

[REVIEW]

# Character Phylogeny in Lepidopteran Embryogenesis: Its Revaluation and Issues to Be Resolved\*

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## 1. Introduction

The order Lepidoptera is the insect group whose embryogenesis has been well investigated. Until about 30 years ago, however, the materials had concentrated on the highest group of this order, or the former suborder Ditryisia, and nothing had been known of the embryogenesis of primitive, non-ditryisian Lepidoptera. In several ditryisian species, for example, *Orgyia antiqua*, *Chilo suppressalis*, *Pieris rapae*, and *Epiphyas postvittana*, it had been known that their embryonic membranes (serosa and amnion) are formed independently, not by the fusion of amnioserosal folds to be described later, and their germ bands or embryos grow in the submerged condition under the yolk until just before hatching irrespective of the shape and size of eggs (Christensen, 1943; Okada, 1960; Tanaka, 1968; Anderson and Wood, 1968). Since such developmental processes are not common to other insects, it had been speculated that these processes are characteristic not only of the Ditryisia but also of the whole Lepidoptera until the time when Ando and Tanaka (1976, 1980) found out a different mode of early embryogenesis in the hepialid moths, *Endoclita*, belonging to the non-ditryisian Lepidoptera.

The discovery of a sac-like germ rudiment formed by invagination of the germ disk into the yolk in this primitive genus encouraged us to investigate the mode of embryogenesis of other primitive Lepidoptera. Since then the mode of embryogenesis has been investigated in several primitive species belonging to different phylogenetic lineages as listed below: *Neomicropteryx nipponensis* (suborder Zeugloptera, Micropterigidae) (Ando and Kobayashi, 1978; Kobayashi and Ando, 1981, 1982, 1983, 1984), *Eriocrania sakhalinella* (former suborder Dacnonypha, Eriocraniidae) (Kobayashi and Ando, 1987), *Mnesarchaea fusilella* (former suborder Exoporia, Mnesarchaeidae) (Kobayashi and Gibbs, 1990, 1995), *Stigmella castanopsiella* (former suborder Monotryisia, Nepticulidae) (Kobayashi, 1996), and *Nemophora albi antennella* (former suborder Monotryisia, Adelidae) (Kobayashi, 1998).

As the results of these extensive researches, it became evident that there exist considerable differences in the mode of embryogenesis not only between the Ditryisia and non-ditryisian lineages but also among the latter lineages. The results also made us possible to extract embryonic characters from the different modes of embryogenesis among these lineages including the Ditryisia, and a phylogenetic tree of relationships among five former suborders, *i. e.*, Zeugloptera, Dacnonypha, Exoporia, Monotryisia, and Ditryisia, was obtained, based on 27 embryonic characters (Kobayashi and Ando, 1988; a revised tree based on 30 characters has been obtained by Kobayashi *et. al.*, 2003). Our study in 1988 was the first attempt in which embryonic data were adopted for the cladistic analysis in insects (probably throughout whole animals). Topology of the resultant cladogram for these five suborders fundamentally coincided with that of phylogenetic trees derived from the cladistic analysis of larval and adult morphological features of major lepidopteran groups (Kristensen, 1984). This means that embryonic characters also are effective in analyzing the

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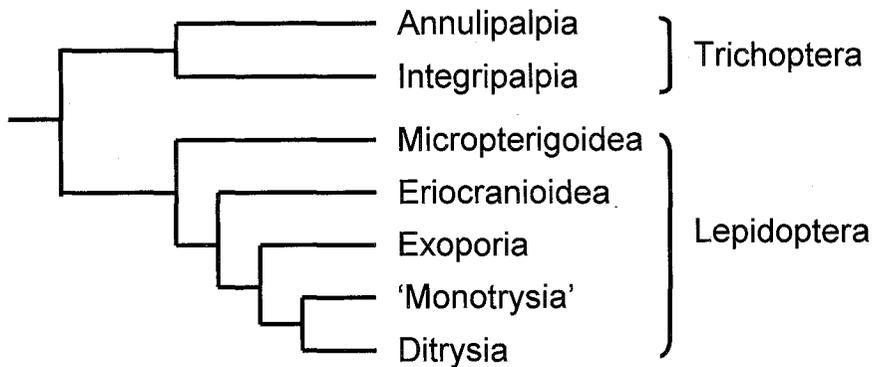


Fig. 1 Phylogenetic relationships among major lepidopteran and trichopteran lineages inferred from the cladistic analysis of 30 embryonic characters [modified from Kobayashi *et al.* (2003)].

phylogenetic relationships among major lepidopteran groups. A method of discriminating between plesiomorphic (primitive) and apomorphic (derived) states in each character, called character phylogeny, was also attempted for the first time in our study of comparative embryology of Lepidoptera.

After our first presentation of the phylogenetic tree of Lepidoptera inferred from embryonic characters in 1988, however, considerable modifications of the classification system of Lepidoptera especially in the subordinal rank have been made (Kristensen and Skalski, 1998; Kristensen, 2003). In the new classification system, former categories of suborders and infraorders have been largely abandoned, and the Lepidoptera are divided into the four following major clades: the Micropterigoidea (the family Micropterigidae only), the Agathiphagoidea (the family Agathiphagidae only), the Heterobathmioidea (the family Heterbathmiidae only), and the Glossata (including remaining 44 superfamilies). The former three superfamilies retain functional mandibles, and are primitively devoid of the coilable proboscis, whereas the huge Glossata comprises the superfamilies formerly included in the traditional suborders Dacnonypha, Monotryisia, Exoporia, and Ditryisia.

Then we have analyzed again the embryonic characters and obtained a phylogenetic tree as shown in Figure 1 (Kobayashi *et al.*, 2003) according to the nomenclature for new taxa. However, the recent drastic changes of the classification system have forced us to revalue the embryonic characters that we had used to analyze the phylogenetic relationships among former lepidopteran suborders, because the mode of embryogenesis of each new suborder (except for the Zeugloptera) cannot be represented by the non-ditryisian species whose embryogenesis we investigated.

Accordingly, several embryonic characters we used for the phylogenetic analysis are now revalued in the context of new classification system of Lepidoptera. In this review I will discuss several embryonic characters and their character phylogeny of important values in considering the evolution and phylogeny of Lepidoptera.

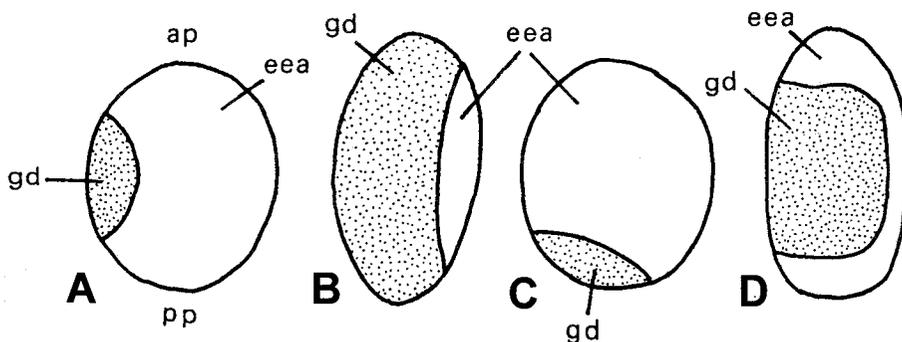


Fig. 2 Shape and size of germ disk in lepidopteran eggs. A. *Neomicropteryx* (Micropterigoidea). B. *Eriocrania* (Eriocranioidea). C. *Endoclitia* (Exoporia). D. *Pieris* (Ditryisia). ap: anterior pole, eea: extraembryonic area, gd: germ disk, pp: posterior pole.

**2. Reevaluation of the mode of the formation of the germ rudiment and embryonic membranes**

In Lepidoptera, soon after the completion of the blastoderm, the cells in the embryonic area actively proliferate, consequently forming a germ disk composed of tall columnar cells. Although the figure and size of the germ disk largely differ according to taxa (Fig. 2), the germ disk then differentiates into a germ rudiment and an amnion, and the extraembryonic area becomes a serosa. In Lepidoptera, as schematically shown in Figure 3, four major types can be distinguished in the manner of formation of these structures; the invaginate type 1 (I-type 1), the invaginate type 2 (I-type 2), the amnio-serosal fold type (AF-type), and the fault type (F-type).

The I-type 1 is known in *Neomicropteryx* (Micropterigidae). In this genus, a small circular germ disk deeply invaginates in the yolk from its posterior and forms a sac-shaped germ rudiment (Fig. 3A). The mouth of invagination then closes, and the extraembryonic area covers the whole egg surface to form the serosa. The sac-shaped germ rudiment separates from the serosa, and sinks completely in the yolk; the concave side of the germ rudiment is a future embryo, and the convex side becomes a thin amnion.

In *Endoclita* (Hepialidae) and *Mnesarchaea* (Mnesarchaeidae), both of which belong to the clade Exoporia in the Glossata in the new system, invagination of the germ disk similar to that of *Neomicropteryx* is also formed, although invagination is shallower than that of the latter species (Fig. 3B). The appearance of the completed germ rudiment assumes a nearly circular, shallow cup-shape. However, in these exoporian species, the cells of the extraembryonic area, from which the serosa later differentiates, are very thick and have two nuclei, and this state persists for a long time after the completion of the serosa; hence these exoporian species are different from *Neomicropteryx* in these aspects. Then we call this type the invaginate type 2 (I-type 2).

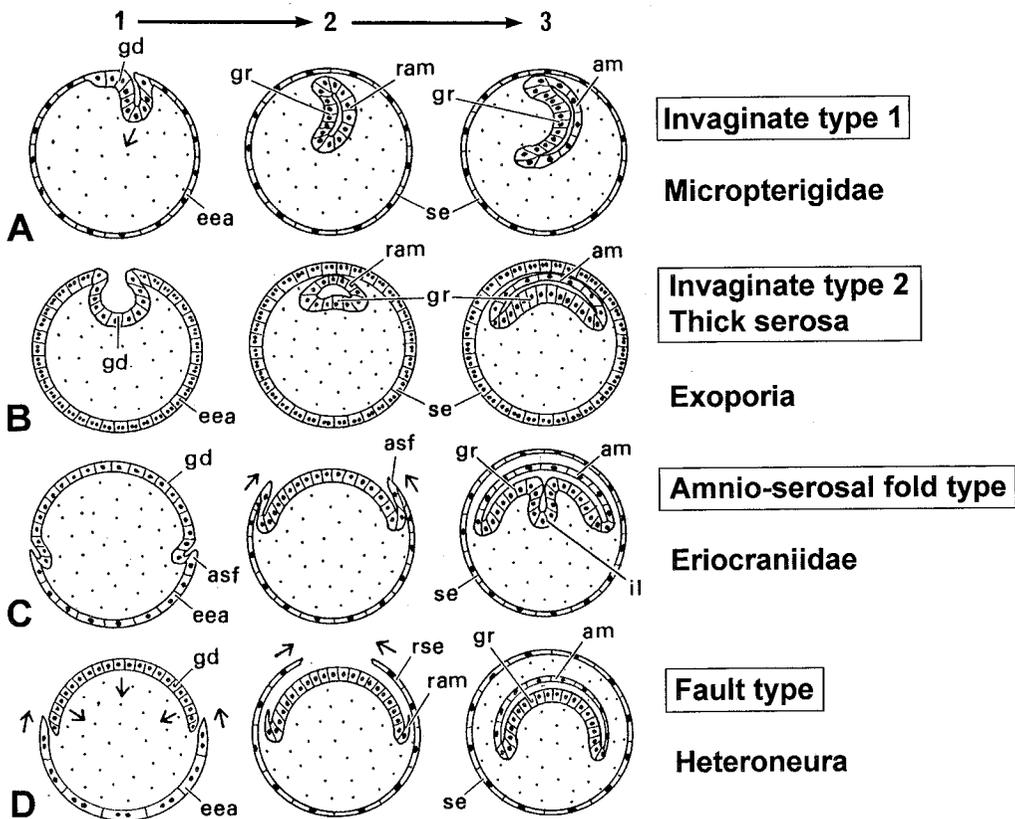


Fig. 3 Comparative diagram showing four types of embryonic membrane formation in major lepidopteran lineages (development proceeds from 1 to 3) [modified from Kobayashi and Ando (1988)]. am: amnion, asf: amnio-serosal fold, eea: extraembryonic area, gd: germ disk, gr: germ rudiment, il: inner layer, ram: rudimentary amnion, rse: rudimentary serosa, se: serosa.

The AF-type was more recently discovered in the eggs of *Eriocrania* (Eriocranioidea) for the first time in Lepidoptera. In this genus, the large germ disk differentiates into a larger germ band or embryo *in situ* without invagination or sinking into the yolk, and embryonic membranes are completed by the fusion of the lips of amnio-serosal folds that extend slowly over the ventral surface of the developing embryo (Figs. 3C, 7A, B). In the earlier works on the ditrysian Lepidoptera, such as *Bombyx* (Toyama, 1902), *Antheraea* (Saito, 1934, 1937), and *Ephesia* (Drummond, 1936), their embryonic membranes are described to be formed by the fusion of amnio-serosal folds. However, as mentioned before, in several other ditrysian Lepidoptera, it has been reported that the amnion and serosa are formed independently, not by the fusion of amnio-serosal folds. In an electron microscopic study, Miya (1984) has clearly demonstrated that the embryonic membranes of *Bombyx* are formed in the manner of the F-type described below, not in that of the AF-type. Hence, we believe the old observations mentioned above to be incorrect.

It is, therefore, possible to regard the F-type as being distributed widely in the Ditrysia. In this type, after differentiation of the large and broad germ disk, it is cut off along the margin of the extraembryonic area, and then sinks slightly into the yolk in like a manner of the formation of a fault in a geological term (Fig. 3D). Next, the rudimentary serosal margins (extraembryonic area) extend over the germ disk, and finally fuse at its ventral surface to complete the serosa. After the completion of the serosa, the edges of the germ disk are flexed ventrally, and spread along the narrow space between the serosa and germ disk, and fuse with each other to form the amnion. In this type, the amnion is thus formed independently of the formation of the serosa. As the germ disk sinks into the yolk, yolk granules enter the space between the completed amnion and serosa, and then the germ disk becomes somewhat compact to form a bilobed germ rudiment.

The F-type was later discovered in *Stigmella* (Nepticuloidea) and *Nemophora* (Incurvarioidea) belonging to the so-called 'monotrysian Heteroneura,' although, in the latter, the germ disk does not sink into the yolk, and hence yolk globules do not enter the space between the amnion and serosa (Kobayashi, 1996, 1998). The F-type is, therefore, probably distributed widely in the clade Heteroneura including the Ditrysia.

Consequently it has been suggested that each of these types is so far restricted to a certain major lineage in the

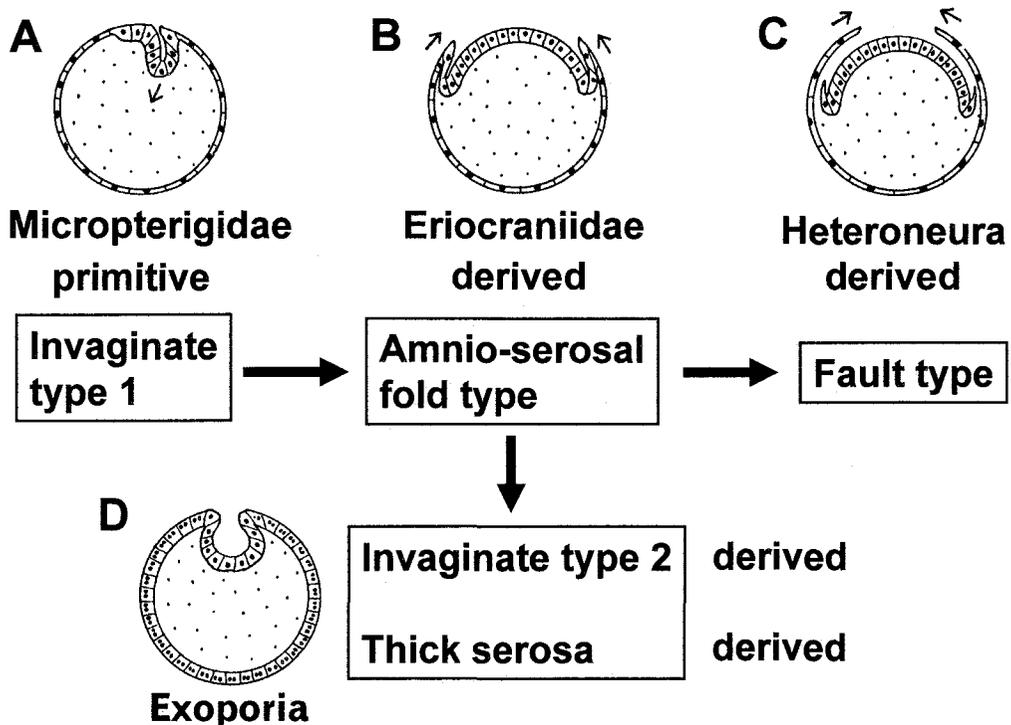


Fig. 4 Supposed changes of four types of embryonic membrane formation, summarized in Fig. 3.

new system of Lepidoptera. Then, as shown in Figure 4, the sequential changes of these types, or their character phylogeny, can be assumed in the context of comparative embryology of insects. The invagination of the germ disk is generally regarded as a primitive condition, because the invagination similar to that of *Neomicropteryx* is often formed in many non-holometabolous insects such as Palaeoptera and Paraneoptera. Thus it can be assumed that the I-type 1, or primitive type, has been retained in the most basal Lepidoptera or the Micropterigoidea (Fig. 4A). It is very interesting to us that an invaginate type similar to the I-type 1 is also known in the egg of *Stenopsyche griseipennis*, one of the most primitive Trichoptera (Miyakawa, 1974). The AF-type is very common in other holometabolous insects, and is presumed to be derived from the I-type 1 during the early diversification of Lepidoptera (Fig. 4B). As mentioned before, however, the AF-type has so far been known only in *Eriocrania* (Eriocranioidea). The F-type is a highly specialized, rare type observed only in the Heteronéura throughout insects, and was probably derived from the AF-type (Fig. 4C). As to the I-type 2 in Exoporia, it is somewhat difficult to assume its origin, because, as mentioned before, an invaginate type is generally regarded as the primitive condition. On the other hand, the formation of a very thick serosa is unique to Exoporia. Then we approve of the idea that the I-type 2 is not primitive and was derived from the AF-type (Fig. 4D).

In spite of these assumptions, however, at present we have no information about whether the I-type 1 is still retained in other mandibulate clades, *i.e.*, the Agathiphagoidea and Heterobathmioidea, and whether the AF-type is restricted within the Eriocranioidea or occurs in other primitive clades such as the Acanthopteroctetoidea, Lophocoronoidea, and Neopseustoidea (Fig. 5). These are important issues to be resolved in future.

### 3. Ancestral germ type in Lepidoptera, short or long germ?

Insect embryos are classified into three categories according to the size or length of the germ disk (or germ anlage) relative to the surface area of the entire egg; that is, short germ, intermediate (half-long), and long germ eggs (Krause, 1939; Sander, 1976). In typical long germ insects such as *Drosophila*, the embryonic area occupies almost all surface area of the blastoderm from the beginning, and all body segments are specified almost simultaneously within the blastoderm prior to the inner layer formation (often called gastrulation). In short germ insects (*e. g.*, crickets), the embryonic area or germ disk is very small at the time of its formation, and this area includes only protocephalon and gnathal segments, whereas the remaining segments of thorax and abdomen form progressively from a posterior growth zone following the inner layer formation. Between these extremities, intermediate germ exists in which anterior body segmentation such as thoracic one occurs first, while the posterior segments originate from a growth zone. In the context of recent molecular developmental biology, a long germ can be defined as an embryo in which three segmentation genes (*gap*, *pair rule*, and *segment polarity*) express almost simultaneously throughout an embryo. In contrast, in short or intermediate germs, the expression of these genes occurs after the posterior segmentation proceeds.

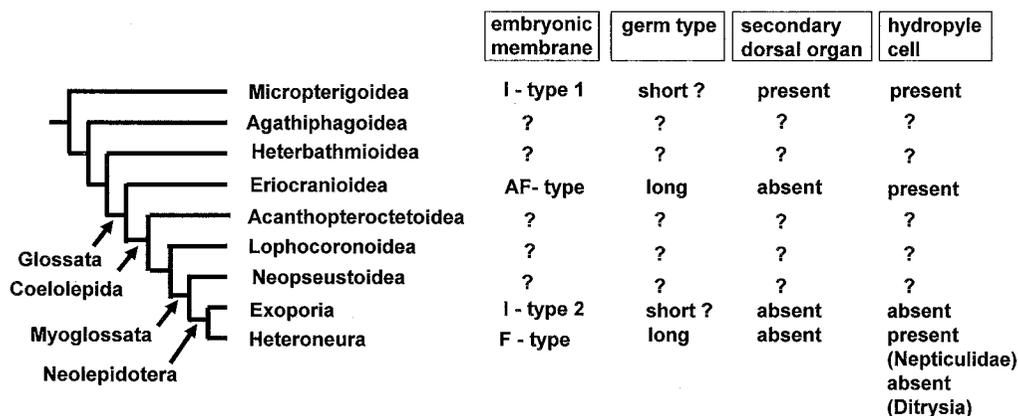


Fig. 5 Mapping type of embryonic membrane formation, germ type, presence or absence of secondary dorsal organ and hydropyle cells on the phylogenetic tree of Lepidoptera [the tree modified from Kristensen (2003)].

The short germ type is observed in many lower insects, *i. e.*, Archaeognatha, Zygentoma, and Hemimetabola, which have panoistic ovaries (Sander, 1976). In contrast, the long germ type is widely distributed in Holometabola such as Diptera and Coleoptera, which have meroistic ovaries. In Lepidoptera, Krause (1939) and Krause and Krause (1964) first evaluated the germ type of two ditrysian species, *i. e.*, *Ephestia kuehniella* and *Bombyx mori*, and they interpreted it as an intermediate type. Nagy *et al.* (1994), however, have described in detail the process of the formation of the early embryo in *Bombyx mori*, and suggested the occurrence of the long germ type. In another ditrysian species, *Manduca sexta*, Kraft and Jackle (1994) have also found that the expression pattern of segmentation genes shows a molecular prepatterning typical of *Drosophila*. These observations are suggesting that the long germ is common in the Ditryisia.

As shown in Figure 2, however, the size and shape of the germ disk in Lepidoptera largely differ according to lineages, and the possibility of the short germ type in two non-ditrysian lineages, *i. e.*, the Micropterigoidea and the Exoporia, has been suggested by Nagy (1995) and Davis and Patel (2002) based on our embryological researches (Fig. 5). In fact, as discussed in the previous section, the mode of the formation of germ rudiments (I-type 1 and 2) in the two lineages largely differs from other Lepidoptera. However, since the small size of a germ disk or rudiment is not necessarily a criterion for defining germ types especially in holometabolous insects, the germ types of these non-ditrysian lineages should be distinguished by molecular approaches in future. Interestingly, the germ disk and early embryo of *Eriocrania* are much larger than those of the Ditryisia (Figs. 2B, 7A, B), thus clearly belonging to the long germ type. The germ disks of monotrysian heteroneurans, *Nemophora* and *Stigmella* are also as large as those of the Ditryisia, then probably belonging to the long germ type. Then, if the Micropterigoidea and the Exoporia belong to short germ insects, the long germ type may occur multiple times in lepidopteran embryogenesis, *i. e.*, in the Eriocranioidea and the Heteroneura (Fig. 5).

In Trichoptera, no reference has been made to the germ type of their embryos. In this order, however, the formation of the inner layer begins simultaneously with the formation of the germ rudiment from the germ disk, or before elongation of the germ band (Miyakawa, 1973; Akaike *et al.*, 1982; Kobayashi and Ando, 1990). Therefore, the trichopteran embryos may belong to the short germ type.

#### 4. Formation of the secondary dorsal organ

As mentioned in section 1, the early embryos of any Ditryisia are immersed in the yolk after the completion of the amnion and serosa. The elongated embryos then accomplish their revolution and the dorsal closure occurs in the immersed condition in the yolk; hence the amnion and serosa are not ruptured and are intact even after the dorsal closure, and fairly amount of yolk remain around the embryo until just before hatching (Fig. 6A). As the result of this developmental process peculiar to the Ditryisia, the secondary dorsal organ, which is temporally formed from the ruptured embryonic membranes in the midgut in many other insects, is not formed in the Ditryisia.

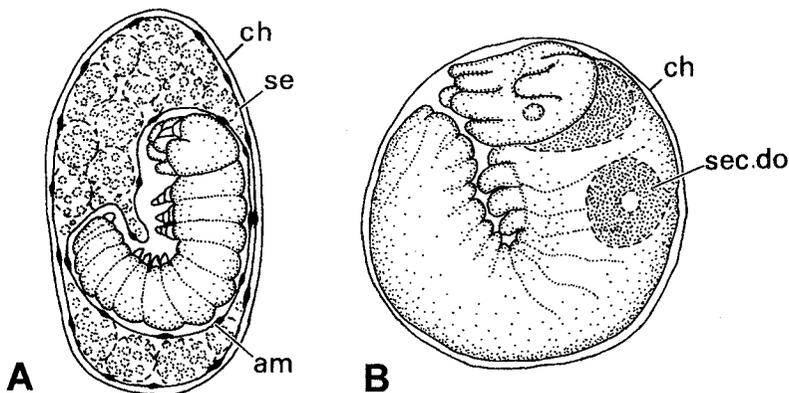


Fig. 6 Lateral view of embryos of *Pieris rapae* (A) and *Neomicropteryx nipponensis* (B) at post-revolution stage. [A: modified from Ando (1978); B: modified from Kobayashi and Ando (1981)]. Note that the secondary dorsal organ is not formed in *Pieris* but formed in *Neomicropteryx*. am: amnion, ch: chorion, se: serosa, sec.do: secondary dorsal organ.

On the other hand, the embryo of *Neomicropteryx* assumes completely superficial position just before revolution, and the secondary dorsal organ is formed soon after the dorsal closure (Kobayashi and Ando, 1981) (Fig. 6B). Although the formation of this organ is common in insects including Trichoptera, it was the first discovery of this organ in Lepidoptera, being so far unknown in any other lepidopteran lineages. It was speculated at first that the absence of the secondary dorsal organ in the Ditrysia may be attributed to the immersed condition of the embryo until just before hatching. The organ, however, is not formed also in the eggs of *Eriocrania* and *Nemophora* whose embryos assume completely superficial position before revolution; that is, their embryonic membranes are not ruptured before and after the dorsal closure. The absence of the secondary dorsal organ, therefore, is not caused by a large quantity of yolk, but might occur together with the differentiation of a certain phylogenetic lineage, probably with the Glossata. However it should be confirmed in future whether the secondary dorsal organ is formed or not in the eggs of other mandibulate clades, the Agathiphagoidea and the Heterobathmioidea.

### 5. Formation of hydropyle cells

In the eggs of *Neomicropteryx*, *Eriocrania*, and *Nemophora*, during the formation of their germ rudiments, a thickened, circular area consisting of about 30 columnar cells appears in the extraembryonic serosal region near the posterior pole (Fig. 7A-C). The serosal cuticle, which is formed from serosal cells and generally covers the whole egg surface below the vitelline envelope, is not formed on the surface of this area; thus the columnar cells remain in contact with the chorion (or the vitelline envelope) even after the completion of the serosa. The columnar serosal cells like these were first described in the locust, *Melanoplus differentialis* (Slifer, 1937; Slifer and Sekhon, 1963), and were termed hydropyle cells since they were confirmed to absorb water in the eggs. Hydropyle cells have also been discovered in many aquatic heteropteran taxa such as *Gerris* (Cobben, 1968; Mori, 1970), but in Holometabola these have been discovered only in the lepidopteran genera above. Although there is no experimental evidence, the facts that the egg volume of these Lepidoptera increases during the early embryonic development, and that the columnar serosal cells adhere directly to the chorion even after the formation of the serosal cuticle, strongly suggest that these cells have function similar to that of hydropyle cells in the eggs of locusts and Heteroptera. Hydropyle cells persist until shortly before revolution.

On the other hand, hydropyle cells have not been found in any ditrysiian and exoporian species, in which the chorion and vitelline envelope are generally thick and tough, and the egg volume is almost constant throughout development. Assuming that the primitive lepidopteran eggs have primarily hydropyle cells, the lack of hydropyle cells might occur independently in both the Exoporia and Ditrysia. This assumption, however, should be verified by the formation of these cells in the Agathiphagoidea and Heterobathmioidea (Fig. 5).

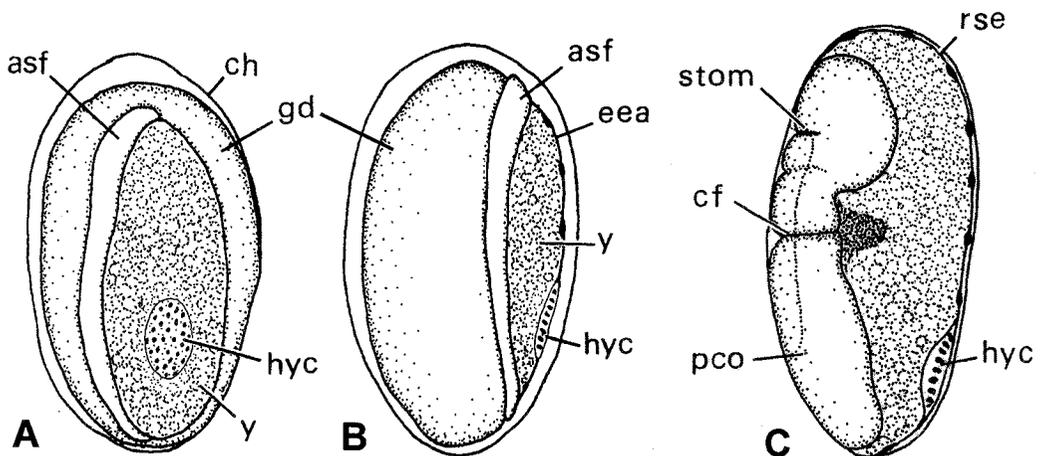


Fig. 7 Dorsal (A) and lateral view (B, C) of *Eriocrania sakhalinella* eggs in early embryonic stages [modified from Kobayashi and Ando (1987)]. asf: amnio-serosal fold, cf: cephalic furrow, ch: chorion, eea: extraembryonic area, gd: germ disk, hyc: hydropyle cell, pco: protocorm, rse: rudimentary serosa, stom: stomodaeum, y: yolk.

Hydropyle cells have not been discovered in trichopteran eggs, although the egg volume increases during the egg period (Miyakawa, 1973; Kobayashi and Ando, 1990).

## 6. Perspectives

Based on the available comparative embryological data, the basal clades of Lepidoptera were discussed and their phylogeny was reconstructed. Our character phylogenetic analyses on lepidopteran embryogenesis have required the current lepidopteran phylogeny in higher levels to be emended, to present a new system as shown in Figure 5. Simultaneously, this well indicates a high potential of comparative embryological analyses in the arguments on phylogenetic issues.

However, we have many things to be resolved for the final goal to the comparative embryological reconstruction of lepidopteran basal clades. The most important tasks in future should be assigned to the study of the embryogenesis of some mandibulate lepidopterans such as Agathiphagoidea and Heterobathmioidea and some lower glossatans such as Lophocornoidea, of which embryogenesis remains still unknown. The embryological knowledge from these works will provide us with a critical insight of high resolution to our comprehensive understanding of lepidopteran basal clades.

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