

Evidence from Embryology for Reconstructing the Relationships of Hexapod Basal Clades

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> Abstract

Hexapod basal clades are discussed and their relationships reconstructed based on comparative embryological evidence. Monophylies of Diplura, Dicondylia and Ectognatha is strongly supported but no embryological evidence supports monophyly of the Entognatha. The developmental potential of the embryonic membrane (it forms part of the dorsal body wall) suggests that proturans may be basal to all other hexapods.

> Key words

Comparative embryology, embryonic membranes, entognathy, Hexapoda, Protura, Collembola, Diplura, Entognatha, Ectognatha, Dicondylia.

1. Introduction

The phylogenetic relationships of hexapod higher taxa have been much debated based on recent evidence from multiple sources, but are still not satisfactorily resolved. For example, doubt has been cast on the reality of the Entognatha-Ectognatha system, especially regarding the status of Entognatha, by KUKALOVÁ-PECK (1987), KOCH (1997), and KRAUS (1998) and by new embryological evidence (IKEDA & MACHIDA 1998).

Protura, although generally dealt with as one of the hexapod basal clades (e.g., KRISTENSEN 1975; BOUDREAUX 1979), have been often regarded as representing a side branch remote from the main line of hexapod evolution (e.g., SHAROV 1966; MANTON 1977), and are thus important in reconstruction of the relationships of hexapod basal clades and elucidation of the origin of hexapods. Furthermore, in recent years some evidence has emerged suggesting a closer affinity between Myriapoda and Chelicerata on the one hand (or of particular lineages within both taxa) and Hexapoda and Crustacea on the other (e.g., AGUINALDO et al. 1997; HWANG et al. 2001; SAKUMA & MACHIDA 2002), so that the long accepted monophyletic status of Atelocerata (= Myriapoda + Hexapoda) and Mandibulata (= Atelocerata + Crustacea) has become

questionable. With this background, a study of the embryology in Protura is desirable more than ever. Our former total ignorance of proturan embryogenesis, in spite of many attempts, is due to the difficulty in rearing proturans. Recently, we have succeeded in rearing them (MACHIDA & TAKAHASHI 2003, 2004) and are currently following their embryonic development. Our ongoing study (FUKUI & MACHIDA in press) has revealed some intriguing features of the embryogenesis of *Baculentulus densus*.

Here, we review recent observations on the embryogenesis of representative species of the basal hexapod clades, and discuss their phylogenetic significance.

2. Embryonic development of Protura

Eggs of *Baculentulus densus* (Imadaté) are spherical to spheroidal with a long axis of about 120 μm and with numerous, variously sized and shaped protuberances scattered over their surfaces (Fig. 1A). Fig. 1B shows an egg in the 4-cell stage. A cell boundary (arrow in Fig. 1B) running along the equator is observed. Cleavage in proturans is holoblastic or

total. Cleavage nuclei proliferate and migrate to the egg surface to form a cellular layer, the blastoderm (Fig. 1C), which secretes a cuticular egg envelope or the blastoderm cuticle on the egg surface soon after its differentiation. The blastoderm becomes more densely cellular, followed by regional differentiation into a long, broad embryonic area and a restricted extraembryonic area or serosa (Fig. 1D). The embryo then begins to segment. In the embryo shown in Fig. 1E, the mandibular, maxillary, labial, thoracic and first abdominal segments are differentiated. Note that the serosal area (Se in Fig. 1E) adjacent to the embryo differentiates into tergum or dorsal body wall (Tg in Fig. 1E); i.e., the primary embryonic membrane (serosa) in proturans retains the ability to participate in definitive dorsal closure and formation of dorsal body wall.

With progressive segmentation, the embryo elongates until its cephalic and caudal ends almost meet (cf. Fig. 1F). Then, blastokinesis occurs. The embryo shown in Fig. 1F has started to bend upward between the thorax and abdomen as shown by the arrow. As blastokinesis continues, the thoracic and abdominal regions further bend dorsally and the caudal end of the embryo recedes from its original location (Fig. 1G), to finally take a position facing the cephalic region (cf. Fig. 1H). Fig. 3 is a sagittal section of an embryo at approximately the same stage as that shown in Fig. 1G. A clump of cells on the dorsum of the embryo next to its cephalic end is the primary dorsal organ (we had failed to discern this structure at the time of my presentation at the 2nd Dresden Meeting). It degenerates soon after blastokinesis. As in pauropod embryos (TIEGS 1947), the primary dorsal organ in proturans seems to be less developed compared to well-developed ones in collembolan (TIEGS 1942a; JURA 1972), dipluran (UZEL 1898; TIEGS 1942b; IKEDA & MACHIDA 2001) and symphyllan embryos (TIEGS 1940).

The embryo continues to grow, its appendages develop strongly (Fig. 1H), and its mouth folds start to form. Though details are still sketchy, the mouth folds in proturans seem to derive from the mandibular, maxillary and labial terga. As the embryo

develops further and elongates, it assumes a twisted configuration (Fig. 2A), and after a little while the prelarva hatches (Fig. 2B).

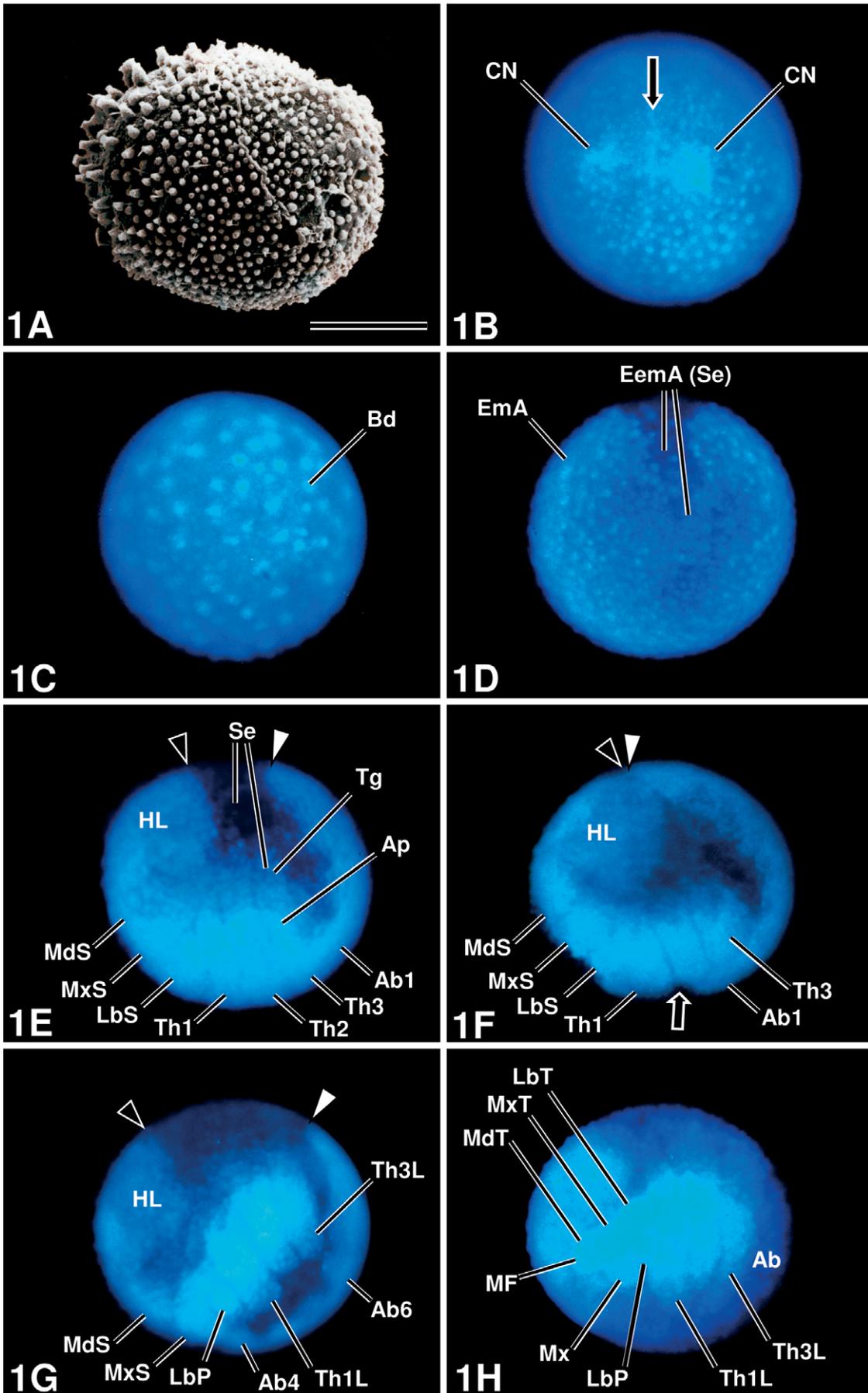
The general features of embryogenesis in this proturan are similar to those in the likewise entognathous collembolans (cf. JURA 1972) and diplurans (cf. IKEDA & MACHIDA 1998, 2001) in that (1) the embryo is long-germ, (2) a simple blastokinesis involving only a minor change of posture occurs, (3) a primary dorsal organ forms, and (4) the embryo participates in and fulfills together with the serosa the secretion of a cuticular egg envelope or blastoderm cuticle. On the other hand, in proturan embryogenesis, the serosa is able to differentiate into dorsal body wall – utterly different from the situation in the other entognathous (and hexapod) taxa, in which the serosa eventually degenerates without participating in body wall formation. The embryonic membrane of Protura is represented only by the serosa. In this respect, Protura resemble Collembola (cf. JURA 1972) but differ from Diplura in which a second embryonic membrane or amnion develops (cf. IKEDA & MACHIDA 1998, 2001).

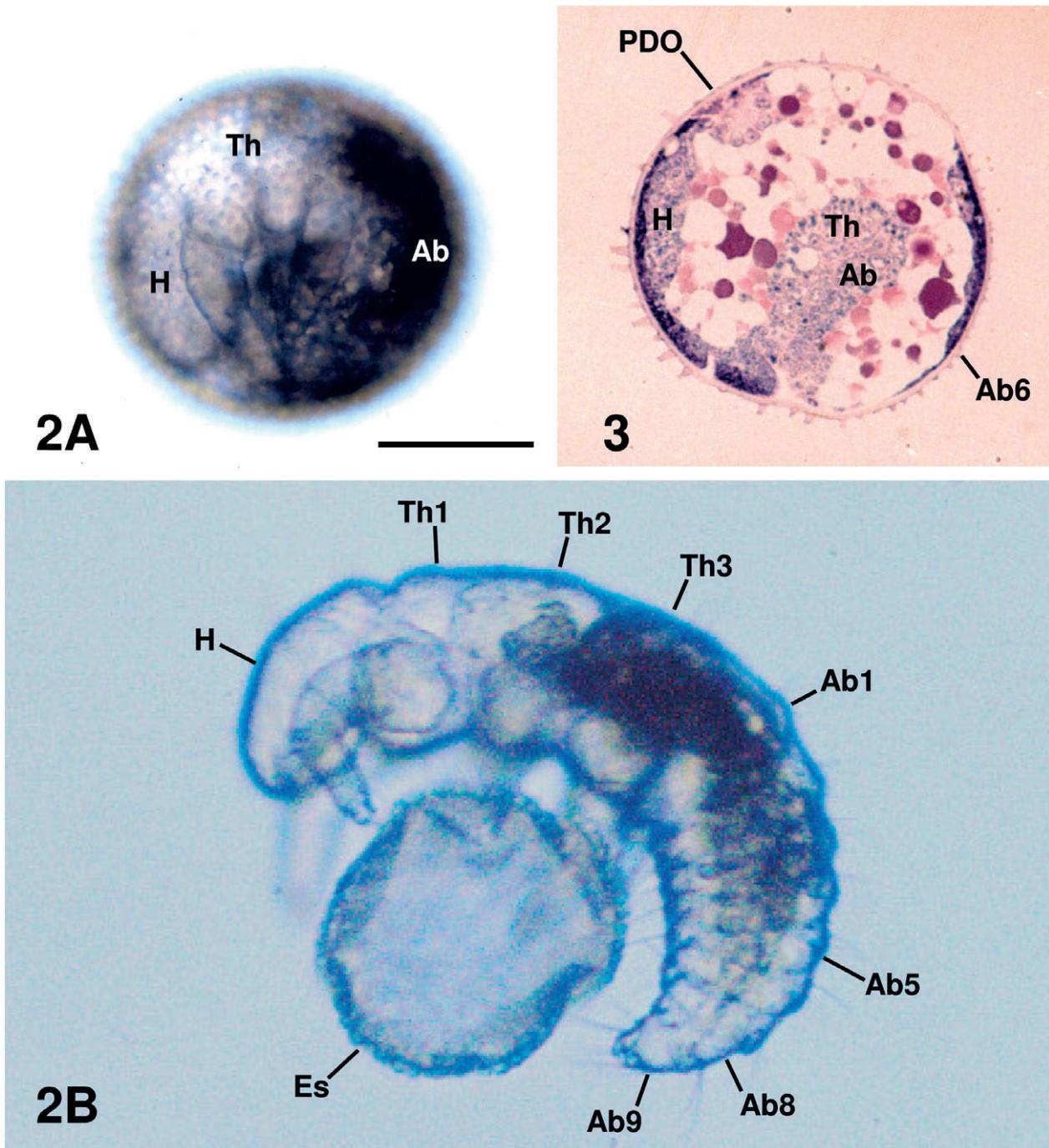
3. Monophyly of Diplura

The comparative morphology of ovarian (cf. BILIŃSKI 1994) and spermatozoal structures (cf. JAMIESON 1987) casts some doubt on the monophyly of Diplura. However, comparative embryological evidence strongly suggests its monophyly as shown by IKEDA & MACHIDA (1998). The evidence concerns formation of entognathy and abdominal metamerism.

The two dipluran suborders Rhabdura (Campodeina) (IKEDA & MACHIDA 1998) and Dicellurata (Japygina) (SILVESTRI 1933) share a peculiar form of entognathy characterized by (1) mouth folds derived only from the mandibular and maxillary terga, (2) partitioning of the posterior part of the maxillary tergum into the admentum, and (3) rotation of the labial appendages involved. These features are unique to Diplura, and are too specific not to postulate their acquisition in an ancestor common to Rhabdura and Dicellurata.

Fig. 1. Development of the proturan *Baculentulus densus* (see text). **A:** Egg (SEM) (after MACHIDA & TAKAHASHI 2003, 2004). **B–H:** Successive stages of development (DAPI staining, UV excitation) (after FUKUI & MACHIDA in press). **B:** Egg at the 4-cell stage. The arrow shows a cell boundary. **C:** Egg under blastoderm formation. **D:** Egg undergoing differentiation of the embryonic and extraembryonic areas. **E:** Egg with an embryo in initial stage of segmentation. **F:** Egg with an embryo in the initial stage of blastokinesis. The arrow shows the bending of the embryo. **G:** Egg with an embryo in the mid stage of blastokinesis. **H:** Egg with an embryo which has completed blastokinesis. Ab = abdomen; Ab1, 4, 6 = first, fourth and sixth abdominal segments; Ap = appendage; Bd = blastoderm; CN = cleavage nucleus; EemA = extraembryonic area; EmA = embryonic area; HL = head lobe; LbP = labial palp; LbS = labial segment; LbT = labial tergum; MdS = mandibular segment; MdT = mandibular tergum; MF = mouth fold; Mx = maxilla; MxS = maxillary segment; MxT = maxillary tergum; Se = serosa; Tg = tergum; Th1–3 = first to third thoracic segments; Th1L, 3L = first and third thoracic appendages; black arrowhead = cephalic extremity of embryo; white arrowhead = caudal extremity of embryo; scale bar = 50 μ m.





Figs. 2, 3. Development of the proturan *Baculentulus densus* (after FUKUI & MACHIDA in press; see text). **2:** Development following the stage shown in Fig. 1H. **2A:** Egg with an embryo just before hatching. **2B:** Newly hatched prelarva. **3:** Sagittal section of an egg at approximately the same stage as that shown in Fig. 1G. Ab = abdomen; Ab1, 5, 6, 8, 9 = first, fifth, sixth, eighth and ninth abdominal segments; Es = eggshell; H = head; PDO = primary dorsal organ; Th = thorax; Th1–3 = first to third thoracic segments; scale bar = 50 μ m.

It is well known that species in both suborders possess only ten abdominal segments (IKEDA & MACHIDA 1998). This has been explained as resulting from intensive reduction of the eleventh abdominal segment so that it is represented only by cerci (cf. MATSUDA 1976). However, this may be misleading. When following the development of abdominal segmentation in dipluran

embryos, it is clear that only ten abdominal segments form; no evidence for an eleventh abdominal segment exists and the cerci originate as paired appendages of the tenth abdominal segment (cf. IKEDA & MACHIDA 1998); KLAAS (2001) arrived at the same conclusion based on a comparison of adult skeletomuscular morphology with that in Ectognatha. This unique

number of abdominal segments can perhaps be taken as another dipluran autapomorphy; the problem, however, is that the number of abdominal segments in the hexapod groundplan is not yet clear.

4. Status of Entognatha

HENNIG's 'Entognatha-Ectognatha System' (1969) has been widely accepted with little challenge. The taxon Ectognatha has support from comparative embryology, as mentioned below, as well as from many other sources of evidence. However, the reality of the taxon Entognatha is suspect because it is supported principally by entognathy. Recent comparative paleontological (KUKALOVÁ-PECK 1987), morphological (KOCH 1997), and our comparative embryological (IKEDA & MACHIDA 1998) research provides evidence discounting the validity of entognathy as a synapomorphy for members of this group.

As mentioned above, entognathy in diplurans develops uniquely. In collembolan embryos, the mouth folds derive from the mandibular, maxillary and labial terga, and neither formation of the admentum nor the rotation of labial appendages occurs (cf. UEMIYA & ANDO 1987). Differences in the development of entognathy in Collembola and Diplura are too pronounced for one to postulate a common acquisition by members of both lineages. Rather, they strongly suggest that entognathy has been acquired convergently and in parallel in members of these two lineages. Thus, entognathy loses its validity as a synapomorphy (IKEDA & MACHIDA 1998).

Collembolans (cf. JURA 1972) and diplurans (UZEL 1898; IKEDA & MACHIDA 1998, 2001; IKEDA 2001) differ also (1) in cleavage type (holoblastic or total in Collembola; superficial in Diplura); (2) in the manner of endodermal and mesodermal segregation (typical in collembolans; unique in diplurans); (3) in abdominal metamerism (unique in embryos of both taxa but in different ways: collembolans have 6 segments and diplurans 10); and (4) in embryonic membrane formation (represented in collembolans only by the primary embryonic membrane or serosa, and in diplurans characterized by an additional second embryonic membrane or amnion).

Embryological information on Protura is still too scanty to incorporate into arguments on the status of Entognatha. However, it should be noted that the single proturan embryonic membrane, the serosa, retains the ability to differentiate into dorsal body wall, which is a feature shared by embryos of other principal arthropod subgroups such as myriapods and crustaceans. In the embryos of all other Hexapoda,

including the other entognathous taxa, the serosa has lost this function (see the following chapter, Fig. 4 and its legend; cf. MACHIDA & ANDO 1998; MACHIDA et al. 2002).

5. Reconstruction of hexapod basal clades based on evolutionary transition of embryonic membranes

MACHIDA et al. (1994) and MACHIDA & ANDO (1998) demonstrated the evolutionary changes in embryonic membranes and their developmental potential (role in provisional dorsal closure and in secretion of the cuticular egg envelope, the blastoderm or serosal cuticle) in different hexapod embryos. Their concept was revised by MACHIDA et al. (2002) and MASUMOTO & MACHIDA (2006), and can be summarized as follows (Protura are not included). Fig. 4 will provide an aid for following the lines.

(1) The most primitive condition is when no functional specialization has taken place between embryo and embryonic membrane in dorsal closure and in secretion of the cuticular egg envelope (Fig. 4A). This condition occurs in embryos of Myriapoda and Crustacea, the two potential sister groups to Hexapoda, and is regarded as plesiomorphic. (2) In hexapod (Protura excluded) embryos, the embryonic membrane (serosa) contributes to provisional dorsal closure but has ceased to participate in definitive dorsal closure (i.e., in dorsal body wall formation) (Fig. 4C–F). This task is exclusively performed by the embryo. (i) In collembolans, the embryonic membrane continues to be represented by the serosa alone (Fig. 4C), but (ii) in diplurans and ectognathans, a second embryonic membrane, the amnion, is acquired, and provisional dorsal closure (i.e., dorsal closure in the embryonic period) is temporally achieved by both serosa and amnion (Fig. 4D–F). (3) In ectognathans, the embryo has lost the ability to secrete a cuticular egg envelope, in contrast to Collembola and Diplura, in which the embryo retains an ability for secreting the cuticular egg envelope (Fig. 4C, D), resulting in the acquisition of the embryonic membrane fold (serosal or amnioserosal fold to secrete cuticle beneath [ventral to] the embryo) (Fig. 4E, F). The plesiomorphic condition in ectognathans is represented by archaeognathans, in which the embryonic membrane fold is ephemeral (Fig. 4E). (4) In dicondylans, the embryonic membrane fold or amnioserosal fold is well integrated into embryogenesis and the 'amnioserosal fold – amniotic cavity system' (ASF-AC) is established (Fig. 4F). The most primitive dicondylan condition is represented

by zygentomans, in which formation of the ASF-AC system is still flexible, but (5) finally in pterygotan embryos, a close linkage between formation of the amnioserosal fold and production of the amnion occurs and the manner of the ASF-AC formation is fixed.

6. Tentative conclusion and perspectives

Fig. 5A is a phylogenetic tree on which critical steps in the evolutionary transition of embryonic membranes summarized in the previous chapter are mapped: each may be considered an autapomorphy for a clade. Both Dicondylia and Ectognatha are supported as monophyletic and a sister group relationship between Diplura and Ectognatha is suggested; however, Entognatha are not monophyletic.

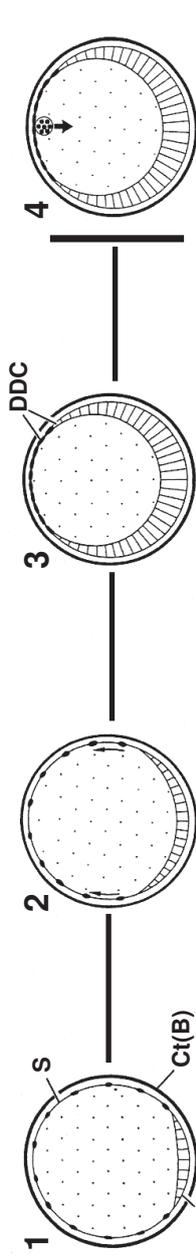
How to incorporate the information from Protura, although premature, in Fig. 5A? I mentioned above that the embryonic membrane (serosa) of proturan embryos is similar to that of myriapodan and crustacean embryos in that no definitive functional specialization between the embryo and embryonic

membrane (serosa) has developed (Fig. 4B); in this respect it differs from that of Collembola, Diplura and Ectognatha (Fig. 4C–F). This feature should thus represent the plesiomorphic stage in the evolutionary transition in developmental potential of embryo and embryonic membranes, as shown in step 1 in the previous chapter (Fig. 4A). Thus, in discussing the affinities of Protura, we should pay more attention to more basal arthropods such as Myriapoda or Crustacea. If such were done, we might revise Fig. 5A as to include Protura.

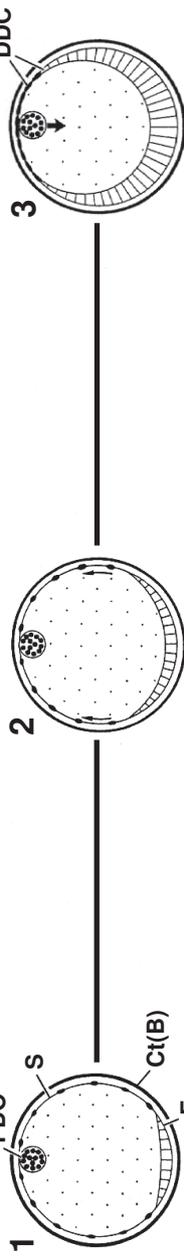
In Fig. 5B, in which this is done, not only is the taxon Entognatha dismissed, but Hexapoda and Ellipura would not be supported either. The most important task now is to accumulate additional, comparative embryological information on Protura. Critical embryological re-examination of Collembola including study of additional species is also desired with such new knowledge to be re-examined and evaluated within the framework followed here. Diplura-Dicellurata likewise deserve comprehensive embryological study since we have little classical embryological knowledge of any species in this lineage.

Fig. 4. Embryonic development of different mandibulate groups, with special reference to embryonic membranes (see text and also refer to MACHIDA et al. 2002 and MASUMOTO & MACHIDA 2006). Tentative embryological information on Protura is included (FUKUI & MACHIDA in press). **A:** Myriapoda and Crustacea. **1.** Blastoderm differentiates into the embryo and serosa, and a cuticular egg envelope or blastoderm cuticle is secreted by the blastoderm or both the differentiated embryo and serosa. In Symphyla, Pauropoda and some higher crustaceans, the primary dorsal organ forms as in Protura, Collembola and Diplura (not drawn in the figure). **2, 3.** With the progression of embryogenesis, definitive dorsal closure (i.e., dorsal body wall formation) proceeds and completes, and the serosa, which was functioning as a 'dorsal closure in the embryonic period,' also participates. **4.** In some forms of crustaceans and myriapods, excessive serosal cells degenerate without participating in definitive dorsal closure. **B:** Protura. **1.** Blastoderm, which secretes a cuticular egg envelope or blastoderm cuticle, differentiates into the embryo and serosa, in which a primary dorsal organ develops at its dorsal side. **2, 3.** With the progression of embryogenesis, the definitive dorsal closure proceeds and completes. The serosa, although its derivative, the primary dorsal organ, degenerates, participates in the definitive dorsal closure without degenerating as in Myriapoda and Crustacea. **C:** Collembola. **1.** Blastoderm differentiates into the embryo and serosa, in which a primary dorsal organ develops at its dorsal side, and a cuticular egg envelope or blastoderm cuticle is secreted. **2, 3.** With the progression of embryogenesis, the definitive dorsal closure proceeds and completes. The serosa, which was functioning as a 'dorsal closure in the embryonic period,' is concentrated dorsally, to degenerate in the same way as the primary dorsal organ, without participating in the definitive dorsal closure. **D:** Diplura. **1.** Blastoderm differentiates into the embryo and serosa, and a cuticular egg envelope or blastoderm cuticle is secreted. The serosa is functioning as a 'dorsal closure in the embryonic period.' **2, 3.** Serosal cells migrate dorsally to form a primary dorsal organ, and the area from which the serosa receded is occupied by an amnion segregated from the embryonic margin. The amnion now functions as a 'dorsal closure in the embryonic period,' instead of the serosa. **4.** With the progression of embryogenesis, definitive dorsal closure proceeds and is completed, but the serosa (primary dorsal organ) and amnion do not participate in it. **E:** Archaeognatha. **1.** Blastoderm differentiates into the embryo and serosa. **2.** Serosa invades beneath (ventrally to) the embryo, to form a serosal fold. **3.** The serosal fold closes beneath the embryo, and generally, soon becomes an amnioserosal one with the production and supplementation of amnion. Now, the whole egg surface is covered by the serosa. Hereby, the secretion of cuticular egg envelope all over the egg is retained, although the embryo has lost the ability to secrete cuticular envelope. **4, 5.** After the secretion of a serosal cuticle, the embryonic membrane fold (an amnioserosal one but rarely a serosal one) is soon regressed, and the serosa, which was functioning as a 'dorsal closure in the embryonic period,' is condensed as a secondary dorsal organ, to degenerate. The amnion replaces the serosa and functions as a second 'dorsal closure in the embryonic period.' **6.** With the progression of embryogenesis, definitive dorsal closure proceeds and completes, but the amnion does not take part in it. **F:** Dicondylia. **1.** Blastoderm differentiates into the embryo and serosa. **2.** By the formation of serosal fold and supplementation of amnion to it in Zygentoma as in Archaeognatha and by the invasion of serosa beneath (ventrally to) the embryo and simultaneous production of amnion from the embryonic margin in Pterygota, an amnioserosal fold forms. **3.** The amnioserosal fold closes beneath the embryo, and an amniotic cavity appears between the fold and embryo, to form the amnioserosal fold - amniotic cavity system (ASF-AC). Now, the whole egg surface is covered by the serosa, and a cuticular egg envelope or serosal cuticle is secreted. **4, 5.** The ASF-AC is maintained for a sufficient time and the embryo develops, concealed by it (the diapause stage). Then, katatrepsis occurs. The amnioserosal fold is regressed, and the serosa, which was functioning as a 'dorsal closure in the embryonic period,' is condensed as a secondary dorsal organ and degenerates. The amnion replaces the serosa and functions as a second 'dorsal closure in the embryonic period.' **6.** With the progression of embryogenesis, the definitive dorsal closure proceeds and completes, but the amnion does not participate in it. A = amnion; AC = amniotic cavity; ASF = amnioserosal fold; ASF-AC = amnioserosal fold - amniotic cavity system; Ct = cuticular egg envelope; Ct(B) = blastoderm cuticle; Ct(S) = serosal cuticle; DDC = definitive dorsal closure; E = embryo; PDO = primary dorsal organ; S = serosa; SDO = secondary dorsal organ; SF = serosal fold.

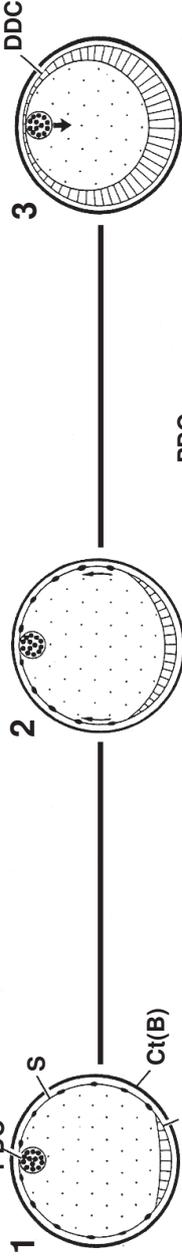
4 A. MYRIAPODA/
CRUSTACEA



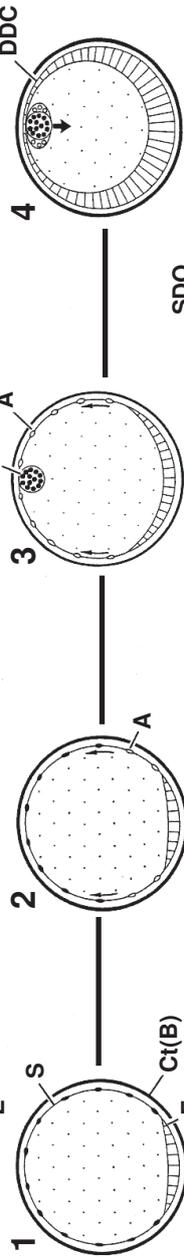
B. PROTURA



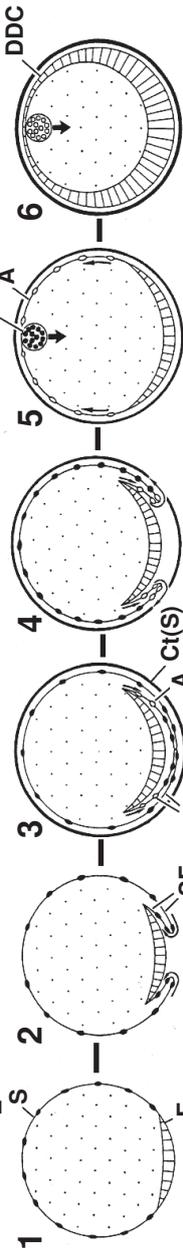
C. COLLEMBOLA



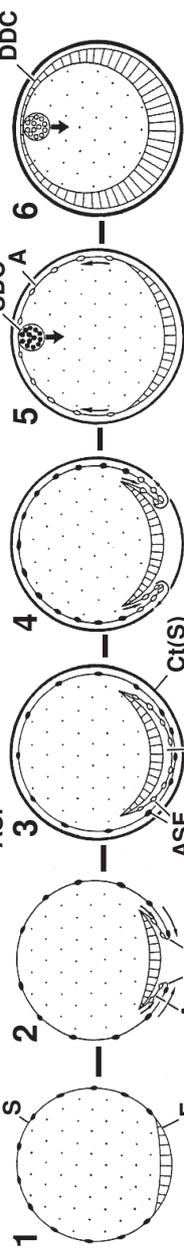
D. DIPLURA



E. ARCHAEOGNATHA



F. DICONDYLIA



▤ embryo
● serosa
○ amnion

ASF-AC

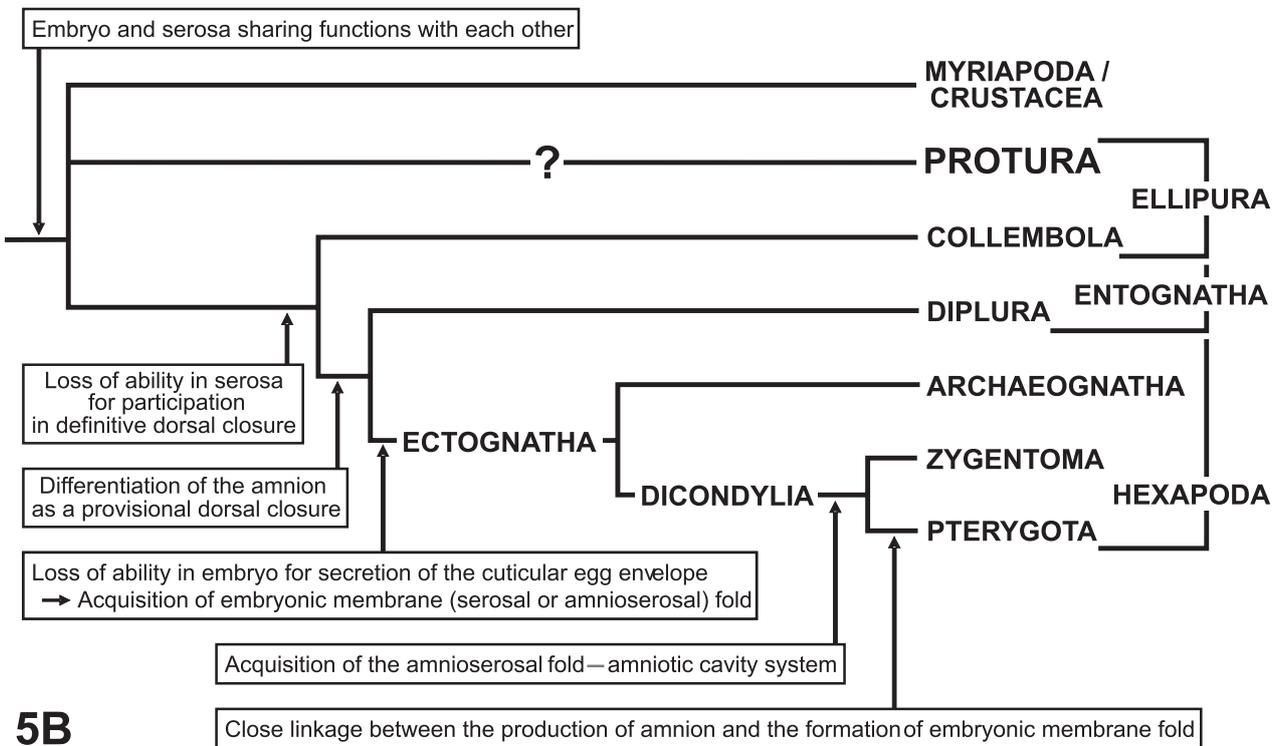
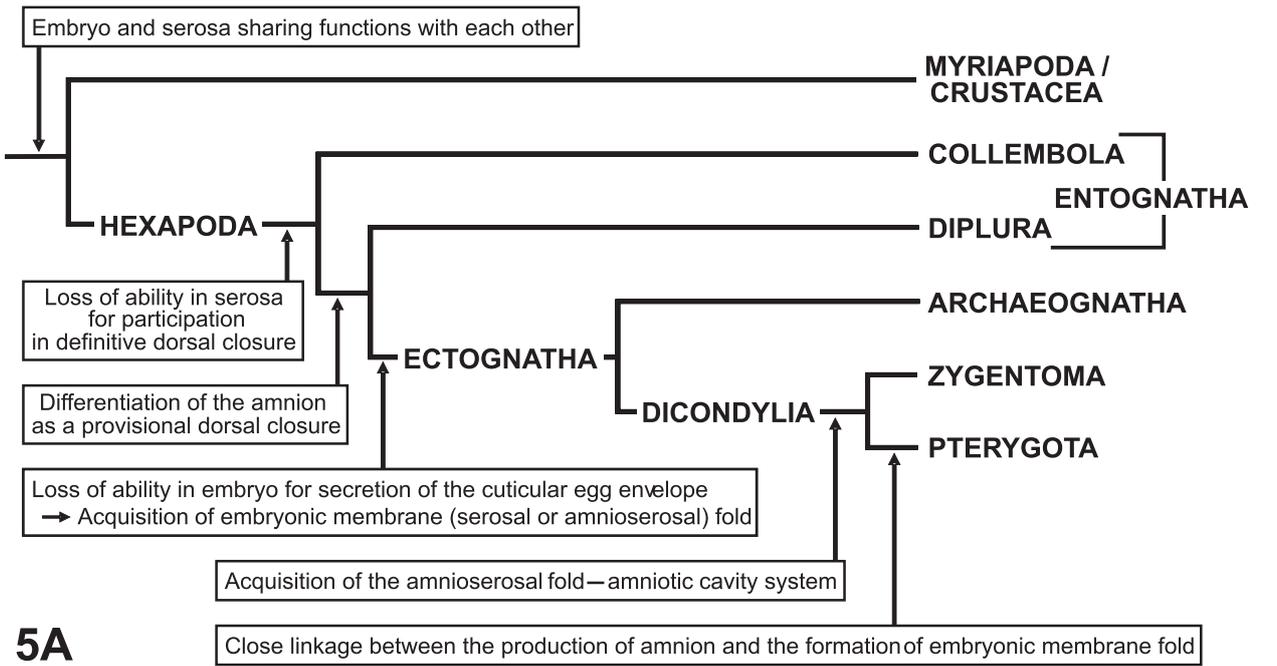


Fig. 5. Reconstruction of hexapod basal clades in the light of evolutionary changes of embryonic membranes and functional specialization in the embryo proper and embryonic membranes. Myriapoda or Crustacea are thought to be the sister group of Hexapoda (see text). **A:** Reconstruction without the embryological data on Protura. **B:** Reconstruction with the tentative embryological information on Protura incorporated.

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