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PATTERNING DEFECTS IN SILKWORM EMBRYOS ANALYSED THROUGH CUTICLE PREPARATIONS

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The mulberry silkworm, *Bombyx mori*, a holometabolous lepidopteran insect, has a metameric body plan. Due to its functional adaptation, *B. mori* presents some unique deviations in its pattern from the evolutionarily advanced dipteran insect, *Drosophila melanogaster*. Previous studies on mutant phenotype analysis in *B. mori* have been carried out in late stages of larval development. Here we employ, the cuticle preparation approach during embryonic development to study morphological landmarks associated with *B. mori*, *Eri*, another race of silkworm, and pattern defects associated with *Ekp* mutant of *B. mori*. The homeotic mutant *Ekp*, generates ectopic abdominal legs, a feature previously documented only during larval development. Using cuticle preparation approach the patterning defects of extra abdominal legs of *Ekp* mutation could be detected as early as 7.5 day old embryo. This approach can be exploited to study patterning defects in *B. mori* mutants affecting early development.

Keywords: *Bombyx mori*, *Drosophila melanogaster*, developmental patterning, *Philosomia cynthia ricini*, cuticle preparation.

INTRODUCTION

The mulberry silkworm, *Bombyx mori*, with the availability of the complete genomic sequence information (Xia *et al.*, 2004) is developing into an excellent system to study the genetic and molecular aspects of patterning similar to the prototype model insect *Drosophila melanogaster*. Classical mutagenesis screens have revolutionized the *Drosophila* genetics by generating a number of mutants exhibiting patterning defects during embryonic development (Nusslein-Volhard *et al.*, 1984). In these studies, the cuticle preparation approach was employed to study pattern defects during embryonic development (Van der Meer, 1977). The rationale was that *Drosophila* embryo has a highly regular and reiterative pattern of cuticular processes, denticles, and naked cuticle on the ventral side of the embryo. Any pattern defect of denticles/ naked cuticle in the mutant background was assigned as the function of that gene. The extended embryonic development period of 10 days in *B. mori*, offers the opportunity to discretely analyse the different patterning events. However, cuticle preparations have not been employed so far in silkworm to study the patterning defects. Despite having a large repository of mutants, there is not enough information about the genes that are involved in early embryonic patterning as most of the mutant phenotypes have been studied only during late larval and pupal

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development. The study of these unique patterning landmarks in *B. mori* using cuticle preparation approach, thus, can be of great significance in understanding genetic basis of patterning in embryo.

We have presented here, the use of cuticle preparation technique to study embryonic patterning in *B. mori*. Comparisons have also been made on the cuticle phenotype of *B. mori* with other wild race like *Philosomia cynthia ricini* (Eri). Even though Eri belongs to the same order and shares common ancestry, they have segregated long ago from *B. mori*. We have also used a homeotic mutant, *Ekp* (member of E complex homeotic mutant of *B. mori*) that show transformation in the fate of segmental identity to demonstrate the utility of our approach. Earlier studies were able to focus on patterning defects in *Ekp* as well as other mutations only during late larval stages (Ueno *et al.*, 1992; 95). However, the present approach can shift the time window of pattern defect analysis to as early as embryogenesis. These cuticle markers can be exploited genetically or experimentally in discerning the roles of genetic loci involved in patterning of the silkworm embryo.

MATERIAL AND METHODS:

Eggs were collected from a synchronous culture maintained at 24°C. Various races used for these studies are: Domesticated strains of mulberry silkworm, Bivoltine strain NB4D2 of *B. mori*, (L) (*Bombycidae*), Eri silkworm, *Philosomia cynthia ricini* (Hutt: *Saturniidae*), a wild species reared on castor leaves (*Ricinus communis*). We also used a bivoltine *Ekp* mutant stock (*Ekp*, Y (yellow blood) and F (flesh cocoon) markers) to study the mutant phenotypes.

Cuticle Preparations:

Embryos were dechorionated following the protocol of Singh and Gopinathan (1997). The dechorionated embryos were rinsed in distilled water. The dechorionated eggs were boiled in 10% KOH for 10 min. and washed with water. The embryos were fixed in acetic acid: glycerol (4:1) for 24h, and were mounted in Hoyer's mountant (recipe in Van der Meer, 1997). Weights were applied on the cover slip to flatten the tissue. The preparations were incubated at 40°C for 2-3 days to allow the clearing of the tissue and were observed under dark field optics of Leica DMRBE Microscope.

RESULTS & DISCUSSIONS

Cuticle Pattern:

B. mori egg is a flattened sphere, 1.2 mm long, 0.95 mm wide and 0.63 mm thick, which gives rise to first instar larva after 10 days of embryonic development. A larval prototype is formed as early as 7.5 days of embryonic development in terms of external morphological landmarks (Tazima, 1978; Singh and Gopinathan, 1995; for review see Gopinathan *et al.*, 1998). *B. mori* larva has a metameric body plan comprising of head, thorax and abdomen (Fig. 1a). The head is chitinous in nature and bear the segments contributing to the mouthparts. The thorax has three segments *viz.*, pro-, meso-, and meta-thorax each bearing a pair of thoracic legs on the ventral side (Fig. 1a). Abdomen is formed of eleven segments (last three are fused to form a single segment). Ventrally, the A3-A6 and A9 bear the abdominal legs or pseudopods (Fig. 1a). Abdominal legs are functional adaptation of silkworm, which are lost during the larval to pupal metamorphosis (Suzuki *et al.*, 2001; Singh *et al.*, 2007). The basic unit of pattern is a segment, which comprises of rows of cuticular bristles along the entire circumference and a naked cuticle. Unlike *Drosophila* where the pattern of bristles on the dorsal and ventral ectoderm is different, silkworm exhibits a regular bristle pattern on both dorsal and ventral surface.

In bivoltine stock NB4D2, the cuticle exhibits clusters of bristles arranged in horizontal rows interspersed with the naked cuticle all along the length of the body.

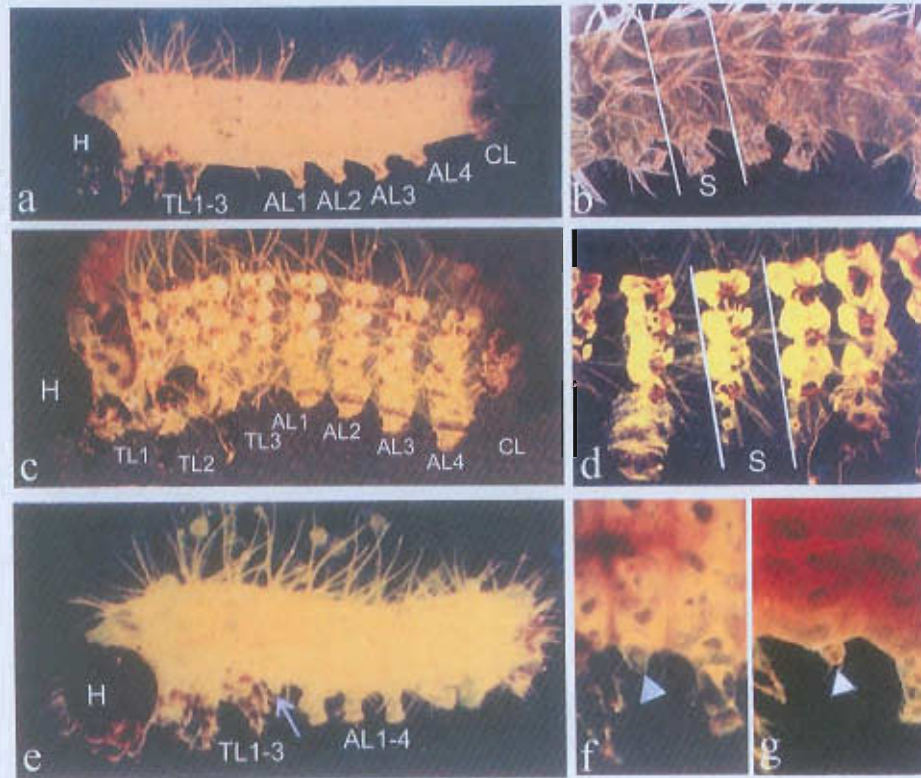


Fig. 1 Embryonic cuticular preparation exhibiting metameric body plan in a 7.5 day old embryo of (a) *B. mori* (NB4D2); (c) *P. ricini*.

Magnified view of cuticle preparations of 7.5 day old embryo of (b) *B. mori* strain (d) *P. ricini*, showing characteristic pattern of the arrangement of bristles and naked cuticle. (e-g) Cuticle phenotypes, in *Ekp* mutant embryo of *B. mori* (e) 7.5 day embryo showing an extra abdominal leg (arrow) in the A2 segment (f, g) magnified view of abdominal segment bearing extra leg (arrowhead).

(H; head; TL; thoracic leg; AL; abdominal leg; CL; caudal leg, S; segment).

Fig. 1. Préparation cuticulaire embryonnaire présentant le plan d'organisation métamérique chez un embryon de 7,5 jours de (a) *B. mori* (NB4D2) ; (c) de *P. ricini*.

Agrandissement des préparations de cuticule d'un embryon de 7,5 jours de la souche *B. mori* (b) de *P. ricini* (d) présentant un schéma caractéristique de disposition des soies et de la cuticule nue (e-g)

Phénotypes de la cuticule, chez l'embryon du mutant *Ekp* de *B. mori* (e) embryon de 7,5 jours présentant une patte abdominale supplémentaire (flèche) dans le segment A2 (f, g), agrandissement du segment abdominal présentant une patte supplémentaire (pointe de la flèche)

(H ; tête ; TL patte thoracique ; AL ; patte abdominale ; CL ; patte caudale ; S ; segment)

The two bands of bristles are separated by a broad band of naked cuticle that is twice in width as compared to the band of bristles (Fig. 1a). Each cluster has 3-4 long serrated bristle like structures, which originate separately from basal cells (Fig. 1b).

Eri silkworm also presents a similar pattern of bristle rows with interspersed naked cuticle (Fig. 1c). However, there is difference in the number of bristles and their number in individual cluster. Each cluster has 4-5 serrated bristles originating from a common basal cell (Fig. 1d), which is a characteristic of Eri. The band of bristles has a characteristic pigmentation all along its width.

Ekp Mutant Phenotype:

The E complex mutants of *B. mori* are characterized by an ectopic leg appendage in the second abdominal (A2) segment (Fig. 1e). Abdominal legs of *B. mori* are functional adaptation and are lost during the larval to pupal metamorphosis (Suzuki *et al.*, 2001; Singh *et al.*, 2007). There is very low penetrance in extra leg phenotype of *Ekp* mutants. The embryonic mutant phenotype can be broadly classified into two types:

1. A small projection from the ventral surface of the A2 segment cuticle. These projections do not bear any distal siphon like cuticular projections as seen in the wild-type abdominal legs (Fig. 1f).
2. The A2 specific legs appearing as miniaturized version of A3. The projection in A2 segments bear rudimentary bristles at their tip and are half the size of the wild-type abdominal leg (Fig. 1g).

The present study clearly reveals that cuticle patterns are characteristic of every species of silkworm. Although Eri silkworm shares a common ancestry with *B. mori*, they have developed a few divergent characteristics over a period of time. The characteristic pattern of pigmentation in each segment in band of bristles in Eri is possibly a functional adaptation of larva to prevent itself from predators. The segmental arrangement in Eri is similar to NB4D2 but the pattern of bristles is dramatically different. The nature of the bristles is characteristic to the races. These studies reveal that despite sharing the common ancestry and being closely related, there is a characteristic cuticle pattern associated with different species of silkworm. These cuticle patterning elements can be exploited as identification characters and can also be used for early detection of the mutant phenotypes associated with the larval and adult structures.

Our studies with E complex mutants revealed that homeotic transformation of A2 to other abdominal segments (A2-A6) can be documented as early as 7.5 days of embryonic development. Interestingly, the mutation in E complex resulted in change of fate of A2 but it did not result in an identical wild-type abdominal leg. This indicated that homeotic transformation pathway and growth are not tightly coupled during development.

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