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CROSSROADS, MILESTONES AND LANDMARKS IN INSECT DEVELOPMENT AND EVOLUTION: IMPLICATIONS FOR SYSTEMATICS

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ABSTRACT

Our understanding of insect development and evolution has increased greatly due to recent advances in the comparative developmental approach. Modern developmental biology techniques such as *in situ* hybridization and molecular analysis of developmentally important genes and gene families have greatly facilitated these advances. The role of the comparative developmental approach in insect systematics is explored in this paper and we suggest two important applications of the approach to insect systematics—character dissection and morphological landmarking. Existing morphological characters can be dissected into their genetic and molecular components in some cases and this will lead to more and richer character information in systematic studies. Character landmarking will be essential to systematic studies for clarifying structures such as shapes or convergences, which are previously hard to analyze anatomical regions. Both approaches will aid greatly in expanding our understanding of homology in particular, and insect development in general.

Key words: *Drosophila*, germ-band formation, insect development, insect evolution, insect systematics

INTRODUCTION

The role of developmental information in systematics has been twofold (Shubin 1994). First, embryological information has been used to establish hypotheses of homology among characters in systematic analyses and second the information has been used to polarize characters (the ontogenetic criterion; Nelson 1978; Patterson 1982). Both of these implementations of developmental data use the principle of recapitulation and observation of embryos during development to establish homology and polarity respectively. While character polarity and homology are essential for clarifying and using existing morphological characters in systematic analyses, more modern molecular tech-

niques can add to systematic analyses in other equally important ways.

In particular, molecular comparative biology of development allows the discovery of new morphological characters and more detailed dissection of existing morphological characters. In order to detail how modern developmental biology can interact with systematic analyses we need to first show what kinds of morphological information this new technology can provide, and to place this new information in the context of systematics. The purpose of this paper, therefore, is twofold. The first objective is to give the reader an indication of the state of the art of developmental biology and genetics in the model organism *Drosophila melanogaster* and to relate this knowledge to insect systematics. The summary presented herein should demonstrate both the great potential of insect development systems and the difficulties associated with collecting the developmental information from a wide variety of taxa for systematic purposes. The second objective of this paper is to make some suggestions as to how developmental analysis can and will be used in future systematic studies of insects.

CROSSROADS AND MILESTONES

Modern developmental biology and evolutionary biology have recently converged at a significant and productive crossroads. Developmental biologists have recently spread out from their favorite model organisms to impart an evolutionary aspect to their understanding of development. *Drosophila* specialists have recently recognized the importance of applying developmental techniques and approaches to organisms other than *D. melanogaster* (Patel 1994*a, b*; Carroll 1994; Tautz 1994; Tautz et al.1994; Akam et al. 1994). Other invertebrate developmental biologists and vertebrate specialists have applied their approaches to questions related to evolution (Kenyon 1994; Davidson 1991, 1994; Tabin 1992; Gurdon 1992; Wolpert 1994; DeRobertis 1994; Duboule 1994; Sander 1994; Laufer and Marigo 1994; Burke et al. 1995; Zuker 1994). Following the insightful lead more than 10 years ago of Raff and Kaufman (1983), evolutionary biologists are again taking a hard look at developmental biology as a source of information in understanding evolutionary mechanisms and pathways and also in systematics (Conway-Morris 1994; Wray and Bely 1994; Dickinson et al. 1993; DeSalle and Grimaldi 1993; Jacobs 1990, 1994; Davidson 1991, 1994; Bitsch 1994).

One of the factors that has most assuredly facilitated this broadening of interests by both developmental and evolutionary biologists is the availability of a wide variety of techniques for developmental analysis. For the most part, these techniques have been unavailable for organisms other than model systems. If not for the model systems though, these tools and techniques would not exist and much of the broadening of the comparative method has these model systems to thank. In insect studies advancement of *Drosophila* as a model system (Rubin 1989; Merriam et al. 1991; DeSalle and Grimaldi 1993) has been a critical step in reestablishing insect development as a part of evolutionary studies. The development of *Drosophila* as a model system has proceeded largely because of three historical events that have or will reach significant anniversaries by 1995. These three milestones are the clarification of the term homeosis (Bateson 1894; McGinnis 1994; Gehring 1993; Lewis 1994; Laufer and Marigo 1994), the classification of early developmental events via massive mutant screens (Nüsslein-Volhard and Weischaus 1980) and the discovery of the homeobox using molecular techniques (McGinnis et al. 1984*a, b*; Scott and Weiner 1984). William Bateson's (1894) coining of the term homeosis[1] and clarification of its role in producing morphological variation in organisms was an important first step in the unraveling of the genetics of development. Undoubtedly, this clarification of the role of homeosis in morphological evolution such as first described by Bateson (1894) has greatly advanced our knowledge of development. One need look no further than Lewis (1978) or Kaufman et al. (1980) and the discovery and characterization of the Bithorax (BX-C) and Antennapedia (ANT-C) Complexes, respectively in *D. melanogaster* to grasp the importance of Bateson's clarification of the term homeosis.

Equally important as the pioneering work done by Bateson (1894) is the second milestone accomplished 15 years ago, the immense mutant screening for maternal effect genes and early embryonic genes conducted by Nüsslein-Volhard and Weischaus (1980). Up to the time of this 1980 study only a handful of maternal effect and embryonic lethal mutants had been characterized. This work established the developmental hierarchy in *Drosophila* discussed below, and is remarkable in that the majority of mutants isolated by

[1] Morphological anomalies were known to be present in a wide variety of organisms in Bateson's time. In particular, plant biologists had characterized several monstrous plant forms (*The Metamorphosis of Plants*: Goethe in Mueller 1952; *Vegetable Teratology*: Masters 1869), and indeed Bateson refers to these works in some detail. Bateson was the first to propose that a particular class of morphological changes that he documented so beautifully in *Materials for the Study of Variation: Treated with Especial Regard to Discontinuity in the Origin of Species* were unique in their expression (Lewis 1994). He suggested that this class of morphological changes was different because it involved not just alterations in the basic morphology of an organism, but transformations of one body part into another: "For the word 'Metamorphy' I therefore propose to substitute the term Homeosis; for the essential phenomenon is not that there has merely been a change, but that something has been changed into the likeness of something else" (Bateson 1894; p. 85).

these workers were embryonic lethal. The clarification and characterization of homeotic transformations in a wide variety of organisms by Bateson (1894) and early developmental genes in *Drosophila* (Nüsslein-Volhard and Weischaus 1981) laid the groundwork for the third milestone alluded to above: the 10th anniversary of the discovery of the homeobox (McGinnis et al. 1984*a, b*; Scott and Weiner 1984). The discovery of this protein motif and its subsequent characterization gave molecular biologists a foothold into developmental processes (reviewed in McGinnis 1994 and Gehring 1993). One significant contribution from these molecular studies is that the homeobox genes in a wide variety of organisms are very similar in sequence. The sequence similarity of these genes allowed a great leap forward in developmental biology because important developmental genes could be obtained relatively simply and compared across large phylogenetic distances. Most importantly for our discussion, the techniques of antibody staining and *in situ* hybridization of probes to embryos were developed as a direct result of these molecular studies. Antibody staining and *in situ* hybridization as well as enhancer trap methods (Bier et al. 1989; Bellen et al. 1989; Wilson et al. 1989) facilitated the direct localization of developmental gene products in developing *D. melanogaster* embryos. These techniques allow the comparison of altered and wild type gene function in the fruit fly by direct determination of the spatial organization of gene products.

More recently, developmental biologists and molecular biologists have begun to broaden the organismal base for spatial expression studies of developmentally important genes. Davidson (1994) offers a lucid review of the growth and the state of the art in the field of "developmental molecular biology." In order to discuss some of the exciting results obtained from the molecular approach to comparative biology and their implications in systematics, some background on the processes involved in the spatial organization of the embryo and its gene products is necessary. Obviously, space restrictions do not allow a thorough discussion of all of the nuances of *Drosophila* development. However, the following discussion should provide an adequate background yet instill in the reader the notion that development of even this relatively simple insect is quite complex.

THE COMPARATIVE MOLECULAR APPROACH

Axes, Germ-Bands and Secondary Fields in Insect Development

One area of the tree of life that has experienced a relatively intense organismal sampling of developmental processes are the Arthropods. In particular, a large number of research programs have exploited the vast knowledge and technical expertise that is centered around the understanding of the development of the model research organism, *D. melanogaster* (Ransom 1982; Ashburner et al. 1978; DeRobertis et al. 1986; Ashburner 1989; Campos-Ortega and Hartenstein 1985; Lawrence 1992; Bate and Martinez-Arias 1993; Lindsley and Zim 1992) to examine other insect systems. In addition, excellent classical treatments of insect morphology and development exist (Sander 1976; Matsuda 1976; Snodgrass 1935; Schwalm 1988; Anderson 1972*a, b,* 1973) that allow the application of developmental principles and patterns obtained from the study of *Drosophila* to a wider array of insect taxa.

The initial determination of the spatial and temporal distribution of gene products in the developmental hierarchy of *D. melanogaster* resulted in several surprising discoveries such as the existence of protein gradients in the early embryo and distinctive "banding" or "striping" patterns of gene products in the developing embryo (Fig. 1). The targets of molecular studies in insect development can be conveniently separated into two areas that roughly coincide with Davidson's (1994) "initial spatial specification mechanisms" and limb "pattern formation" subdivisions. These two areas also coincide with the primary embryonic axes determining systems and the secondary fields within these primary embryonic axes that give rise to limbs (Williams and Carroll 1993).

Studies emphasizing analyses of the primary embryonic axes concern analyses of zygotic and early embryonic groundplan. These studies involve the examination of early developmental events such as determination of germ-band formation, segmental identity, and segment polarity. Maternally and zygotically active genes determine the Anterior-Posterior (AP), and the Dorsal-Ventral (DV), embryonic axes. Three gene systems determine the AP axis and one determines the DV axis (Nüsslein Volhard 1980; Govind and Steward 1991; Lawrence and Sampedro 1993; Sprenger and Nüsslein-Volhard 1993). Zygotically active pattern genes then refine the AP and DV axes by appearance in a regulatory hierarchy (Nüsslein-Volhard and Wesichaus 1980; Pankratz and Jäckle 1990, 1993; Carroll 1990) that includes gap genes, pair-rule genes, segment polarity genes and homeotic genes (Fig. 1). Each segment can be thought of as being divided into an anterior and posterior compartment and the pair-rule genes and segment polarity genes are essential for this compartmentalization in the developing segments of the embryo.

Germ-band formation (Fig. 2) has been a topic of discussion in insect development since Krause (1939) first characterized embryos as having long, intermediate or short-germ-bands (Sander 1976, 1988; Tautz et al. 1994; French 1988; Tear et al. 1988; Patel 1994*a,*

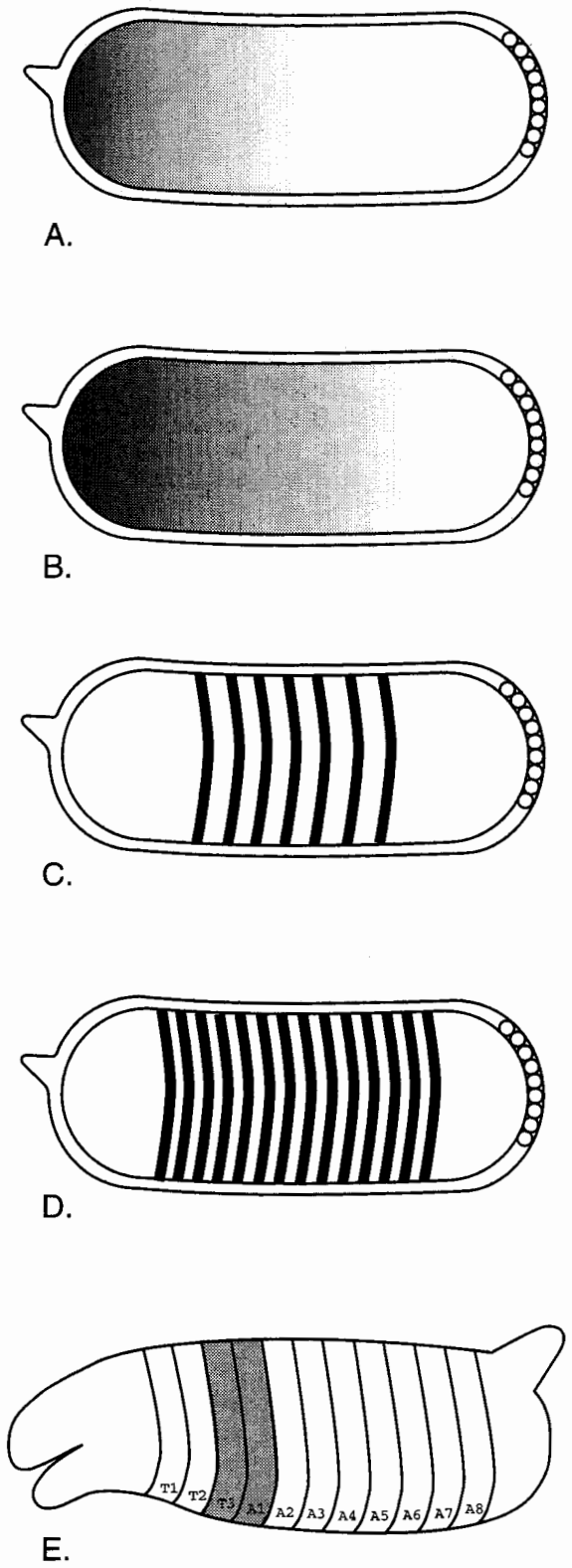

Fig. 1. Schematic drawings of antibody and *in situ* staining of *Drosophila melanogaster* embryos to show the embryonic location of gene products from the five classes of genes in the developmental hierarchy. A representative gene from each class in the hierarchy is presented in these schematics. In general, antibody and *in situ* experiments give the same results for the genes depicted. For a more detailed description of these types of experiments see Patel *b, c*). These terms describing the embryo are meant to differentiate functional aspects of insect embryogenesis. At the germband stage all insects have the same basic segmental organization which consists of a head with three gnathal segments (the mandibular, maxillary, and labial segments) and a procephalic region. Three thoracic segments follow the head with 8–11 abdominal segments completing the posterior portion of the germ-band stage embryo. Long-germ-band embryos appear to lay down the entire segmental pattern by the onset of gastrulation; short-germ-band insects deploy body segments during a post-blastoderm growth phase, and intermediate band insects have segments as far posterior as the thorax and even the anterior abdomen established at the blastoderm stage, while segments posterior to these are established after gastrulation in a processional fashion. The utility of the germ-band terminology with respect to systematics and homology is discussed below.

The secondary fields mentioned by Carroll (1994, 1995) and Davidson (1994) involve, among other things, the development of limbs. Holometabolous insects such as the Diptera and Lepidoptera develop through larval stages where limb anlagen called imaginal discs are formed on the inside of the larvae. These discs form as epidermal invaginations in genetically and developmentally predetermined positions along the anterior posterior axis of the larva. In *Drosophila* there are 19 imaginal discs that correspond to the eye-antennal pair, the three pairs of leg discs, a pair of wing discs, a pair of haltere discs, a pair of labial discs, a pair of clypeo-labral discs, a pair of dorsal prothoracic discs (humeral discs) and a genital disc (Russel 1982; Oberlander 1985). Like the segments in the developing embryo, each imaginal disc can also be divided into compartments that are established by the interaction of developmental gene products discussed below.

Development of secondary fields in the larvae of holometabolous insects is extremely complex, but recent work (Williams and Carroll 1993; Cohen 1990,

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(1994*a*).—A. The distribution of the maternal effect gene *bicoid* in a newly oviposited egg demonstrating the anterior-posterior gradient that is indicative of this classical morphogenetic gene product.—B. The distribution of the gap class gene *hunchback* in a syncistial blastoderm embryo, showing the 60%-40% egg length distribution of this gene product.—C. The distribution of the pair-rule gene product *evenskipped* in a post-blastula embryo showing the seven stripe pattern that marks the seven pairs of segments in the developing embryo.—D. The distribution of the *engrailed* gene product in a post-blastula embryo showing the 14 stripe pattern indicative of the segment polarity class of genes. Each stripe marks the posterior compartment of the segments in the developing embryo.—E. The distribution of the *Ultrabithorax* gene product in a first larval instar showing a typical homeotic gene product distribution. The staining in the third thoracic segment and the first abdominal segment is specific for this locus.

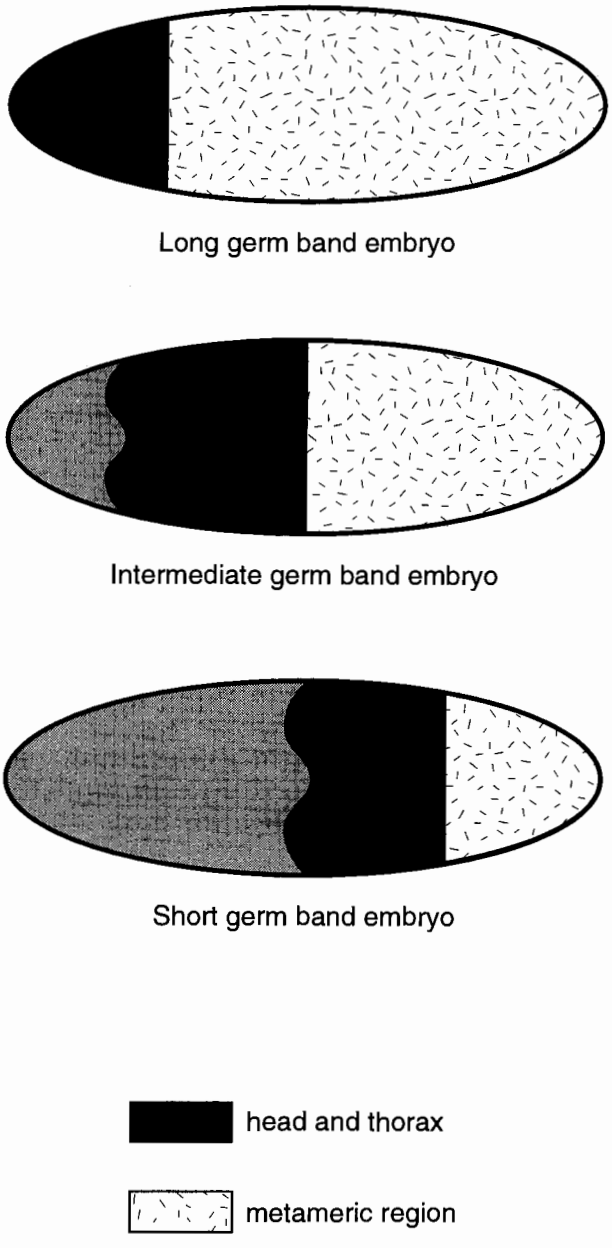


Fig. 2. Schematic representation of a long-germ-band embryo (top), an intermediate germ-band embryo (middle) and a short-germ-band embryo (bottom). The black regions will eventually develop into head and thoracic structures. The metameric region is stipled and will eventually develop into the abdominal and terminal regions of the insect. For more details see the text.

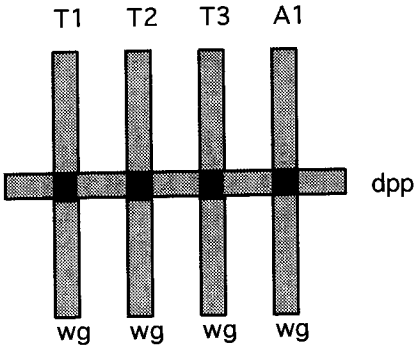
1993) has somewhat clarified our understanding of the processes that occur at the genetic and molecular levels. In this section we will concentrate on a brief review of the events responsible for wing development, as this particular limb has been used as a paradigm for limb development by several authors (Williams and Carroll 1993; North and French 1994; Davidson 1994). We divide the events for limb differentiation into five, more or less, distinct stages that correspond roughly to the stages listed in Williams and Carroll (1993). Although we list these as distinct stages, gene products and interactions required in one stage are often essential in other stages (Fig. 3).

The five stages are: 1) determination of the position of the wing disc along the AP axis of the larval body plan; 2) determination of limb fate, i.e., whether the disc will become a wing, a leg, a haltere, etc.; 3) the establishment of the three fields of orientation in the imaginal disc: anterior-posterior (AP), dorsal-ventral (DV), and proximal-distal (PD); 4) surface elaboration including the determination of the position of the notum versus the wing proper and differentiation of the dorsal and ventral wing surfaces; and 5) the implementation of venation and sensilla formation by a wide variety of genes and genetic interactions. Unique evolutionary questions can be assigned to each of these different stages. Most of the evolutionary questions addressed to date have been concerned with limb fate and position, but studies on pattern elaboration of wings show great potential (Carroll 1994).

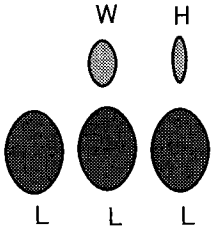
Comparative Molecular Insights: The Primary Developmental Field

Studies examining the early embryonic development of insects have for the most part used early-acting segment polarity genes and pair-rule genes, and have in general been instrumental in forming a picture of segmentation in developing insect embryos as well as a better understanding of the evolution of the germ-band developmental strategies discussed in Fig. 2. The strategy used in these studies is to directly compare the patterns seen in *D. melanogaster* with other taxa and to place the observed differences in an evolutionary context using the current hypotheses concerning insect phylogeny (Patel 1994*a, b, c*; Tautz et al. 1994; Akam et al. 1994). Table 1 summarizes these types of comparative molecular studies done for insects. The types of insect developmental evolution questions concerning the primary fields of development that have been addressed using this comparative approach include the evolution of mesoderm formation, formation of the gut, the evolution of oogenesis and maternal gene function (Tautz et al. 1994; Patel 1994*a*), the universality of parasegments and the origins of pair-rule organization in insect embryos (Patel et al. 1994; Patel 1994*a, b, c*)

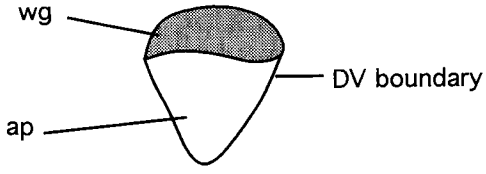
Germ-band formation in insects has been the major subject of comparative molecular studies. *Drosophila melanogaster,* a long-germ-band insect, has been examined for several early segmentation genes (Table 1). The general approaches to examining this problem involve the determination of the position and timing of expression of *engrailed* (*en)* stripes in a variety of long- and short-germ-band insects (Patel 1994*a, b*; Tautz et al. 1994), in conjunction with information ob-



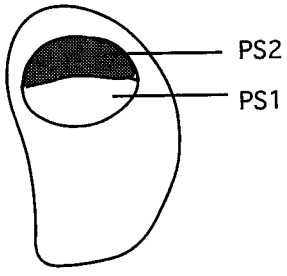
A. *al, dpp, wg*



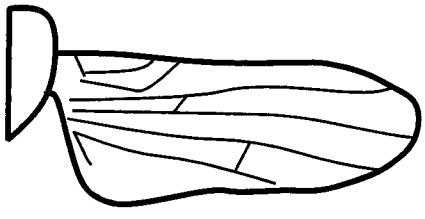
B. BX-C, *Dll, vg*



C. *al, ap, Dll, dpp, en, hh, pd, sd, vg, wg*



D. *ap,* PS1, PS2, *sd, vg*



E. *Dll, hh, N, rho, wg,* etc. For others, see Garcia-Bellido and de Celis (1992); Sturtevant and Bier (1995).

Fig. 3. Cartoons of the five stages involved in the determination of the secondary developmental fields of the wing redrawn after Williams and Carroll (1993). Only specific events in each stage are represented and lists of genes involved in events at each stage are given to the right of each cartoon.—A. The position of imaginal discs in the embryo and larval stage are established late in embryogenesis

tained from studies using pair-rule and segment polarity genes (Patel et al. 1994).

The spatial and temporal distribution of the *en* gene product can conveniently be used to determine the position of the posterior compartment of each segment during development. In this sense, *en* expression can refine our understanding of germ-band extension at the molecular level by actually demonstrating whether or not the signals for segmentation are present at various developmental stages in insects with different germ-band strategies. In *D. melanogaster* there are 14 *en* stripes in the blastoderm embryo, three representing the head segments, three for the thoracic segments and eight for the abdominal segments. These data suggest that the signals necessary for all segments are present in this long-germ-band insect at the blastoderm stage.

Patel (1994*a*) suggests that there are two possibilities for the formation of segments in the short-germ-band insects; first, all segments could be established at the blastoderm stage as in long-germ-band insects and expand during the growth phase, or, second, the proliferative zone could generate the information needed for segments and the segments would then form sequentially after the growth phase. Two short-germ-band insects have been used to determine the mode of segment determination. Using the *en* antibody to detect segmental position in developing embryos, the short-germ beetle, *Tribolium castaneum,* shows sequential establishment of segments. In particular, at the beginning of gastrulation a single *en* stripe appears that corresponds to the mandibular segment. The remaining *en* stripes corresponding to the rest of the segments in this insect then appear sequentially during the rest of embryogenesis. A second short-germ insect, *Schistocerca,* shows establishment of the first *en* stripes in the thorax, with no apparent evidence of *en* activity in the area where abdominal cells will eventually proliferate. Later in embryogenesis, the abdominal *en* stripes begin to appear sequentially from an area of new cell proliferation. Examination of the distribution of other developmental gene products in embryos of short-germ-band insects supports the contention that these insects develop by sequential differentiation of segments from zones of cell proliferation (*wingless [wg]* in *T. castaneum*: Nagy and Carroll 1994; *Antennapedia [Antp], Ultrabithorax [Ubx], abdominal-A [abd-A]* and *Abdominal-B [Abd-B]* in *Schistocerca*: Tear et al. 1990, Kelsh et al. 1993, 1994, Patel 1994*b*; *hairy [h]* and *even skipped [eve]* in *T. castaneum*: Sommer and Tautz 1993, Patel et al. 1994; *eve* in *Schistocerca*: Patel et al. 1992; Patel 1994*a*).

Although *en* staining at various developmental stages in a wide variety of organisms has been instrumental in determining the modes of germ-band elongation (Patel 1994*a*; Sommer and Tautz 1993) an explanation for the phylogenetic distribution of germ-band strategies comes from other sources. Figure 4 shows the phylogenetic distribution of long-, short- and intermediate-germ-band strategies for several orders of insects and demonstrates that germ-bandedness, per se, shows a homoplasious distribution.

Characterization of the spatial distribution of pair-rule genes that are responsible for the "transient double segmental organization" of the developing *D. melanogaster* embryo (Patel et al. 1994) has added to the understanding of germ-band formation in insects. More detailed examination of this pair-rule gene expression in the four insect orders discussed in Table 1 (Diptera, Coleoptera, Lepidoptera and Orthoptera) render a high degree of interpretability of germ-banded-

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and are initially specified by the original metameric AP and DV positional information by the intersection of two pattern determining genes (Cohen et al. 1993), *wingless* (*wg;* horizontal lined area) and *decapentaplegic* (*dpp*; vertical lined area). The interaction of a third gene product, *aristaless* (*al*) is also essential for this determination of the position of the wing discs in the developing embryo.—B. This stage concerns the determination of the identity of the appendage to arise from the disc. Leg imaginal disc (represented by the dark gray large ovals) identity is determined by the interaction of Bithorax complex genes (BX-C) and the *Distal-less* (*Dll*) gene (reviewed in Williams and Carroll, 1993; Davidson, 1994; Cohen, 1993; Vachon et al., 1992; Blair, 1995). Wing and haltere imaginal disc (represented by the light gray small ovals) formation results from the dorsal migration of cells, estimated at about 30 in number, from the leg disc area (the mesothracic segment). The expression of a putative transcription factor (*vestigial*; *vg*) is responsible for the determination of these cells as wing disc cells. The future position of the legs (L), wings (W) and halteres (H) are depicted under the points of *dpp* and *wg* intersection shown in 3A.—C. This cartoon represents the specific interactions involved in dorsal ventral pattern formation in the wing. The interaction of several genes is important in the differentiation of the wing disc into compartments to impart upon the developing disc these three polarity systems (anterior-posterior [AP], dorsal-ventral [DV] and proximal-distal [PD]). Dorsal compartmentalization is accomplished by the activity of the *apterous* (*ap*) gene product (shown in white). Further determination of the DV axis in the early developing wing disc is established by the restriction of the *wg* gene product (shown in gray) to the ventral compartment (Struhl and Basler 1993; Basler and Struhl 1994). The distribution of this gene product in later stages of wing disc development is extremely complicated. Other genes involved are dpp, Dll, en, hedgehog (hh), scalloped, vg, and al.—D. The fourth stage in the transformation of the wing disc to adult wing structures involves the differentiation of the notum from the wing proper implemented by the activity of *vg* and *sd* as mentioned above. This cartoon represents a third instar larval wing disc with the distribution of the two integrin gene products. Further differentiation of the dorsal and ventral wing surfaces are mediated by the activity of two integrin gene products designated PS1 (shown in white) and PS2 (shown in gray; also known as inflated [*if*]). *vg, sd* and *ap* have also been implicated in the determination of identities in the dorsal and ventral wing blades as possible regulators of the dorsal and ventral integrins (Williams and Carroll, 1993).—E. The final stage concerns the venation of the wing (shown in cartoon) and has been reviewed in great detail genetically by García Bellido and de Celis (1992) and at the molecular level by Williams et al. (1994) and Sturtevant and Bier (1995). Work at this stage has attempted to explain the genetic and molecular basis for wing sensila and wing venation.

Table 1. Summary of the antibody staining (plain text) and *in situ* hybridization (bold text) studies done on the various classes of genes in the regulatory hierarchy.[1]

	Taxa						
	Drosophilidae	Other Diptera	Lepidoptera	Coleoptera	Orthoptera	Thysanura	Other Arthropods
MATERNAL							
bicoid		**25, 27, 28, 34**					
nanos		**14, *39***					
snail				**25**			
twist				**25**			
GAP							
hunchack	9, **23, 29**	**23, 25, 28**	**37**				
knirps		**23, 25**					
Krüppel	22	**23, 25**	**37**	**26**			
tailless	29	**22, 25**					
PAIR RULE							
even-skipped	22			8, 13, 20	8, 15, 20		
fushi tarazu				18			
hairy	21	**23, 25, 28, 32**	**26**				
patched	**29**		**34**				
runt			**37**				
SEGMENT POLARITY							
Dax					2		
engrailed	**29**	6, **23, 25, 28**	8	6, 8, 12, 38	8, 16, 17	8, 11	16, 17, 20
gooseberry-b	**29**						
wingless	**29**		**34, 37**	**35**			
HOMEOTIC							
Antennapedia			19				
abdominal-A	**29**		19, **34**	5, 20	1, 4, 20	7	
Abdominal-B					3		
fushi tarazu				**31**			
Sex combs reduced			19				
Ultrabithorax		**23, 25, 28**	19, **34**		1	7	
SECONDARY FIELD							
apterous			**34**				
decapentaplegic			**34**				
Distall-less		**30**	**34**				
invected			**34**				
scalloped			**34**				
OTHERS							
Fasciclin IV					14		
Trg				10	10		

[1] The individual genes used in the studies are indicated in the left hand column. The studies are also listed so that the particular type of insect on which the experiments were performed are indicated in the row at the top. References in bold refer to *in situ hybridization,* otherwise to antibody hybridization. 1 Kelsh et al. 1994; 2 Dawes et al. 1994; 3 Kelsh et al. 1993; 4 Tear et al. 1990; 5 Stuart et al. 1993; 6 Schmidt-Ott et al. 1994; 7 Carroll et al. 1995; 8 Patel 1994b; 9 Treier et al. 1989; 10 Kispert et al. 1994; 11 Scholtz et al. 1994; Brown et al. 1994; 13 Patel et al. 1994; 14 Kolodkin et al. 1992; 15 Patel et al. 1992; 16 Patel et al. 1989a; 17 Patel et al. 1989b; 18 Brown et al. 1994; 19 Warren et al. 1994; 20 Patel 1994a; 21 Tautz and Sommer 1995; 22 Reuter et al. 1989; 23 Sommer and Tautz 1991; 24 Lukowitz et al. 1994; 25 Sommer and Tautz 1994; 26 Sommer and Tautz 1993; 27 Schröder and Sander 1993; 28 Tautz and Sommer 1995; 29 Dickinson et al. 1993; 30 Panganiban et al. 1994; 31 Brown et al. 1994; 32 Carroll et al. 1994; 33 Langeland and Carroll 1993; 34 Carroll 1994; 35 Nagy and Carroll 1994; 36 Webster et al. 1994; 37 Kraft and Jäckle 1994; 38 Fleig 1994; 39 Curtis et al. 1995.

ness in these insects. The domains of segment polarity genes such as *en* and *wg* are established by pair-rule genes in *D. melanogaster.* When examined for *eve* expression, this long-germ-band insect has a seven stripe pattern in the early embryo that modulates the fourteen stripe *en* pattern. The short-germ-insect *Schistocerca* displays no segmental pattern of *eve* expression with the domain of expression of this gene product being primarily in the posterior portion of the gastrulating embryo. Sequential *en* expression that marks the segments in the embryo (see above) proceeds without the expression of *eve* to regulate position as the *eve* signal remains in the posterior domain of the developing embryo. In the second short-germ-insect *Tribolium, eve* stripes appear as the embryo elongates and, in particular, appear to modulate *en* expression. The lepidop-

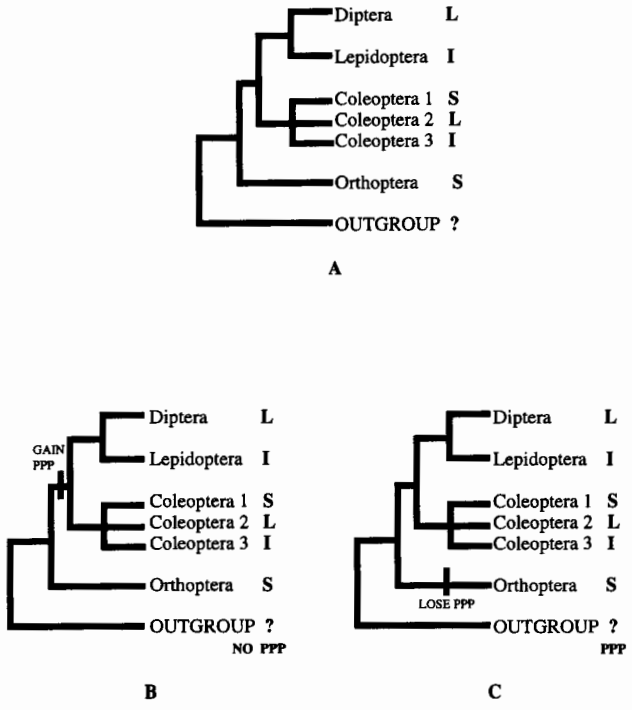

Fig. 4. A. The phylogenetic distribution of long-, short- and intermediate-germ-band modes of development among Diptera, Coleoptera, Orthoptera and Lepidoptera are shown. The germ-band character state for the outgroup is presumed unknown.—B, C. Dissection of germ-band character state using the pair-rule prepatterning (PPP) criterion established by Patel (1994*a, b*). These cladograms suggest two character reconstructions due to the unknown character state for PPP in the ancestor to the four insect orders examined. In one reconstruction (B) the ancestor is presumed to lack PPP and hence the gain of PPP is a synapomorphy for Diptera, Coleoptera and Lepidoptera. In the second reconstruction (C) the outgroup is presumed to have PPP and hence the loss of PPP is an apomorphy for the Orthopteran lineage. Either reconstruction would reinterpret the homoplasy of the short- and long-germ-banded character states depicted in A and suggest a lack of homology for character designations such as long, short, and intermediate.

teran *Manduca sexta,* can be used as an example of an intermediate band insect (Broadie et al. 1991; Sander 1976; Kraft and Jäckle 1994). *Manducas sexta* shows an early embryonic morphology that resembles short-germ-band insects in that only head lobes and a region that resembles a growth zone are present in the developing embryo. The major difference between *M. sexta* and a short-germ-band insect is that instead of undergoing cell proliferation in the growth zone as short-germ-band insects will do, *M. sexta* develops by elongation of the germ-band through tissue reorganization. When *M. sexta* was examined using the pair-rule gene *runt* and the segment polarity gene *wg,* 8 and 16 stripes, respectively, were observed in the early embryo (Kraft and Jäckle 1994). These results suggest a pattern of pair-rule regulation of segment polarity expression in this insect also. Two other Coleoptera have been examined (Patel et al. 1994) due to their variable germ-band designations and it is evident that there is pair-rule prepatterning in these taxa similar to that of *Tribolium.* These comparative molecular studies demonstrate that a complex morphological character such as germ-bandedness can be interpreted in molecular terms. Germ-bandedness can be dissected into the presence or absence of pair-rule prepatterning and the implications for this dissection with respect to character evolution in insects will be discussed below.

Comparative Molecular Insights: The Secondary Developmental Field: Limbs

Carroll (1994; 1995) has divided the problem of insect appendage development and evolution into three major questions concerning differences in appendage number, differences in appendage type, and differences in pattern on the appendage. The evolution of appendage number has recently been examined by Carroll et al. (1995). This study used the observation that wing primordia can first be visualized in the developing embryo as discrete clusters of *snail* expressing cells and *vestigial* producing cells using antibodies for these two gene products (Alberga et al. 1991). In *D. melanogaster* development of wings in all segments but T2 is repressed by homeotic gene activity (*Ubx, abdA, AbdB, Scr* and *Antp*; for a concise review see Carroll et al. 1995). Although homeotic gene repression is the mechanism by which loss of wing formation is implemented in the various segments, these genes are not at all involved in the actual development of adult wings (Carroll et al. 1995). This is an important piece of information because it suggests that the sole role of homeotic genes in the formation of wings in *D. melanogaster* is to repress expression of *vestigial* and *snail* in the wing primordia and hence to repress the development of the wing.

In developing embryos of *Thermobia domestica* (a primitive apterygote Thysanurid insect that would represent the condition prior to the "invention" of insect wings), *abdA* and *Ubx* are expressed in the posterior thoracic segments and the anteriormost abdominal segments just as in the more derived pterygote insects described above. Carroll et al. (1995) use these data and the fossil record to suggest a plausible scenario for the evolution of insect wings. They suggest that the first stage in the evolution of wings in pterygote insects occurred on all segments of the insect, because there was no homeotic gene input into the repression of these structures on these segments. Subsequently, certain elements of wing formation must have evolved response mechanisms to homeotic (ANT-C or BX-C) gene regulation. The *Thermobia* results suggest that homeotic gene structure and function have been conserved in the ancestral taxa and hence evolution of *Scr* responsive elements implement the reduction or elimination of wings in the first thoracic segment. In a

similar way, the evolution of *Ubx* and *abdA* responsive elements resulted in the repression of wing development on abdominal segments and further in diptera (two-winged pterygotes) the reduction of wings on the third thoracic segment.

Carroll (1994, 1995) suggests that the second class of questions one can approach with respect to limb development concerns determination of appendage type. He uses the evolution of halteres versus wings as an example of these differences and concludes that the regulation of homeotic target genes is involved in this class of differences. This mode of regulation should be contrasted with the appendage number differences where control of those differences is through regulation by homeotic repression.

A recent study of the systematics of the enigmatic insect order Strepsiptera (twisted-wing parasites) suggests that regulation of appendage type and position may have been a factor in the evolution and diversification of this group (Whiting and Wheeler 1994). Strepsiptera are a small order of insects (520 species) that are exclusively parasitic on a wide range of insect taxa. Adult male Strepsiptera have wings fully developed on the third thoracic segment and wings on the second thoracic segment that are reduced to structures similar morphologically and functionally to the halteres (reduced hind wings) of Diptera. Strepsiptera have been posited as the sister group to nearly every other order of insects, but recent work using 18S rDNA and 28S rDNA sequences and morphological characters strongly support the placement of Strepsiptera as the sister group to Diptera (Whiting and Wheeler 1994). This phylogenetic conclusion is important in that it raises the possibility that the same developmental mechanism responsible for the modification of the haltere in Diptera is also operating in Strepsiptera but on a different thoracic segment. It is plausible that this mechanism arose in the dipteran-strepsipteran ancestor, and its expression was subsequently switched to a different thoracic segment, presumably in the strepsipteran lineage, after their divergence. Studies into the expression patterns of *Ubx, abdA, AbdB, Scr* and *Antp* in Strepsiptera, primitive Diptera, and other mecopteroid insects, will bring further insight into the evolution of these genes in a phylogenetic context.

The final aspect of appendage development, pattern formation on the appendage, has used the excellent background work of Nijhout (1991, 1994) on butterfly wings. In particular, the buckeye, *Proecis coenia,* has been used as a model system. The unique aspect of Lepidoptera wings concerns the elaborate coloring and patterning of the colors on the wing, that although present in other orders of insects, is by no means as extreme as in the Lepidoptera. Carroll (1994) summarizes the developmental genes that have been isolated and characterized from this insect, and in particular these genes represent DV axis determining genes (*apterous*), PD axis determining genes (*scalloped, Distal-less*), AP axis determining genes (*invected, engrailed* and *decapentaplegic*) and wing margin genes (*wingless*) in the developing limb. Not surprisingly, Carroll et al. (1994) showed that the expression of these genes in *P. coenia* is very similar to the expression patterns in *D. melanogaster.* On the other hand, the startling result arose that in the fifth instar *P. coenia,* these coordinate systems have been co-opted to produce a second pattern of transcription. Although the transcription patterns of most of the genes used do not correspond directly to pigment patterns in the adult wings, Carroll et al. (1994) suggest that they, "do reflect a fundamental dynamic patterning system within each wing cell". *Dll* is one exception to this general noncorrespondence in that it has been suggested to be responsible for the initial proximodistal restriction of pigments, midline rays, posterior enlargements and the posterior eyespot (Carroll et al. 1994; Carroll 1995). The success stories that arise from studies in the three major areas of limb development discussed by Carroll (1994, 1995) demonstrate the plausibility of using the developmental approach to examine limb characters in systematic studies.

PHYLOGENETIC ANALYSIS AND PARALLELISMS

The deciphering of the evolution of germ-band formation (Patel 1994*a, b*; Tautz et al. 1994) and the telling of the evolution of insect appendages (Carroll 1994, 1995) are indeed exciting, but these topics are not necessarily questions on which the typical insect systematist might capitalize. From the standpoint of systematics we are struck with very different group-specific problems that the developmental comparative approach can aid in deciphering. An examination of the systematics of the Drosophilidae will assist in demonstrating the kinds of problems that may arise in systematic analysis at the generic level. Although the morphologies discussed below are highly specific for this family of Diptera, the methods used to pinpoint them and, in general, their distribution should be familiar to most systematists.

Figure 5 shows a total evidence (Miyamoto 1985; Kluge 1989) hypothesis for several genera of Drosophilidae based on DNA sequences and morphological data next to the morphology-only hypothesis. The details of this analysis have been discussed at length in DeSalle and Grimaldi (1991, 1992, 1993) and DeSalle (1994). Note that the Hawaiian *Drosophila* could be construed as the taxon that causes much of the incongruence between the total evidence analysis and the morphological analysis. In short, addition of the molecular characters to the data set calls into question the morphological-character support for the *Hirtodroso-*

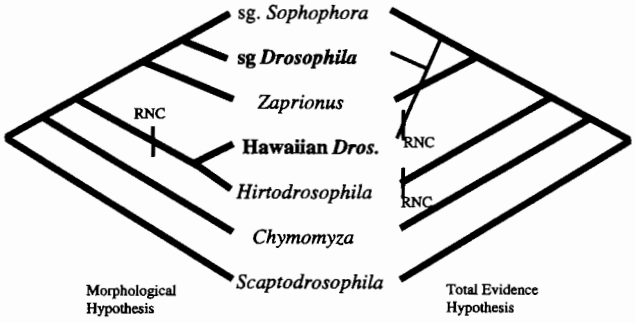

Fig. 5. Right panel shows the total evidence cladogram for several genera of Drosophilidae taken from DeSalle and Grimaldi (1991). The left panel shows the cladogram obtained when only morphological characters are used to infer phylogeny. The single character that supports the *Hirtodrosophila*-Hawaiian *Drosophila* sister grouping in the morphological hypothesis (raised nasal carina: RNC) is shown mapped on both cladograms and implies that this character is homoplasious in the total evidence hypothesis.

phila-Hawaiian *Drosophila* sister group relationship. There is a single morphological character in the DeSalle and Grimaldi (1991; originally described in Grimaldi 1990) analysis that hypothesizes these two groups as sister taxa: a raised nasal carina. This example, points out the type of morphological character that will concern most systematists.

We suggest that the first step in an integrative approach to development and evolution is the construction of a cladogram from all available evidence that approximates the genealogical history of a group in question. Morphological characters are then mapped (Maddison and Maddison 1992; Brooks and McLennan 1991; Harvey and Pagel 1991; Miller and Wenzel 1995) on the cladogram and convergences and parallelisms are pinpointed for further scrutiny. One caveat that must be made clear concerning this character-mapping approach is that character optimization (Maddison and Maddison 1992; Swofford and Maddison 1992) on the tree can have a profound effect on how the evolution of the character is interpreted.

Several authors in the systematics literature have indicated an interest in this approach. Saether (1983) and Sluys 1989) both suggest that these types of characters can be used in phylogenetic analyses. Saether (1983) coined the term "underlying synapomorphy" and defined the phenomenon as a "close parallelism" produced by the same underlying genetic factors in a monophyletic group. Sluys (1989) examined this idea of underlying synapomorphy in more detail and suggested that under cases of "rampant parallelism" the principle of parsimony is not appropriate. Although we disagree with the use of underlying synapomorphy as a foible for debunking parsimony (DeSalle and Grimaldi 1993), some interesting and important ideas about "rampant parallelism" are discussed by these authors. For instance, Sluys (1989) rightly points out that homology is the cornerstone of these types of analyses. He also interestingly suggests that rampant parallelism is intricately connected to canalization and the organization of genetic and epigenetic systems controlling morphology. In other words, characters with common epigenetic potential and canalization patterns or similar genetic elements are more prone to rampant parallelism. In the context of developmental genetics, Sluys (1989 p. 366) suggests that "a back mutation in the regulatory mechanism would switch on again the gene complex that remained unexpressed in the predecessors of a species."

The rationale for the general approach we suggest for pinpointing interesting morphological phenomena is explained best by referring to Sluys's (1989) differentiation of parallelism versus convergence. Systems where homologous genes and developmental processes are involved are most appropriate for understanding the interface of development and evolution. In order for homologous developmental processes to be involved in morphological change, examination of parallelisms is most appropriate. Convergence in its most strict definition does not necessarily involve the reappearance of morphologies caused by the homologous genetic elements or developmental mechanisms. Sluys (1989) and Saether (1983) both indicate this difference and suggest that recognition of parallelisms versus convergence is important. Whereas they suggest that parallelisms are grounds for reinterpretation of characters in parsimony analysis we feel that rampant parallelisms are grounds for further character dissection using modern molecular and developmental biology.

An excellent case in point occurs again in the Drosophilidae and concerns a morphological trend called hypercephaly (Grimaldi and Fenster 1989; summarized in DeSalle and Grimaldi 1993). Within the family Drosophilidae, hypercephaly or broad headedness has arisen at least eleven independent times, twice within the genus *Chymomyza,* twice within the subgenus *Drosophila,* once within the genus *Mulgravea* and six times within the genus *Zygothrica* (Grimaldi 1987). DeSalle and Grimaldi (1993) discuss this hypercephalic trend in *Zygothrica* and suggest that the head region of the fly be used as a "starting point" for understanding the bridge between development and evolution. Their suggestion concerning the importance of the head in systematic studies of flies arose not only from the analysis of parallelisms and convergences in the group but also from the observation that the majority of the characters used by Drosophilidae systematists concerned the morphology of the head (DeSalle and Grimaldi 1992, 1993).

CHARACTER DISSECTION

Once characters have been examined for parallelism it is possible for further examination of their genetic

and molecular basis. Genetic dissection of complex traits is a difficult task, and usually entails application of rather sophisticated genetic approaches (linkage analysis, allele sharing methods, association studies in human populations, and genetic analysis of large crosses in model organism studies; Lander and Schork 1994). In light of the fact that most systematists are interested in typically nonmodel organisms and hence will be unable to apply these techniques, we feel that the use of the phyletic phenocopy paradigm (Bassile and Stebbins 1985; DeSalle and Carew 1992) often times can allow an educated guess as to the genetic basis for certain characters. Examination of a parallelism concerning a morphological character used in Drosophilidae generic-level systematics called interfacetal setae (Grimaldi 1990) is a good example of this preliminary approach. The character arises at least four times in the family Drosophilidae (DeSalle and Grimaldi 1992). A close examination of the morphology involved in this character indicates that the loss of interfacetal setae is a phenocopy of simple mutations found in *D. melanogaster* (*Hairless* and *deltoid*). It should be noted that these two mutant forms in *D. melanogaster* are a simple point mutation and an allele produced by the insertion of a transposable element into the *Notch* locus, respectively. Other *D. melanogaster* loci can produce the phenotype (such as *Shibire* and *DOA*) but these are embryonic lethals and it is difficult to suggest that these mutants are responsible for the loss of interfacetal setae. The phyletic phenocopy approach may result in valuable suggestions as to which loci could be examined to further dissect characters.

Some character systems are more complicated in that they do not have an obvious phenocopy or in that the phenocopies are produced by more complex genetic interactions. An example using an admittedly poor morphological character has been discussed at length by DeSalle and Grimaldi (1992, 1993). This example takes advantage of the interest in the *bobbed* (*bb*) phenotype in *D. melanogaster* and the fact that the *bb* morphology appears as a rampant parallelism with respect to *Drosophila* phylogeny. When the underlying molecular and genetic mechanisms involved in the production of the morphology are examined a phylogenetically informative molecular character is revealed and the causes of the multiple independent morphological arisals can be explained (DeSalle and Grimaldi 1992).

Germ-band determination in insects can be used as an example of character dissection at higher taxonomic levels. As explained in Fig. 2, the character germbandedness can be coded as long, intermediate or short. The phylogenetic distribution of the character states for this character is depicted in Fig. 4. This figure also shows two possible character reconstructions using these data that reinterpret the underlying homoplasy of the germ-band character states. In the first scenario, it is assumed that the ancestral outgroup lacked pair-rule prepatterning, so that such prepatterning becomes a synapomorphy for Coleoptera, Diptera and Lepidoptera. In the second scenario, the ancestral outgroup is assumed to have pair-rule prepatterning and hence the loss of this prepatterning is seen as an apomorphy that diagnoses the Orthoptera. Either character reconstruction reinterprets the homoplasious nature of germ-band designation via the dissection of germ-bandedness using pair-rule genes.

LANDMARKING

Wheeler (1981, p. 4) made the insightful observation that many of the major groups of Drosophilidae are diagnosed by, "whether a certain bristle is present or absent, or if it is directed forward or backward". In addition, in the morphological revision of the family Drosophilidae (Grimaldi 1990) nearly 70% of the female characters and 55% of male characters concern morphological variation of the head region. The concentration of Drosophilidae morphological systematics on the head and on sensilla is the result of the occurrence of readily identifiable morphological variation of these structures, and new sources of characters are highly desirable.

The vast majority of the morphological characters used by *Drosophila* systematists are adult characters (Grimaldi 1990), however, there exists a wealth of larval characters in other dipteran systems (Meier 1995). In addition, ultrastructure offers a source of characters in the Drosophilidae that has only recently been exploited (Grimaldi 1990). Developmental approaches offer yet another source of characters for the morphological systematist. Use of well-defined molecular markers of development may allow for a more concise delineation of morphological structures in developmental stages that to this point have been considered lacking in structure.

In Drosophilidae larvae, a major part of the head involutes as part of the developmental process. This involution "obscures" many of the fine larval head structures that can be used in systematic studies of the group. Molecular landmarking using well-characterized developmental genes such as *en,* other segment polarity genes and homeotic genes can "uncover" hidden morphological structures. One extreme example concerns the nature of segmentation in the head. The drosophilid head has classically been enigmatic concerning segment number. Several researchers have argued that the drosophilid head has only six segments while others argue seven. Studies using the *en* antibody clearly show seven "stripes" in the drosophilid head (Schmidt-Ott and Technau 1992, 1994 Schmidt-

Ott et al. 1994*a*). Since *en* is a marker for the posterior compartment of segments, this result is taken as strong evidence for the existence of seven segments in the drosophilid head (Schmidt-Ott and Technau 1992). Other difficult morphological characters that have been ignored in the past because of our inability to determine homology or even to discern structure may now be examined using an approach similar to this. Localization of the *en* gene product in developing *D. melanogaster* embryos, as well as other important head development genes such as *Dfd* (Chadwick and McGinnis 1987; Mahaffey et al. 1989; Regulski et al. 1987), *lab, Scr* and *pb* (Mahaffey et al. 1989), *spalt* (Kuhnlein et al. 1994), *hh* (Mohler and Vani 1992, *crumbs* (Tepass et al. 1990; Tepass and Knust 1990) and *cap and collar* (Mohler et al. 1995) among others are examples of the potential of the approach. In fact, Younossi-Hartenstein et al. (1993) have used the position of *crumbs* antibody staining in conjunction with patterns from two *en*-enhancer trap lines to determine the embryonic origin of imaginal discs (see also Bate and Martinez-Arias 1991) in the drosophilid head. Data such as these on the origin of imaginal discs are essential for addressing the important problem of homology of imaginal discs in the Holometabola (Svacha 1992) and to allow the utilization of information on discs in systematic studies.

Another excellent example of the application of this approach that concerns the discovery of several new structures in the drosophilid head is the use of the 22C10 antibody to visualize structures in the head of *D. melanogaster* embryos. Schmidt-Ott et al. (1994*a* p. 8365) show in their Fig. 2 staining patterns with this antibody and demonstrate the delineation of several new sensory organs in the larval head region. In addition, they were able to use these staining patterns in combination with mutant analysis to determine the genes that are responsible for these structures. In all, seven new sensory organs were identified and their fasciculation patterns were established.

Another source of characters using these well-defined molecular markers has been discussed by Collazo and Fraser (1995) and is also relevant to our discussion of discovering new *Drosophila* characters. He has described systems where the researcher can follow developmental markers and this allows the visualization of developmental and morphogenetic events and hence morphologies in developmental stages that have been ignored. Certain cell lineages in the developing embryo can also be marked using developmental techniques. Schmidt-Ott and Technau (1992, 1994) have used horse-radish peroxidase (HRP) staining by injection to mark cells in the developing head of *D. melanogaster* embryos. Their Figure 2 (Schmidt-Ott and Technau 1994*b* p. 368) shows staining of specific cells in the developing head at various stages. These data allow the characterization of morphogenetic movements and changes in the head of developing *D. melanogaster* and are potentially a great source of character information.

SUMMARY

The recent explosion of molecular techniques and approaches in developmental biology has added a new dimension to our approach to comparative biology. The pioneering work of Bateson (1894) on homeosis is the cornerstone to all subsequent work on homeotic phenomena in insects and opened the way for the classical genetic work of Lewis (1978) Kaufman et al. (1980) and Nüsslein-Volhard and Wieschaus (1980) on the BX-C, ANT-C and maternal and early embryonic loci respectively. These genetic studies were essential for the molecular analysis of homeotic genes and early developmental genes and resulted in molecular cloning of homeobox genes (McGinnis et al. 1984*a*; Scott and Weiner 1984) and other developmentally important genes. The cloning of homeotic loci and other developmentally important genes allowed technological advances such as antibody staining, *in situs* and enhancer traps that are essential for analysis of developmental pathways in insects. It is interesting to note that the entire chain of events described above make Bateson's (1894) ideas concerning homeosis even more timely. The genetics and technology that arose as a result of his work can now be looped back to clarify and enlighten our ideas about homeosis in insects in particular, and insect evolution, in general.

Several aspects of insect evolution have been examined using the new approach of comparative developmental biology (Patel 1994*a, b*; Carroll 1994, 1995; Tautz et al. 1994; Akam et al. 1994). While these kinds of studies are both elegant and highly informative, the importance of the application of these techniques to systematic analysis is somewhat limited. Systematists can, however, benefit from the elegant advances made by developmental biologists and, in particular, from the comparative molecular approach. The problems that systematists face that can benefit from developmental analyses are quite different from those currently addressed by the comparative method. Classically, development has been used to examine and refine character homology assessment and polarity. We suggest that developmental approaches can add in two additional important areas of systematics. The first concerns the use of this approach to dissect complex morphological characters or even morphogenetic events, the second encompasses the generation of new characters using landmarking techniques. In order for these approaches to be viable sources of character information in systematics, though, culturing techniques and collection techniques will have to be developed so

that embryonic stages of a wide variety of taxa can be examined.

ACKNOWLEDGMENTS

We thank Dr. Elliot Meyerowitz for two clarifying comments on the content of this talk. The first concerns Bateson's role in defining the term homeosis and the second concerns the fact that some of the eye setae mutants (*Shibire* and *DOA*) that produce visible morphological change are embryonic lethals. They manifest the visible phenotype only through genetic mosaic and recombination techniques, but when they occur naturally produce lethality at early developmental stages. Any misinterpretations of the literature even after being made aware of these suggestions are of course ours. We also thank Elizabeth Bonwich for help with the manuscript, and Chris Fisher for drawing the figures. Finally, we thank Dr. Elizabeth Zimmer for the invitation to speak at the symposium and for her support during the writing of the manuscript.

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