911; Bruckmoser, 1965; Uemiya and Ando, 1987a). These investigations were, however, exclusively based on the light microscopical observations on the external embryology and lacking in resolution. Hereat, I endeavored to thoroughly figure out the formation of entognathy in Collembola and reconstruct its groundplan, employing various, critical techniques, *i.e.*, the fluorescence and scanning electron microscopies of external morphology, histological light microscopies on the serial semi-thin sections, and transmission electron microscopy. In the present study, I succeeded in full documentation of the entognathy formation in Collembola, using *Tomocerus cuspidatus* as materials. The groundplan of entognathy formation in Collembola can be characterized by (see Fig. 37A, A’): 1) the mouth fold is formed by the fusion and ventral extension of terga of intercalary, mandibular, maxillary and labial segments (cf. Figs. 23A, 26B, B’, 27A, 37A), and 2) the postgnathal region of

entognathy is fulfilled by the fusion of labial tergal elements of mouth folds extended from the both sides (cf. Figs. 24B-D, 28B, D, 29A, B, 30A, B, 37A').

According to Bruckmoser (1965) in *Orchesella villosa* (Entomobryidae) and Uemiya and Ando (1987a) in *Tomocerus ishibashii*, the mouth folds are mandibular, maxillary and labial tergal in origin. In the present study, the mouth fold of *Tomocerus cuspidatus* was revealed to communicate with the clypeolabrum, passing through the region of intercalary tergum (cf. Fig. 26B, B'). This implies that the intercalary tergum should be one of the constituents of the mouth fold. Folsom (1900) also mentioned the participation of the intercalary element in the formation of mouth fold in *Anurida maritima*.

Hoffmann (1911) reported in *Tomocerus plumbeus* (Tomoceridae) that the postgnathal region of entognathy is derived from the mouth folds extended from the both sides, whereas Bruckmoser (1965) assigned the origin of the postgnathal region to the labial sternum in *Orchesella villosa*, and Uemiya and Ando (1987a) concluded that postgnathal region of *Tomocerus ishibashii* is established by the fused labial subcoxae or postmentum. In the present study on the entognathy formation of *Tomocerus cuspidatus*, it was clearly revealed that the labial sternum is totally covered by the medial extensions of the labial subcoxae, which are themselves soon concealed with the medial extensions of the labial tergal elements of the mouth folds (cf. Figs. 24C, 29A, B).

In the Entognatha-Ectognatha System, the entognathy has been regarded as the most reliable synapomorphy for members of Entognatha. Fukui and Machida (2009) and Fukui (2010) investigated the formation of entognathy in Protura in detail and characterized it as (see Fig. 37B, B'): 1) the mouth fold is derived from the intercalary, mandibular, maxillary and labial tergum, and 2) the

postgnathal region is covered by labial tergal elements of the mouth folds. In the present study, I reconstructed the groundplan of entognathy formation in Collembola, and we know that the entognathy of Collembola is constructed in the groundplan exactly coincident with that in Protura. The manner of entognathy formation shared with Collembola and Protura may be regarded as their synapomorphy, which can be named the "entognathy of ellipuran type".

Ikeda and Machida (1998) for Rhabdura and Sekiya and Machida (2009, 2011) for Dicellurata investigated in detail the formation of entognathy of Diplura, which comprises two suborders Rhabdura and Dicellurata. Rhabdura and Dicellurata were revealed to have the same groundplan in the formation of entognathy, which can be characterized as (see Fig. 37C, C'): 1) the mouth fold is formed by the intercalary, mandibular, maxillary and labial terga plus the anterior part of labial coxae, 2) the postgnathal region of entognathy is established by the postmentum, 3) the labial appendages undergo a 90-degree rotation during the entognathy formation, and 4) the admentum, which is labial coxal in origin, is partitioned from the posteromedial part of the mouth fold. The manner of entognathy formation of Diplura is absolutely different from that of Collembola and Protura, *i.e.*, the entognathy of ellipuran type, in major aspects. The entognathy of the former should be discriminated from of latter as the former's autapomorphy, being named the "entognathy of dipluran type". The homology between entognathies of the ellipuran and dipluran types is suspect. Hence, being established with only one autapomorphy "entognathy", "Entognatha" in "Entognatha-Ectognatha System" is not always substantiated, whereas Ellipura is strongly supported, in light of the critical comparative analyses of the entognathy formations in Entognatha.

4. 7. Evolutionary changes of hexapodan groundplans

Hennig's "Entognatha-Ectognatha System" formulated as "Entognatha (= Ellipura (= Protura + Collembola) + Diplura) + Ectognatha" has been the most widely accepted phylogenetic relationship for hexapod basal splitting (Hennig, 1953, 1969; Kristensen, 1975; Boudreaux, 1979). As has been addressed, comparative embryological analyses dismiss the Entognatha, but on the other hand strongly suggest the affinity of Diplura and Ectognatha, supporting the monophyly of Cercophora as well as the monophyly of Ellipura. Recent molecular phylogenetic analyses based on 18S and 28S ribosomal RNA datasets proposed the Nonoculata hypothesis, in which the monophyly of Entognatha is supported, and Protura and Diplura are allied (Luan *et al*., 2005; Gao *et al*., 2008; Dell'Ampio *et al*., 2009). On the other hand, from the analysis of three nuclear protein-coding genes, Sasaki *et al*. (2013) rejected the monophyly of Entognatha, placed Protura at the sister position to all other hexapods, and suggested affinity between Collembola and Diplura, the assemblage of which is allied to Ectognatha. However, my comparative embryological evidence does not match any of these molecular phylogenies, unless I could postulate the convergent loss of the ability for serosa to participate in definitive dorsal closure and parallel acquisition of amnion in Diplura and Ectognatha, as well as the convergent acquisition of the entognathy of Ellipura type in Protura and Collembola.

In the background the basal splitting of Hexapoda remains highly controversial, an international huge project "1000 (1K) Insect Transcriptome Evolution (1KITE)" was launched in 2011, aiming at inferring a robust

phylogeny of Hexapoda, based on transcriptomes of more than 1,000 species encompassing all major insect orders and lineages. As the first contribution from the 1KITE, analyzing 1,478 protein-coding genes of 103 hexapod species, Misof *et al*. (2014) proposed a phylogeny ("1KITE Phylogeny") robust enough for future comparative analyses of hexapod evolution to rely on. In it, the basal splitting of Hexapoda was reconstructed as: "Hexapoda = Ellipura (= Protura + Collembola) + Cercophora (= Diplura + Ectognatha)".

The present study was made in order to figure out the groundplan of Collembola, using *Tomocerus cuspidatus*, focusing on the development of embryonic membranes and their developmental potentials as well as the formation of entognathy. On the 1KITE Phylogeny, I will map the results discussed in the above sections on the embryonic membranes and entognathy (Fig. 38). In the section "4. 6. 2. Formation of entognathy in Collembola and other two entognathan orders", I concluded that: 1) Collembola and Protura share the "entognathy of ellipuran type" as the autapomorphy of their assemblage "Ellipura", and 2) the entognathy of Diplura, which should be discriminated from the "entognathy of ellipuran type", is a autapomorphy of the group "entognathy of dipluran type". In the 1KITE Phylogeny the monophylies of both Ellipura and Diplura are ensured, and the "entognathy of ellipuran type" and entognathy of dipluran type" are correctly put on the lineages of Ellipura and Diplura as their autapomorphies, respectively (open stars in Fig. 38).

In the section "4. 5. 2. Dorsal closure and participation of serosa to it", I re-examined the developmental potential of serosa in Collembola. I concluded that the serosa of Collembola maintains ability to differentiate into body elements. In the section "4. 5. 3. Evolutionary changes of the embryonic

membranes and their developmental potentials in Hexapoda", I attributed the trait "loss of ability in serosa for differentiating into body elements" in addition to the assemblage of Diplura and Ectognatha as another synapomorphy, reinforcing the monophyly of Cercophora, which has been suggested by another comparative embryological trait "differentiation of amnion" (*e.g.*, Machida and Ando, 1998; Machida *et al.*, 2002; Machida, 2006). The 1KITE Phylogeny ensures the sister group relationship of Diplura and Ectognatha, *i.e.*, suggesting the monophyly of Cercophora, which is correctly backed by the comparative embryological autapomorphy newly suggested or the "loss of ability in serosa for differentiating into body elements" (solid star in Fig. 38) in addition to another comparative embryological autapomorphy "differentiation of amnion" which has been hitherto proposed from the hexapod comparative embryology (asterisk in Fig. 38).

Reconstructing the groundplan of each group and its evolutionary changes, I have critically examined the embryological features of Collembola and other groups of hexapods. As addressed above, the conclusions deduced from the comparative embryology were correctly followed and mapped on the 1KITE Phylogeny. This strongly suggests the validity and legitimacy of the critical comparative embryological approach. In turn, the 1KITE Phylogeny has got one more, additional support from the comparative embryology.

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7. TABLE

Table 1. Major developmental events in each stage in *Tomocerus cuspidatus*.

Stages	Developmental events
Stage 1	Cleavage, formation of yolk cells and primordial germ cells
Stage 2	Formation of blastoderm, differentiation of mesoderm
Stage 3	Formation of primary dorsal organ
Stage 4	Secretion of blastoderm cuticles, commencement of differentiation of embryo and serosa
Stage 5	Differentiation of cephalic segments and appendages
Stage 6	Rupture of chorion, differentiation of thoracic segments and appendages, elongation of cephalic appendages
Stage 7	Blastokinesis, differentiation of abdominal segments and appendages
Stage 8	Completion of blastokinesis, commencement of definitive dorsal closure
Stage 9	Completion of six paired ommatidia, completion of appendage articulation
Stage 10	Elongation of third and fourth antennal segments, eye regions deeply pigmented
Stage 11	Completion of definitive dorsal closure, acquisition of definitive form by embryo, secretion of larval cuticle

8. FIGURES

Fig. 1. An adult of *Tomocerus cuspidatus* Börner, 1909. Dorsal view.

An1-4: first to fourth antennal segments, Th1L: foreleg.

Scale bar = 1 mm.

Fig. 2. Rearing case for *Tomocerus cuspidatus*.

Scale bar = 5 cm.

An4
An3
An2
Th1L
An1
1

2

Fig. 3. Eggs of *Tomocerus cuspidatus*. A. An egg just after oviposition. B. An egg 17 days after oviposition.

BdC: blastoderm cuticle, Ch: chorion; S: spine.

Scale bars = 100 µm.

3A

Ch
BdC
S
3B

Fig. 4. Successive stages of embryonic development of *Tomocerus cuspidatus*. Lateral views, DAPI-staining. A. Stage 1. B. Stage 2. C. Stage 3. D. Stage 4. E. Stage 5. F. Early Stage 6. G. Late Stage 6. H. Stage 7. I. Stage 8. J. Stage 9. K. Stage 10. L. Stage 11. An asterisk in Figure 4E shows the blastoderm yet to differentiate into the embryonic and extra-embryonic areas. Arrows show the position of primary dorsal organs. Black and white arrowheads show the cephalic and caudal ends of embryo, respectively.

1-6: first to sixth abdominal segments, An: antenna, Bd: blastoderm, Bm: blastomere, Cllr: clypeolabrum, E: eye, Em: embryo, Fu: furca, HC: head capsule, HL: head lobe, Lb: labium, Lr: labrum, Md: mandible, Mx: maxilla, Pd: proctodeum, PDO: primary dorsal organ, Se: serosa, Te: tenaculum, Th1L: foreleg.

Scale bar = 200 µm.

4A
Bm
4B
Bd
4C
PDO
Bd
4D
Bd
4E
HL
Cllr
Em
An
Md
Mx
Lb
Se
*
4F
Cllr
Em
Md
Se
4G
HL
Cllr
Em
An
Md
Mx
Lb
Th1L
Se
4H
HL
Th1L
Se
Cllr
An
Md
Te
2
1
Pd
4I
HL
Th1L
1
2
3
4
5
An
Lr
Fu
6
Se
4J
HC
Th1L
E
1
2
3
4
An
4K
HC
Th1L
E
1
2
3
4
An
6
5
4L
HC
Th1L
E
1
2
3
4
An

Fig. 5. A. A transverse section of an egg of *Tomocerus cuspidatus* in Stage 2. B. Enlargement of boxed area in Figure 5A, showing a mesodermal cell undergoing a radial division. C. A section through a different plane on the same egg as in Figure 5A. Arrow represents a blastoderm cell undergoing a tangential division to form the mesoderm. Arrowheads represent mesodermal cells, black ones showing the mesodermal cells undergoing radial division.

Bd: blastoderm, Ch: chorion, PGC: primordial germ cell, Y: yolk, YC: yolk cell.

Scale bars = A: 50 µm, B,C: 10 µm.

Ch
Bd
YC
Y
PGC
5A

Bd
Y
5B

Bd
Y
5C

Fig. 6. A transverse section of an egg of *Tomocerus cuspidatus* in Stage 3.

Arrowheads represent mesodermal cells.

Bd: blastoderm, Ch: chorion, PDO: primary dorsal organ, PGC: primordial germ cell, Y: yolk, YC: yolk cell.

Scale bar = 50 µm.

PDO
Ch
Bd
YC
Y
PGC
6

Fig. 7. Sections of eggs of *Tomocerus cuspidatus*. A. A horizontal section of an egg under secretion of the first blastoderm cuticle in early Stage 4. A'. Enlargement of boxed area in Figure 7A, showing the protrusion of blastoderm. B. A transverse section of an egg under secretion of the second blastoderm cuticle in late Stage 4. B'. Enlargement of boxed area in Figure 7B, showing the filamentous outgrowth of primary dorsal organ or tendrils. Arrows represent filamentous outgrowth of primary dorsal organ.

Bd: blastoderm, BdC1 and 2: first and second blastoderm cuticles, Ch: chorion, Me: mesoderm, PDO: primary dorsal organ, PGC: primordial germ cell, Y: yolk, YC: yolk cell.

Scale bars = A, B: 100 µm, A': 10 µm, B': 20 µm.

BdC1
Ch
Y
Bd
YC
7A
BdC1
Ch
Me
Bd
Y
7A'
PDO
Ch
BdC1
Bd
YC
Y
PGC
7B
Ch
BdC1
PDO
Bd
BdC2
Y
7B'

Fig. 8. Transverse sections of an egg of *Tomocerus cuspidatus* in late Stage 4.

A. A section through the posterior gnathal region. B. A section through a plane a little posterior to that shown in Figure 8A. Arrowheads represent mesodermal cells.

Bd: blastoderm, Ec: ectoderm, EEmA: extra-embryonic area, EmA: embryonic area, PDO: primary dorsal organ, PGC: primordial germ cell, Se: serosa, Y: yolk, YC: yolk cell.

Scale bars = 50 μm.

Se
PDO
YC
EEmA
Y
EmA
EEmA
EmA
PGC
Ec
8A

Bd
YC
Y
PGC
8B

Fig. 9. Embryos of *Tomocerus cuspidatus* in late Stage 6. A. Lateral view, DAPI-staining. An arrow shows primary dorsal organ. B. Ventral view, SEM.

Ab: abdomen, An: antenna, Cllr: clypeolabrum, HL: head lobe, InA: intercalary appendage, LbCp: labial coxopodite, LbP: labial palp, Md: mandible, MxCp: maxillary coxopodite, MxP: maxillary palp, Se: serosa, Sd: stomodaeum, Th1, 2Cp: coxopodites of fore- and midleg, Th1, 2Tp: telopodites of fore- and midleg, Th3L: hindleg.

Scale bars = 100 µm.

Se
Cllr
Ab
HL
Sd
InA
An
Md
MxP
LbP
LbCp
Th1Tp
Th1Cp
Th2Tp
Th2Cp
Th3L
9A

LbP
LbCp
An
InA
Cllr
Sd
Md
MxP
MxCp
Th1Tp
Th2Tp
9B

Fig. 10. A. A transverse section of an egg of *Tomocerus cuspidatus* in late Stage 6 through the labial and abdominal segments. Chorion and blastoderm cuticles removed. B. Enlargement of serosa in Figure 10A. C. Another enlargement of Figure 10A. Black and white arrowheads show the newly differentiated serosal cells and attenuated ones, respectively.

CS: coelomic sac, Ec: ectoderm, Lb: labium, LbG: labial ganglion, Me: mesoderm, MMe: median mesoderm, Nb: neuroblast, PGC: primordial germ cell, Y: yolk, YC: yolk cell.

Scale bars = A, C: 50 µm; B: 20 µm.

Me
Ec
YC
Y
PGC
Lb
CS
LbG
MMe
Nb
10A
Ec
Y
10B
MMe
Y
Lb
CS
Nb
LbG
10C

Fig. 11. Successive stages of blastokinesis in *Tomocerus cuspidatus*, Stage 7 (A-D; D' is an enlargement of D, showing the antennal annulation). Lateral views, DAPI-staining. Arrows show the position of primary dorsal organs.

1-6: first to sixth abdominal segments, Ab: abdomen, An: antenna, An1-4: first to fourth antennal segments, Cllr: clypeolabrum, Fu: furca, HL: head lobe, InA: intercalary appendage, LbP: labial palp, Md: mandible, MxP: maxillary palp, Pd: proctodaeum, Sba: subanal lobe, Se: serosa, Spa: supraanal lobe, Te: tenaculum, Th1-3L: fore-, mid- and hindleg, VT: ventral tube.

Scale bars = A-D: 100 μm; D': 50 μm.

HL
Se
Pd
Cllr
Ab
InA
Md
An
MxP
LbP
Th1L
VT
1
11A
Th1L
Se
HL
Cllr
InA
An
Md
Te
3
2
1
Pd
11B
Th1L
HL
1
2
Cllr
An
Md
3
4
Fu
Se
Pd
11C
Th1L
HL
An
1
2
Cllr
Md
3
4
Fu
Sba
Pd
Spa
6
5
Se
11D
Th1L
Th2L
An1
An2
An3
Th3L
An4
Cllr
Fu
11D'

Fig. 12. A. A sagittal section of an egg of *Tomocerus cuspidatus* in late Stage 7. Chorion and blastoderm cuticles removed. B. Enlargement of boxed area in Figure 12A, showing the serosal cell undergoing mitosis. White arrowheads show the serosal cells which have been strongly attenuated. Black arrowheads show the serosal cell undergoing mitotic division.
6: sixth abdominal segment, AbG: abdominal ganglia, BlC2: second blastoderm cuticle, Fu: furca, Pc: protocerebrum, PDO: primary dorsal organ, PGC: primordial germ cell, SoG: suboesophageal ganglion, ThG: thoracic ganglia, Y: yolk, YC: yolk cell.
Scale bars = A: 50 µm; B: 10 µm.

YC
PDO
Pc
ThG
SoG
Y
AbG
Fu
6
BIC2
PGC
12A
Y
12B

Fig. 13. A. An embryo of *Tomocerus cuspidatus* in Stage 8. Lateral views, DAPI-staining. An arrow shows the position of primary dorsal organ. B. Enlargement of hindleg in Figure 13A.

Ab1-6T: terga of the first to sixth abdominal segments, An: antenna, Cp: coxopodite, Fe: femur, Fu: furca, HL: head lobe, LbT: labial tergum, Lr: labrum, Th1L: foreleg, Th1-3T: terga of the first to third thoracic segments, Tr: trochanter, Tta: tibiotarsus.

Scale bars = 100 µm.

Fig. 14. A. An embryo of *Tomocerus cuspidatus* in Stage 9. Lateral views, DAPI-staining. An arrow shows the position of primary dorsal organ. B. Enlargement of hindleg in Figure 14A.

Ab1-5T: terga of the first to fifth abdominal segments, An: antenna, Cx: coxa, E: eye, Fe: femur, HC: head capsule, LbT: labial tergum, Scx: subcoxa, Th1L: foreleg, Th1-3T: terga of the first to third thoracic segments, Tr: trochanter, Tta: tibiotarsus.

Scale bars = 100 µm.

LbT
Th1T
Th2T
Th3T
HL
Ab1T
An
Ab2T
Lr
Fu
Ab3T
Ab6T
Ab5T
Ab4T
13A

Th2T
Th3T
Cp
Tr
Fe
Tta
13B

Th1T
LbT
Th2T
HC
Th3T
E
Ab1T
An
Ab2T
Ab3T
Th1L
Ab4T
Ab5T
14A

Th2T
Th3T
Scx
Cx
Tr
Fe
Tta
14B

Fig. 15. An embryo of *Tomocerus cuspidatus* in Stage 10. Lateral view, DAPI-staining. An arrow shows the position of primary dorsal organ. Ab1-6T: terga of the first to sixth abdominal segments, An1-4: first to fourth antennal segments, E: eye, Fu: furca, HC: head capsule, LbT: labial tergum, Th1L: foreleg, Th1-3T: terga of the first to third thoracic segments.
Scale bar = 100 µm.

Fig. 16. An embryo of *Tomocerus cuspidatus* in Stage 11. Lateral view, DAPI-staining. An arrow shows the position of primary dorsal organ. Ab1-4T: terga of the first to fourth abdominal segments, An1-4: first to fourth antennal segments, E: eye, HC: head capsule, LbT: labial tergum, Th1L: foreleg, Th1-3T: terga of the first to third thoracic segments.
Scale bar = 100 µm.

Fig. 17. A sagittal section of an embryo of *Tomocerus cuspidatus* in Stage 11. An arrow shows the filamentous outgrowths of primary dorsal organ yet to degenerate in the final stage of dorsal closure.
Fg: foregut, H: heart, LaC: larval cuticle, LbT: labial tergum, M: musculature, MgE: midgut epithelium, Pc: protocerebrum, PDO: primary dorsal organ, Th1G: ganglion of the first thoracic segment, Th1, 2T: terga of the first and second thoracic segments.
Scale bar = 50 µm.

LbT
Th1T
Th2T
HC
E
Th3T
An1
Ab1T
An2
Ab2T
An3
Th1L
An4
Ab3T
Fu
Ab4T
15
Ab6T
Ab5T

HC
LbT
Th1T
Th2T
E
Th3T
An1
Ab1T
An2
An3
Ab2T
Th1L
Ab3T
An4
16
Ab4T

Fg
LbT
Th1T
H
MgE
LaC
Th2T
M
PDO
Pc
17
Th1G

Fig. 18. The first (A), second (B), third (C) and fourth (D) instar larvae of *Tomocerus cuspidatus*. Dorsal views.

An1-4: first to fourth antennal segments, Th1L: foreleg.

Scale bar = 500 µm.

An4
An3
An2
Th1L
18A
An4
An1
An3
An2
Th1L
18B
An4
An3
An1
An2
Th1L
18C
An4
An3
An1
An2
Th1L
18D

Fig. 19. Dorsal closure of *Tomocerus cuspidatus*. DAPI-staining. Dorsal views of the second thoracic to the fourth abdominal segments of embryos in Stage 8 (A), Stage 9 (B), Stage 10 (C), and Stage 11 (D), respectively. Arrows show the position of primary dorsal organ.

Ab1-4T: terga of the first to fourth abdominal segments, SeC: serosal cell, Th2, 3T: terga of the second and third thoracic segments.

Scale bar = 100 µm.

Th2T
SeC
Th3T
Ab1T
Ab2T
19A
Ab3T
Th2T
SeC
Th3T
Ab1T
Ab2T
19B
Th2T
SeC
Th3T
Ab1T
Ab2T
Ab3T
19C
Ab4T
Th2T
Th3T
Ab1T
Ab2T
Ab3T
Ab4T
19D

Fig. 20. Transverse sections of dorsal regions of the embryos of *Tomocerus cuspidatus* in Stage 8 to Stage 11. A. The first abdominal segment of an embryo in Stage 8. B. The first abdominal segment of an embryo in Stage 9. C. The first thoracic segment of an embryo in Stage 10. D, D'. The first (D) and second (D') thoracic segments of an embryo in Stage 11. An arrow show the serosal cell located in the lateralmost portion of serosa. Arrowheads show the medialmost dorsal epidermal cells or the cells at the frontier of dorsal closure.

Ab1T: tergum of the first abdominal segment, BlC2: second blastoderm cuticle, H: heart, M: musculature, MgE: developing midgut epithelium, PDO: primary dorsal organ, Se: serosa, Th1T, 2T: terga of the first and second thoracic segments, Y: yolk.

Scale bar = 10 μm.

MgE
Se
BIC2
Y
Se
20A
Se
Ab1T
Y
20B
MgE
Se
Th1T
Y
20C
MgE
Th1T
PDO
M
20D
Th2T
H
MgE
Y
M
20D'

Fig. 21. A. A transverse section through the dorsal part of the first abdominal segment of an embryo of *Tomocerus cuspidatus* in Stage 9. TEM. B. Enlargement of boxed area in Figure 21A. The black arrowhead shows the overlapping of the epidermal cell onto the serosal cell.

Ab1T: tergum of the first abdominal segment, MgE: developing midgut epithelium, Se: serosa, Y: yolk.

Scale bars = A: 10 µm; B: 5 µm.

Se
Ab1T
Ab1T
MgE
Y
21A

Se
Ab1T
Y
MgE
21B

Fig. 22. 3D reconstructions of embryos of *Tomocerus cuspidatus* during the dorsal closure. A-A”. Embryo in the initial stage of dorsal closure, Stage 8. Dorsolateral view of the cephalic to the second thoracic segments (A), the second thoracic to the second abdominal segments (A’), and the second to sixth abdominal segments (A”). B-B”. Embryo in the late stage of dorsal closure, Stage 10. Dorsolateral view of the cephalic to the third thoracic segments (B), the second thoracic to the second abdominal segments (B’), and the first to fourth abdominal segments (B”). Serosa, its nuclei, primary dorsal organ, and embryo are shown in yellow, red, green, and blue, respectively.

Ab1-6T: terga of the first to sixth abdominal segments, HC: head capsule, HL: head lobe, LbT: tergum of the labial segment, PDO: primary dorsal organ, Th1-3T: terga of the first to third thoracic segments.

HL
PDO
Th1T
Th2T
22A
Th2T
Th3T
Ab1T
Ab2T
22A'
Ab2T
Ab3T
Ab4T
Ab5T
Ab6T
22A"
HC
PDO
LbT
Th1T
Th2T
Th3T
22B
PDO
Th2T
Th3T
Ab1T
Ab2T
22B'
Ab1T
Ab2T
Ab3T
Ab4T
22B"

Fig. 23. Development of cephalic region of *Tomocerus cuspidatus*. Lateral views. Antennae omitted. Stage 7 (A), Stage 8 (B), Stage 9 (C), Stage 10 (D). An arrowhead shows the posterior tentorial pit.

An: antenna, Cl: clypeus, Cllr: clypeolabrum, E: eye, HC: head capsule, HL: head lobe, InA: intercalary appendage, In-MxT: intercalary to maxillary terga, La: lacinia, LbCx: labial coxa, LbP: labial palp, LbScx: labial subcoxa, LbT: labial tergum, Lr: labrum, Md: mandible, MF: mouth fold MxCx: maxillary coxa, MxP: maxillary palp, MxScx: maxillary subcoxa, Prm: prementum, Sli: superlingua.

Scale bar = 50 μm.

23A

23B

23C

23D

Fig. 24. Development of cephalic region of *Tomocerus cuspidatus*. Ventral views, Stage 7 (A), Stage 8 (B), Stage 9 (C), Stage 10 (D). Arrowheads show the invagination of labial glands.

Cllr: clypeolabrum, Ga: galea, Gl: glossa, Ic: incisor, La: lacinia, LbCx: labial coxa, LbP: labial palp, LbScx: labial subcoxa, LbT: labial tergum, Li: lingua, Lr: labrum, Mo: molar, MxP: maxillary palp, Pgl: paraglossa, Prm: prementum, Sd: stomodaeum, Sli: superlingua.

Scale bar = 50 μm.

Ic
Ga
MxP
LbP
Pgl
Mo
Gl
Sli
La
Cllr
Sd
24A

LbCx
LbScx
LbP
LbT
Pgl
La
Ic
Gl
Sli
Li
Lr
24B

LbScx
LbCx
LbP
LbT
Pgl
Gl
Sli
Li
Lr
24C

Prm
LbP
MxP
LbT
Pgl
Gl
Sli
Li
Lr
Ga
24D

Fig. 25. A. Cephalic region of an embryo in early Stage 7. Lateral views, DAPI-staining. Antenna removed. B. Enlargement of boxed area in Figure 25A. B'. The same frame as Figure 25B, showing the boundaries of intercalary, mandibular, maxillary and labial terga by dotted lines. An: antenna, Cllr: clypeolabrum, HL: head lobe, InA: intercalary appendage, InT: intercalary tergum, LbCx: labial coxa, LbP: labial palp, LbScx: labial subcoxa, LbT: labial tergum, Md: mandible, MdT: mandibular tergum, MxCx: maxillary coxa, MxP: maxillary palp, MxScx: maxillary subcoxa, MxT: maxillary tergum, PDO: primary dorsal organ.
Scale bars = 50 μm.

Fig. 26. A. Cephalic region of an embryo in middle Stage 7. Lateral views, DAPI-staining. Antenna removed. B. Enlargement of boxed area in Figure 26A. B'. The same frame as Figure 26B, showing the boundaries of intercalary, mandibular, maxillary and labial terga by dotted lines. An: antenna, Cllr: clypeolabrum, HL: head lobe, InT: intercalary tergum, LbP: labial palp, LbT: labial tergum, Md: mandible, MdT: mandibular tergum, MF: mouth fold, MxCx: maxillary coxa, MxP: maxillary palp, MxT: maxillary tergum, PDO: primary dorsal organ, Th1-3L: fore-, mid- and hindleg, Th1T: tergum of the first thoracic segment.
Scale bars = 50 μm.

25A
HL
PDO
Cllr
An
InA
LbScx
LbCx
Md
MxScx
MxP
MxCx
LbP
25B
HL
LbT
MxT
InT
MdT
LbScx
LbCx
Md
MxScx
25B'
HL
LbT
MxT
InT
MdT
LbScx
LbCx
Md
MxScx
26A
PDO
HL
Th1T
An
Cllr
Th2L
Th3L
Md
MF
MxCx
MxP
Th1L
26B
LbT
HL
MdT
MxT
InT
Th1L
Cllr
MF
LbP
26B'
LbT
HL
MdT
MxT
InT
Th1L
Cllr
MF
LbP

Fig. 27. Cephalic region of an embryo of *Tomocerus cuspidatus* in middle Stage 7. SEM. Antennae removed. A. Lateral view. B. ventral view. C. Posterior view of labium.

An: antenna, Cllr: clypeolabrum, Ga: galea, Gl: glossa, HL: head lobe, Ic: incisor, InA: intercalary appendage, In-MxT: intercalary to maxillary terga, La: lacinia, LbCx: labial coxa, LbP: labial palp, LbScx: labial subcoxa, LbT: labial tergum, Md: mandible, MF: mouth fold, Mo: molar, MxCx: maxillary coxa, MxP: maxillary palp, MxScx: maxillary subcoxa, Pgl: paraglossa, Sd: stomodaeum, Sli: superlingua, Th1L: foreleg.

Scale bars = 50 μm.

In-MxT
HL
LbT
An
Cllr
Th1L
LbSx
InA
LbCx
MF
LbP
Md
MxScx
MxCx
MxP
27A

MxP
LbP
Ic
Mo
Ga
Pgl
La
Cllr
Gl
Sd
Sli
27B

LbSx
LbCx
Gl
Pgl
LbP
La
Ga
Ic
MxP
27C

Fig. 28. Cephalic region of an embryo of *Tomocerus cuspidatus* in Stage 8. SEM. Antennae removed. A. Lateral view. B. Posterolateral view. C. Ventral view. D. Posterior view of labium. Black and white arrowheads show the invagination of labial gland and the frontier of extending labial terga, respectively.

An: antenna, Cl: clypeus, Ga: galea, Gl: glossa, HL: head lobe, Ic: incisor, In-MxT: intercalary to maxillary terga, La: lacinia, LbCx: labial coxa, LbP: labial palp, LbScx: labial subcoxa, LbT: labial tergum, Li: lingua, Lr: labrum, Md: mandible, MF: mouth fold, MxP: maxillary palp, Pgl: paraglossa, Sli: superlingua, Th1L: foreleg.

Scale bars = 50 μm.

In-MxT
LbT
HL
An
Th1L
Cl
LbCx
Lr
Md
MF
MxP
LbP
28A
In-MxT
LbT
Th1L
MF
Md
LbScx
LbCx
MxP
LbP
Pgl
Gl
28B
LbP
Ic
An
Ga
Pgl
La
Gl
Lr
Li
Sli
MxP
28C
LbT
LbScx
LbCx
Pgl
LbP
Gl
28D

Fig. 29. Postgnathal region of embryos of *Tomocerus cuspidatus*. SEM. A. The embryo in early Stage 9. B. The embryo in late Stage 9. Black and white arrowheads show the invagination of labial gland and the frontier of medially extending labial terga, respectively.

An: antenna, Gl: glossa, LbCx: labial coxa, LbP: labial palp, LbScx: labial subcoxa, LbT: labial tergum, Li: lingua, Lr: labrum, Pgl: paraglossa, Th1L: foreleg, Th1S: sternum of the first thoracic segment. Scale bars = 50 µm.

Gl
Pgl
Lr
LbP
Li
LbCx
LbT
LbT
Th1L
LbScx
29A
Th1S

An
Pgl
Gl
LbP
Lr
LbCx
Li
LbT
LbT
Th1L
Th1S
29B

Fig. 30. Transverse sections of embryos *Tomocerus cuspidatus* through the base of labium. A. An embryo in early Stage 9. Light microscopy. White arrowheads show the frontier of medially extending labial terga. B. An embryo in late Stage 9. TEM.

CDLG: common duct of labial gland, DLG: duct of labial gland, Hyp: hypopharynx, LbScx: labial subcoxa, LbT: labial tergum, VT: ventral tube.

Scale bars = 10 µm.

DLG
Hyp
DLG
LbT
30A
LbScx
LbT
Hyp
CDLG
LbT
LbT
VT
VT
30B

Fig. 31. Cephalic region of an embryo of *Tomocerus cuspidatus* in Stage 10. SEM. Antenna removed. A. Lateral view. A dotted line represents dorsal limit of the mouth fold. B. Ventral view. Low vacuum SEM of the same specimen as that shown in Fig. 31A, before ion-sputtering. Arrows show the developing ventral groove. Black and white arrowheads indicate the anterior and posterior tentorial pits, respectively.
An: antenna, CDLG: common duct of labial gland, Cl: clypeus, Gl: glossa, HC: head capsule, In-MxT: intercalary to maxillary terga, LbP: labial palp, LbT: labial tergum, Lr: labrum, MF: mouth fold, MxP: maxillary palp, Pgl: paraglossa, Prm: prementum, Th1L: foreleg.
Scale bars = 50 µm.

In-MxT
HC
LbT
An
Cl
Th1L
An
LbT
Lr
MxP
MF
Prm
31A

Prm
LbP
Pgl
Th1L
Gl
LbT
Lr
CDLG
LbT
An
Th1L
31B

Fig. 32. Sections of the cephalic region of embryos of *Tomocerus cuspidatus* in Stage 10. A. A transverse section through the antennal base, showing the anterior tentorial pit. B. A horizontal section through the plane including the posterior tentorial pit. Black and white arrowheads indicate the anterior and posterior tentorial pits, respectively.

An: antenna, Cl: clypeus, GP: gnathal pouch, LbGl: labial gland, LbT: labial tergum, MAn: musculature of antenna, Md: mandible, MF: mouth fold, MMx: musculature of maxilla.

Scale bars = 10 µm.

An
MAn
MF
Cl
GP
Md
32A

LbT
MMx
LbGl
LbT
32B

Fig. 33. A first instar larva of *Tomocerus cuspidatus*. SEM. A. Cephalic region. Lateral view. B. Cephalic and thoracic regions. Ventral view. Arrows show ventral groove.

An: antenna, Cl: clypeus, E: eye, HC: head capsule, In-MxT: intercalary to maxillary terga, LbP: labial palp, LbT: labial tergum, Lr: labrum, MF: mouth fold, MxP: maxillary palp, Prm: prementum, Th1-3L: fore-, mid- and hindleg, VT: ventral tube.

Scale bars = 50 μm.

HC
In-MxT
LbT
E
An
Th1L
Cl
MF
Lr
LbT
Prm
33A
MxP
LbP

Prm
LbT
Th2L
Th3L
Th1L
An
VT
33B

Fig. 34. A section of a first instar larva of *Tomocerus cuspidatus* through the antennal base, showing the anterior tentorium. Arrowhead and black and white arrows indicate the anterior tentorial pit and two branches of the anterior tentorium, respectively.

An: antenna, AT: anterior tentorium; GP: gnathal pouch, Md: mandible, MF: mouth fold, Sd: stomodaeum.

Scale bar = 10 µm.

Fig. 35. Sections of a first instar larva of *Tomocerus cuspidatus* through the plane including the posterior tentorial pit (A) and that including the maxillary base, slightly anterior to Figure 35A (B), showing the posterior tentorium. The arrowhead and asterisks indicate the posterior tentorial pit and ducts of the labial gland, respectively.

BGP: blind end of gnathal pouch, LbGl: labial gland, LbT: labial tergum, MMx: musculature of maxilla, MxT: maxillary tergum, PT: posterior tentorium.

Scale bars = 10 µm.

An
AT
GP
Sd
MF
Md
34

MMx
MxT
LbGl
LbT
35A

MMx
MxT
BGP
PT
LbGl
LbT
35B

Fig. 36. 3D reconstructions of the head of a first instar larva of *Tomocerus cuspidatus*. A. Lateral view. A'. The same frame as Figure 36A. Head capsule, antennae, mandibles, maxillae, prementum, labial glands and hypopharynx are made partly transparent to show anterior and posterior tentoria. B. Dorsal view. Head capsule, antennae, mandibles, maxillae, prementum, labial glands and hypopharynx are made partly transparent to show anterior and posterior tentoria. Anterior and posterior tentoria are shown as yellow and green, respectively. Black and white arrows show two branches of the anterior tentoria. Black and white arrowheads indicate the anterior and posterior tentorial pits, respectively.
An: antenna, AT: anterior tentorium, Cl: clypeus, HC: head capsule, Lr: labrum, MF: mouth fold, MxP: maxillary palp, Prm: prementum, PT: posterior tentorium.

HC
An
Cl
MF
Lr
MxP
Prm
36A

HC
AT
An
Cl
Lr
MxP
PT
Prm
36A’

HC
PT
AT
Cl
AT
PT
36B

Fig. 37. Diagrams representing the construction of entognathy in three entognathan orders, Collembola (A: lateral, A’: ventral), Protura (B: lateral, B’: ventral), and Diplura (C: lateral, C’: ventral). Colorations show the different origins of the parts – red: intercalary tergum, yellow: mandibular tergum, blue: maxillary tergum, green: labial tergum, orange: labial subcoxa, purple: labial coxa. Blue, green and red stars represent the glossa, paraglossa and labial palp, respectively. See the text.

Adm: admentum, An: antenna, E: eye, Lr: labrum, Md: mandible, Mx: maxilla, Pm: postmentum, Prm: prementum.

E
An
Lr
37A
Md
Mx
Prm
An
Lr
37A'
Lr
37B
Md
Mx
Prm
Lr
37B'
An
Pm
Lr
Adm
37C
Md
Mx
Prm
Pm
An
Lr
Adm
37C'

Fig. 38. Mapping the comparative embryological traits concerning on the embryonic membranes and entognathy, on the 1KITE Phylogeny (Misof *et al*., 2014). See the text.

Crustacea / Myriapoda
Entognathy of ellipuran type
Collembola
Entognatha
ELLIPURA
Protura
Hexapoda
Entognathy of dipluran type
Nonoculata
Diplura
CERCOPHORA
Ectognatha
Loss of ability in serosa
for differentiating
into body elements
Differentiation of amnion

38