Characterization of abdominal appendages in the sawfly, *Athalia rosae* (Hymenoptera), by morphological and gene expression analyses

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Abstract

Larvae of the sawfly, *Athalia rosae*, have remarkable abdominal prolegs. We analyzed the morphogenesis of appendages and the expression of *decapentaplegic* and *Distal-less* genes during embryonic development to characterize the origin of prolegs. Proleg primordia in abdominal segments A1–A9 appeared shortly after the inner lobes (endites) of gnathal appendages were formed. These were located on the ventral plates, medioventral to the appendages of the other segments in light of serial homology. Nothing was seen where the main axis of the appendage should develop in abdominal segments. The primordia in A1 and A9 disappeared before larval hatching. Anal prolegs appeared separate from cerci, the main axes of appendages, which were formed temporarily in A11. The expression of *decapentaplegic*, which reflects the primary determination of appendages, was detected in the lateral juxtaposition with the prolegs. *Distal-less* was expressed in the main axes of appendages, protruding endites and the cerci, but not in prolegs and anal prolegs or the gnathal endites which do not protrude. These findings suggest a possibility that the abdominal and anal prolegs of *A. rosae* are outgrowths of ventral plates which derived from coxopodal elements, but not main axes of appendages.

Keywords

Proleg • Appendage • Endite • *decapentaplegic* • *Distal-less* • Hymenoptera

Introduction

Larval forms of insects vary, reflecting their lifestyle. Modifications of appendages in each body segment contribute to diversity, although the appendages are serially homologous structures (Angelini and Kaufman 2005). Analyses to elucidate how these modifications take place and the molecules involved have focused mostly on cephalic and thoracic appendages. Less attention has been paid to abdominal appendages since the absence of appendages in abdomen is one of the most outstanding derived features of insects; however, abdominal appendages appear during embryonic development in most insect orders and usually disappear before larval hatching (Matsuda 1976). Nevertheless, primitive insects retain abdominal appendages even in adults: Embryonic abdominal appendages develop to adult structures, such as styli and eversible sacs, in bristletails (Archaeognatha) and silverfish (Zygentoma). Some holometabolous species have abdominal appendages in the larval stage. The larvae of sawflies (Hymenoptera), moths, and butterflies (Lepidoptera) have remarkable abdominal appendages, known as prolegs. Although these two groups have similar caterpillar-like larval forms, the arrangement of prolegs is different. Insect abdomens generally consist of 11 metameric segments (A1–A11) and the telson. Most sawfly larvae bear prolegs in the abdominal segment A2–A8 and a pair of anal prolegs in the last segment, A11, whereas lepidopteran larvae usually have prolegs in segments A3–A6, and anal prolegs in A11.

Appendage development in the abdominal segments is regulated primarily by the Hox genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*) (Hughes and Kaufman 2002; Angelini and Kaufman 2005). *Ubx* and *abd-A* share functions to repress *Distal-less* (*Dll*) expression, resulting in the suppression of appendages from the anterior to middle abdominal segments. *Abd-B* specifies the identity of

posterior abdominal segments and suppresses appendage development. These Hox genes basically suppress appendage development in the abdomen, although their function differs between species having abdominal appendages. Although Ubx and Abd-A are distributed similarly in embryos of sawflies and lepidopterans, the prolegs of sawflies are considered to be the proximal parts (coxae) of the main axes of appendages lacking distal regions (Suzuki and Palopoli 2001) and those of lepidopterans to be the whole limb, consisting of both proximal and distal regions (Warren et al. 1994). Previous molecular studies on abdominal appendage development seem to take little account of the origin of the structures finally formed, and the underlying mechanisms forming these structures are not well understood. It is therefore necessary to identify the structure occurring in each abdominal segment for correct understanding of the molecular regulatory mechanisms and evolution of abdominal appendages.

In the present study, we examined the morphogenesis of appendages during embryonic development in the sawfly, *Athalia rosae*, by scanning electron microscopy and sectioning the body segments and analyzed gene expression patterns of *decapentaplegic* (*dpp*) and *Dll*. Our findings provide insights into the origin of the prolegs of the sawfly, which have previously been considered as the proximal parts (coxae) of appendages.

Materials and methods

Sawfly stocks

Laboratory stocks of *A. rosae* were maintained at 25°C under 16 -h light/8-h dark conditions (Sawa et al. 1989). Embryos with synchronized

developmental stages were obtained by artificial activation of mature unfertilized eggs, as described previously (Sawa and Oishi 1989). These embryos are haploid males and complete embryonic development in 120 h. Embryos were periodically collected and sacrificed for examinations.

Microscopy

Embryos were dissected out of eggs, and vitelline membranes and embryonic membranes were removed in 0.15 M NaCl for scanning electron microscopy. The embryos were fixed overnight at room temperature with alcoholic Bouin's fixative, transferred to 70% ethanol, cleaned with an ultrasonic cleaner for 10 s, post-fixed with 1% OsO_4 for 2 h, dehydrated in an ethanol series, and immersed in *t*-butyl alcohol. These were dried in a *t*-butyl freeze drier (VFD-21S; Vacuum Devise), coated with gold, and examined under a scanning electron microscope (SM300; Topcon).

For light microscopy, embryos were fixed with alcoholic Bouin's fixative, transferred to *n*-butyl alcohol, soaked, and embedded in Histosec tissue-embedding medium (Merck). Sections were cut at 5-µm thickness, stained with hematoxylin and eosin, and examined under a light microscope (BH2; Nikon).

Isolation of the *Distal-less* orthologue of *A. rosae*

Distal-less (*Dll*) orthologous cDNA was cloned by PCR-based methods. Total RNA was extracted from 72-h-old embryos using an RNeasy Midi kit (Qiagen). Poly(A)+ RNA purified with an mRNA purification kit (GE Healthcare) was used to prepare the RACE-ready cDNA libraries using a SMART RACE cDNA library Amplification kit (Clontech). Degenerate primers corresponding to the homeodomain of Dll were designed

(5’-GNAARGGNAARAARATGMGNAARCC-3’ and 5’-CTTCATCATYTTYTTRTAYTT-3’, where M=A+C, N=A+G+C+T, R=A+G, Y=C+G). PCR was performed using the RACE-ready cDNA libraries as template and an Advantage 2 PCR kit (Clontech). Adjacent 5’ and 3’ regions were obtained by the RACE method using gene-specific primers and the RACE-ready cDNA libraries as template.

PCR products were cloned into pCRII-TOPO vector plasmid using a TOPO TA Cloning kit Dual Promoter (Invitrogen). The inserted DNA fragments were sequenced using a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare) and analyzed with a DNA sequencer (ABI Prism 377; Perkin Elmer). The sequence of *A. rosae Dll* (*Ar Dll*) cDNA was deposited in the DDBJ/EMBL/GenBank database with accession no. AB378321.

Whole-mount *in situ* hybridization

The RNA probes used were a 252 bp-long fragment corresponding to highly conserved region in the *Athalia rosae decapentaplegic* (*Ar dpp*) cDNA (DDBJ/EMBL/GenBank database, AB121072; Yamamoto et al. 2004) and a 304 bp-long fragment corresponding to a region spanning a part of ORF and the 3’ UTR of *Ar Dll* cDNA (nucleotide positions 1,097–1,400). Digoxigenin-labeled RNA probes were prepared using a DIG RNA labeling kit (SP6/T7; Roche). Embryos from which vitelline membranes, embryonic membranes, and yolks had been removed were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), transferred to PBT (0.1% Tween-20 in PBS), dehydrated with an ethanol series, and then rehydrated and subjected to hybridization. Pre-hybridization, hybridization with 2 μg/ml each of probes, washing, and detection of signals were performed as described by Yamamoto et al. (2004). The specimens were observed under a binocular microscope

(MZ16F; Leica).

Results and discussion

Morphogenesis of appendages

We first examined the morphogenesis of appendages in detail by scanning electron microscopy (SEM). In *A. rosae* germ band formation begins at about 24 h after egg activation (24-h AEA) and dorsal closure completes at about 70-h AEA (Sawa et al. 1989). Segmentation and appendage formation occur during this period, so we focused on the developmental stages from 22-h through 70-h AEA.

Segmentation started in the head and thorax at 22-h AEA, and the cephalic (preantennal and antennal), three gnathal (mandibular, maxillary, and labial), and three thoracic (T1–T3) segments were formed. Primordia of appendages in each segment appeared shortly after segmentation. Primordia of antennae developed in the head lobe, and the mandible, maxilla, and labium developed in the respective gnathal segments. The clypeus was formed anterior to the mouth opening (stomodeum). A pair of limb buds appeared in each thoracic segment. Segmentation of the abdomen began at 24-h AEA, and 11 abdominal segments were formed.

Maxilla, labium, and thoracic legs elongated and segmented into proximal coxopodites and distal telopodites (28- to 32-h AEA, Fig. 1a, b). Mandibles enlarged, but lacked telopodite regions. Telopodites of the maxilla and labium differentiated into maxillary and labial palpi, respectively. During 32- to 38-h AEA, the inner lobes (endites) of the maxilla (lacinia and galea) and labium (glossa–paraglossa composite) began appearing from their coxopodites (Fig. 1a). Each telopodite region of the thoracic legs further differentiated into two regions:

trochanter+femur and tibia+tarsus+pretarsus. The appendage primordia were yet to be detectable in abdominal segments at this stage (Fig. 1c, d).

The medial region of the mandible swelled to form two endites, and they were clearly distinguished as molar and incisor during 38- to 52-h AEA (Fig. 1e). The molar and incisor corresponded in position to the lacinia and galea of the maxilla, respectively. Paired labral anlagen appeared on the clypeus, resulting in the formation of clypeolabrum. Segmentation of thoracic legs proceeded to differentiate four telopodal segments: trochanter, femur, tibia+tarsus, and pretarsus (Fig. 1f). A pair of swellings appeared in the first through 11th abdominal segments (A1–A11), except for the tenth segment (A10, Fig. 1g, h). The appearance of a pair of swellings in A11, most probably the cerci, was slightly delayed (Fig. 1h, arrows).

During katatrepsis (52- to 60-h AEA), subdivision of the coxopodites into proximal subcoxa and distal coxa took place in the main axes of ventral appendages (mandible, maxilla, labium, and thoracic legs; Fig. 1i, j, open arrows). The demarcation of mandibular endites, molar and incisor, became ambiguous (Fig. 1i). The maxillary endites (lacinia and galea) protruded, whereas the labial endites (glossa–paraglossa composite) enlarged without protrusion (Fig. 1i). Subdivisions did not occur in endites. Segmentation of the thoracic legs completed as the tibia and tarsus separated (Fig. 1j), although the tibia and tarsus later fused to become the tibiotarsus. Abdominal swellings in A2–A8 enlarged without any subdivisions to become prolegs (Fig. 1k, l). In contrast, the swellings in A1, A9, and A11 (cerci), which were initially prominent, gradually degenerated and disappeared before larval hatching (Fig. 1k, l). As dorsal closure proceeded (60- to 76-h AEA), A10 was reduced, being restricted in a small dorsal area. In segment A11, a pair of swellings developed in the area distinct from where the cerci had appeared temporarily (Fig. 1l inset, asterisks). These swellings enlarged and finally became anal prolegs.

Our observations do not conflict with the currently accepted consensus that the prolegs are appendicular in origin or a part of appendages. The prolegs resemble the developing endites of the gnathal appendages because they do not show the subdivision of the coxopodite that is common to all appendages except for the antenna. This suggests that the prolegs consist of appendicular components but are not the main proximal–distal axis of the appendages, but rather are serially homologous to endites. The anal proleg is probably not the main axis of appendages in A11, either because the main appendage in this segment is reduced before the formation of the anal proleg bud.

Allocation of the proleg in an abdominal segment

SEM examination of embryos, which had begun katatrepsis, revealed that abdominal prolegs in segments A2–A8 were positioned medioventrally compared to the thoracic legs (Fig. 1k). We ascertained the position of prolegs in an abdominal segment by histological examination, comparing the position to that of a thoracic leg. Cross-sections of the second thoracic (T2) and fourth abdominal (A4) segments of a 53-h AEA embryo were examined (Fig. 2a, b). The spiracles and ventral ganglia were located at comparable positions in the T2 and in the A4 segment. The telopodite of the thoracic leg developed from the coxopodite located below the spiracle opening (Fig. 2a). The proximal ventral area of the thoracic leg expanded toward the ventral midline to form the eusternum (Fig. 2a, arrowheads). The eusternum is the secondary sternum derived from coxopodal elements, and it covers most of the venter of the segment. There is evidence that the abdominal ventral plate is homologous to the eusternum in thoracic segments (Uchifune and Machida 2005). The proleg was located medioventrally in comparison to the thoracic leg (Fig. 2b), positioned at the expanded ventral plate (eusternum). There was no appendicular

structure in the corresponding area where the telopodite of the thoracic leg developed (Fig. 2b, asterisk). These findings suggest that the abdominal prolegs are outgrowths from the ventral plates and thus homologous to endites.

Abdominal appendages of the primitive insects, bristletails (Archaeognatha), are retained in adults as styli and eversible sacs. The styli are regarded as remnants of the telopodites of appendages and the eversible sacs as coxopodal endites (Bitsch 1994). The presence of endite-like abdominal appendages has been reported in some holometabolous insects: caddisflies (Trichoptera; Kobayashi and Ando 1990), scorpionflies (Mecoptera), and hangingflies (Mecoptera; Du et al. 2009). Two pairs of swellings, termed ventral and medial swellings, appear in abdominal segments A1–A8 during embryogenesis of the caddisfly, *Nemotaulius admorsus* but all disappear before larval hatching. Kobayashi and Ando (1990) interpreted that ventral swellings correspond to the coxopodites of appendages and medial swellings to the coxopodal endites based on their arrangement. Similarly, in some mecopterans, two pairs of swellings, termed lateral and median processes, develop in the first eight segments during embryogenesis. Lateral processes are considered to be homologous to coxopodites of thoracic legs. Du et al. (2009) demonstrate that the lateral processes of a scorpionfly, *Panorpa emarginata*, finally flattened to become part of the lateral plates (pleura) in larval abdomen. In contrast, the median processes formed along the medioventral line become the abdominal prolegs that remain during larval stages in some mecopterans (Du et al. 2009). The prolegs of sawflies could be considered to correspond to the medial swellings of caddisflies and the median processes of mecopterans. It therefore seems not unusual for abdominal prolegs to be swollen from ventral coxopodites in Holometabola.

Expression patterns of *decapentaplegic* and *Distal-less*

We have demonstrated that the expression of the *decapentaplegic* (*dpp*) gene reflects the primary determination of appendage development in *A. rosae* embryos (Yamamoto et al. 2004). We reexamined *Ar dpp* gene expression in each segment in detail to determine the exact position of its expression (Fig. 3a–e). Whole-mount *in situ* hybridization of a 48-h AEA embryo revealed that *Ar dpp* was expressed in the clypeolabrum, antennae, mandibles, maxillae, labia, and the distal tips of each thoracic leg. In addition, the *Ar dpp* signals were detected in the proximal dorsal regions of the main axes of gnathal appendages (Fig. 3b). Although *Ar dpp* was expressed in abdominal segments A1–A9 and A11, the exact position was not in the prolegs. *Ar dpp* expression was in the ectoderm, laterally juxtaposed to the proleg primordia in A2–A9 (Fig. 3c, d). We suggest that this lateral expression spots in the abdomen correspond to the spots of expression in the proximal dorsal region of the gnathal appendages. This interpretation further supports our conclusion that the prolegs are not the main proximal–distal appendage axis, but are homologous to ventral coxopodite outgrouwth (endites). *Ar dpp* was expressed in temporarily formed cerci in A11 (Fig. 3e), but not in the anal prolegs, again indicating that the cerci but not the anal prolegs are the main appendage axis in this segment.

The expression pattern of another key gene, *Distal-less* (*Dll*), was examined. We cloned the *Dll* orthologue of *A. rosae* (*Ar Dll*; DDBJ/EMBL/GenBank database, AB378321). Whole-mount *in situ* hybridization using a 48-h AEA embryo showed *Dll* gene-specific signals. *Ar Dll* was expressed in the clypeolabrum and antennae (Fig. 3f). There was no *Ar Dll* expression in the mandible (Fig. 3g), while maxillary and labial palpi, the main axes of appendages, showed *Ar Dll* expression (Fig. 3h, i). *Ar Dll* was expressed in the maxillary endites (lacinia and galea),

whereas it was not expressed in the mandibular endites (molar and incisor) and the labial endites (glossa–paraglossa composite; Fig. 3g–i). *Ar Dll* expression appeared in the thoracic legs as a stripe at the trochanter–femur joint and the region distal to the tibia, tarsus, and pretarsus (Fig. 3f). These were the typical expression patterns of *Dll* in insects, described as ring and sock. No *Ar Dll* expression was detected in the proleg primordia in abdominal segments except for A11 in which it reflected the development of cerci (Fig. 3f, j). In summary, *Ar Dll* was expressed in the main axes of appendages and the protruding endites, whereas it was not expressed in the prolegs, anal prolegs, and endites that did not protrude.

In many insects, the expression of *Dll* is detected in the distal region of appendages, so the *Dll* expression has been often used as a telopodite marker; however, this is unlikely. *Dll* expression other than in telopodites has been reported in several species. Giorgianni and Patel (2004) showed that *Dll* is expressed in the endites in the red flour beetle, *Tribolium castaneum* (Coleoptera) and the grasshopper, *Schistocerca americana* (Orthoptera). *Dll* expression is detected in the caudal filament of the firebrat, *Thermobia domestica* (Zygentoma; Ohde et al. 2009). Although the origin of the caudal filaments is debated (prolongation of the dorsal plate of A11 (Matsuda 1976) vs. elongation of the telson (Tojo and Machida 1997)), there is consensus that the caudal filaments are not homologous to appendages. *Dll* is also expressed in the horns of beetles belonging to the genus *Onthophagus* (Moczek et al. 2006). On the other hand, *Dll* expression is absent in the mandible and its endites (molar and incisor) in insects examined to date. A common feature of *Dll*-expressing structures is that they protrude in a certain direction rather than simply outgrow. These findings taken together corroborate that *Dll* is expressed in protruding structures irrespective of their origin. Absence of *Dll* expression in the prolegs of *A. rosae* would not be a reason to conclude that the prolegs are the proximal parts (coxae) of the main appendage axis.

Alternatively, the lack of *Dll* expression could be linked to the fact that the prolegs grow only little and do not protrude very much.

Possible interpretation of the origin of sawfly prolegs

Appearance of abdominal prolegs in the insect lineage is thought to have occurred independently by derepression of gene regulatory networks to suppress appendage development (Nagy and Grbic 1999). Modification of the regulatory interactions between suppressive Hox genes and the downstream target genes will allow prolegs to remain in the abdomen. Whole limb-type prolegs of Lepidoptera are considered to be the secondary appearance of appendages with proximal and distal parts in specific abdominal segments. This is caused by redeployment of appendage development processes, while the gene interactions to form final structures seem to vary among species: permissive *Dll* expression in the absence of Ubx/Abd-A (Warren et al. 1994; Suzuki and Palopoli 2001) and the proleg promoting function of *abd-A* (Tomita and Kikuchi 2009).

The abdominal prolegs of sawflies have been considered as the coxopodites of main axes of appendages (Suzuki and Palopoli 2001). In contrast, our findings suggest that sawfly prolegs are the outgrowths of coxopodites, having possible endite nature. Abdominal segments of insects seem to have potential to develop appendicular components during embryogenesis as the ground state since swellings representing appendage primordia appear in embryonic stages in most insect orders (Matsuda 1976). We assume that sawflies as well as the mecopterans and trichopterans developing endite-like swellings recruit the mechanisms to develop endites in gnathal segments to the abdomen, though another possibility that they are novel structures has not been eliminated. In sawflies and some mecopterans, after the recruitment of the endite-like swellings to the abdomen, these structures would escape later repression

and would thus remain after larval hatching to give rise to abdominal prolegs. The anal prolegs are swollen from ventral plates and apparently not the main appendage axes (cerci) of A11; however, a question whether they are the serial homologues to abdominal prolegs remains.

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Figure legends

Fig. 1

SEM micrographs of appendage morphogenesis in *A. rosae* embryos. Embryos of 28- to 38-h AEA (**a**–**d**), 38- to 52-h AEA (**e**–**h**), and 52- to 60-h AEA (**i**–**l**) are presented. Each panel shows cephalic regions (**a**, **e**, **i**), thorax (**b**, **f**, **j**), anterior abdomen (**c**, **g**, **k**), and posterior abdomen (**d**, **h**, **l**). *Arrowheads* in **b** indicate the boundary between telopodite and coxopodite. *Inset* in **h** is the lateral view of A11 segment, and *arrows* indicate temporarily appearing cerci. *Open arrows* in **i** and **j** indicate the boundary between coxa and subcoxa. *Inset* in **l** shows the lateral view of posterior segments of 70-h AEA embryo, and *asterisks* mark a pair of anal prolegs. *A1–11*: abdominal segments 1–11, *An*: antenna, *Lb*: labium, *Lr*: clypeolabrum, *Md*: mandible, *Mx*: maxilla, *T1–3*: thoracic segments 1–3, *cx*: coxa, *fe*: femur, *ga*: galea, *gpc*: glossa–paraglossa composite, *in*: incisor, *la*: lacinia, *lbp*: labial palp, *mo*: molar, *mxp*: maxillary palp, *pta*: pretarsus, *ta*: tarsus, *ti*: tibia, *tr*: trochanter.

Fig. 2

Cross-sections of the second thoracic (**a**) and fourth abdominal (**b**) segments of a 53-h AEA embryo show the allocation of thoracic legs and abdominal prolegs. *Arrowheads* indicate expanding ventral plates (eusterna) derived from coxopodites in the thoracic segment. Abdominal proleg (*plg*) positioned medially to thoracic leg (*tlg*). Proleg develops in the location corresponding to the expanding eusternum in the thoracic segment. *Asterisks* indicate the relative position where main axis of appendage is to appear in the abdominal segment. *vg*: ventral ganglion, *sp*: spiracle.

Fig. 3

Whole-mount *in situ* hybridization of embryos (48-h AEA) using antisense-RNA probes for *Ar dpp* (**a**–**e**) and *Ar Dll* (**f**–**j**). *Ar dpp* is expressed in all cephalic appendages, thoracic legs and abdominal segments A1–A9 and A11 (**a**). *Ar dpp* expression in gnathal segment (mandible) is restricted to proximal dorsal region (**b**). Signals in A2–A9 are located on the lateral juxtaposition (*arrowhead*) of the prolegs (*arrow*)(**a**, **c**). Transverse view of A4 shows *Ar dpp* signal on the ectoderm (**d**). In A11, *Ar dpp* is expressed in the cerci (**e**). Expression of *Ar Dll* is detected in cephalic appendages: antennae, clypeolabrum, maxillary palp, labial palp, and thoracic legs (**f, h**, **i**). There is no expression in abdominal segments except for the cerci in A11 (**f**, **j**). Signals are detected in protruding endites of maxilla (lacinia and galea) (**h**), while signals are absent in mandible (**g**) and labial endite (glossa–paraglossa composite) (**i**). *A1–11*: abdominal segments 1–11, *An*: antenna, *Lb*: labium, *Lr*: clypeolabrum, *Md*: mandible, *Mx*: maxilla, *T1–3*: thoracic segments 1–3, *ga*: galea, *gpc*: glossa–paraglossa composite, *in*: incisor, *la*: lacinia, *lbp*: labial palp, *mo*: molar, *mxp*: maxillary palp, *vm*: ventral midline.

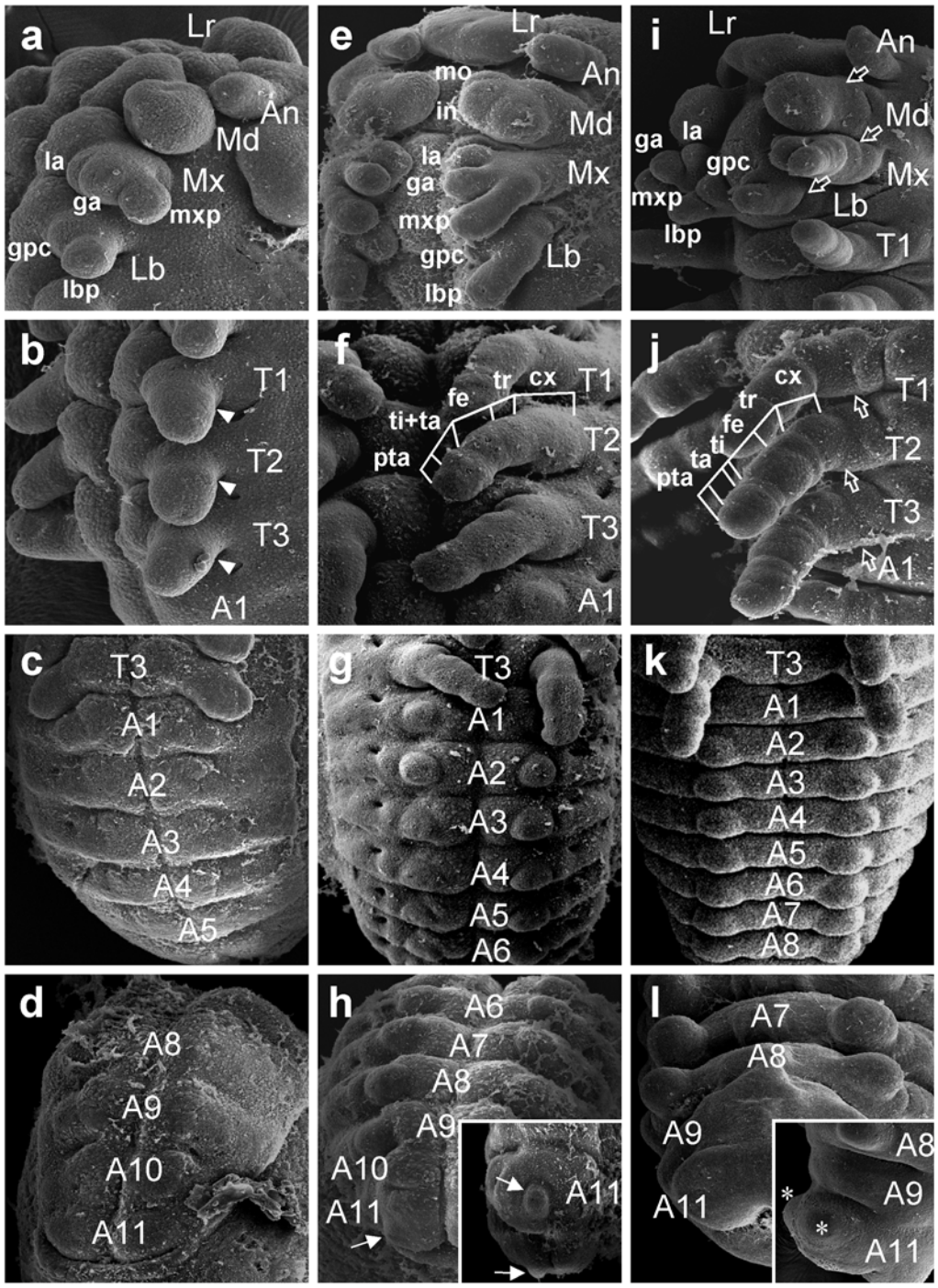

Fig. 1, Oka et al.

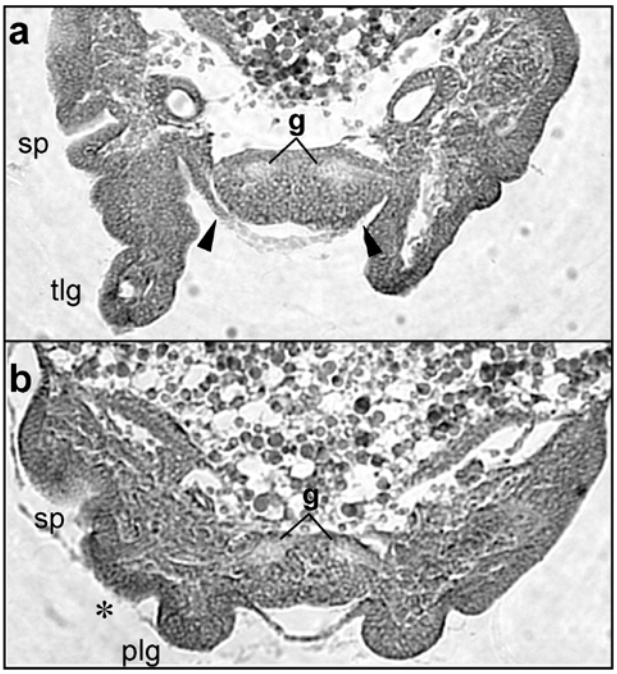

Fig. 2, Oka et al.

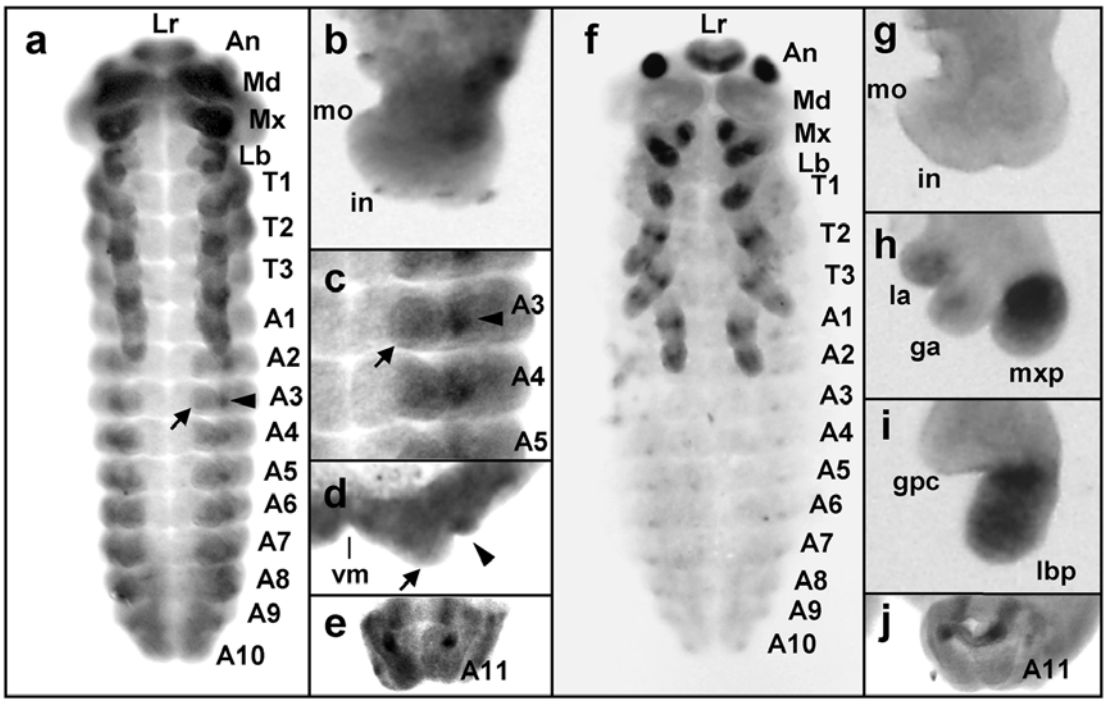

Fig. 3, Oka et al.