

Resolving arthropod relationships: Present and future insights from evo-devo studies

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ABSTRACT

In the past two centuries, the field of arthropod phylogeny has been the subject of intense discussion. Traditionally, relationships based on morphology and fossil evidence of the four major arthropod lineages have suggested a closer relationship between myriapods and insects to the exclusion of crustaceans. It was also generally recognized that the chelicerates branched off as a basal group. However, recent molecular studies analyzing sequence data strongly contradict these groupings, and instead suggest the following relationships: (i) [insects + crustaceans], and (ii) [chelicerates + myriapods]. As is evident from this lack of congruence, future resolution of arthropod relationships must rely upon a re-evaluation of traditionally assigned morphological homologies. The field of evolutionary developmental biology (evo-devo) has the potential to accomplish this by emphasizing the developmental mechanisms governing formation of particular morphological features. For example, previous studies of gene expression patterns have revealed that all arthropod mandibles are gnathobasic. As a result of these analyses, this feature (mandibular composition) can no longer be used to group myriapods and insects. Future investigations should shift the focus to delineating the genetic mechanisms of structural development down to their most specific events, encompassing regulatory mechanisms to the level of individual target genes. These detailed genetic networks can then be used to establish true homologies of complex morphological traits such as tracheal systems and Malpighian tubules.

1 INTRODUCTION

Arthropods represent the most diverse animal phylum and the origins of this vast diversity have fascinated scientists for decades. Such high levels of morphological variation have also enabled arthropods to successfully inhabit nearly every ecological niche on earth. Although all arthropods share unifying features such as segmented bodies, jointed appendages, and a hard exoskeleton, they also exhibit distinct differences in the organization of their body plans. Based

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on these differences, we can recognize four major extant arthropod lineages: chelicerates, myriapods, crustaceans and insects. Even when these members of groups exhibit a shared feature such as a defined head region, selection has acted to modify the structures rendering them unique to each species in their respective sub-phyla. A full understanding of the origins of such complexity has the potential to enhance our understanding of the relationships among these main lineages.

For the past two centuries, arthropod phylogeny has been subject to vigorous discussion. Traditionally, relationships between the different arthropod sub-phyla have been primarily determined by analysis of fossil evidence and by comparison of adult structures. Although all combinations of relationships among the four groups have been proposed at one time or another, only the mandibulate theory and the “TCC” view have been seriously considered (Kukalova-Peck 1992; Telford & Thomas 1995). The mandibulate theory unites the insects, myriapods, and crustaceans into the Mandibulata, with trilobites and chelicerates branching off as basal lineages (Fig.1A). In contrast, the “TCC” view unites the trilobites, crustaceans, and chelicerates, thereby separating them from the insects and myriapods. Although these two hypotheses differ substantially, they share a close relationship between the myriapods and insects. The grouping of the insects and myriapods as monophyletic is based on the presence of five shared derived characteristics: a tracheal system, Malpighian tubules, absence of appendages homologous to the second antennae of crustaceans, unbranched legs, and a mandible composed of a whole limb (Manton 1964). Based on these morphological similarities, insects and myriapods were thought by (Sharov 1966) to be sister groups and in the united into Atelocerata.

In the past decade, this close relationship between insects and myriapods has been challenged by molecularly based phylogenies (Boore et al. 1995; Cook et al. 2001; Friedrich & Tautz 1995; Gonzalez-Crespo & Morata 1996; Kusche & Burmester 2001; Regier & Shultz 1997). In 1995, a pair of studies comparing nuclear ribosomal gene sequences and gene rearrangements within mitochondrial genomes concluded that insects may be a sister group to crustaceans (Fig. 1B), not myriapods (Boore et al. 1995; Friedrich & Tautz 1995). This finding attracted a lot of attention, because it was the first in recent times to question the existence of Atelocerata. Subsequent analyses of mitochondrial gene rearrangements, and additional sequence comparisons between both nuclear elongation factors and homeotic (Hox) genes, all strongly supported a close insect/crustacean relationship (Boore et. al. 1998. Cook et. al. 2001, Reiger & Schultz 1997) . These analyses also suggested that myriapods and chelicerates may be sister groups (Fig. 1B). This second conclusion has been further corroborated by additional studies of a variety of gene sequences (Cook et al. 2001; Friedrich & Tautz 1995; Kusche & Burmester 2001).

Despite intense research on resolving arthropod relationships, a universally accepted phylogeny remained elusive. As is evident from the above summary, the emerging view from molecular based phylogenies is in direct conflict with the previously favored groupings, including the Atelocerata. This underscores the need to revisit the various morphological traits/structures used to argue for traditionally assigned homologies. The emerging field of evolutionary developmental biology (evo-devo) can offer unique insight into the possible resolution of arthropod relationships by focusing on developmental processes governing the formation of structure(s) under study. Comparing these genetic mechanisms in a wide array of

arthropod taxa can therefore provide a significant contribution to the field of arthropod systematics.

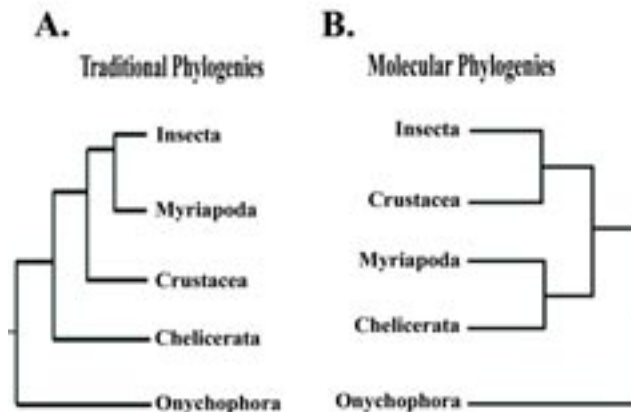


Figure 1. Two models of arthropod relationships. (A) Classical textbook model with Insecta and Myriapoda forming “Atelocerata” (Brusca & Brusca 1990). (B) An emerging view based on recent molecular data (Boore et al. 1998; Hwang et al. 2001; Kusche & Burmester 2001).

2 EVO-DEVO SUCCESS STORY: THE ORIGIN OF ARTHROPOD MANDIBLES

Arthropod mandibles are feeding appendages functioning as “jaws” and are used to bite and chew food (Manton 1964). The specific part of this appendage that directly manipulates food (ie. limb base versus limb tip) has been used to support a close phylogenetic relationship among myriapods, crustaceans, and insects. As shown in figure 2A, mandibles can be composed of either a whole limb or the basal portion only (Manton 1964). The jaws of insects and myriapods have been considered to be whole limbs (composed of both coxopodite and telopodite), manipulating food with the tip of the appendage (Manton 1964; Telford & Thomas 1995). In contrast, crustacean mandibles were thought to be gnathobasic (composed of coxopodite only), the result of either a reduction or absence of the distal portion of the appendage (Manton 1964; Telford & Thomas 1995). Thus, the structure of mandibles has been recognized as a key morphological feature uniting insects and myriapods into Atelocerata (Sharov 1966).

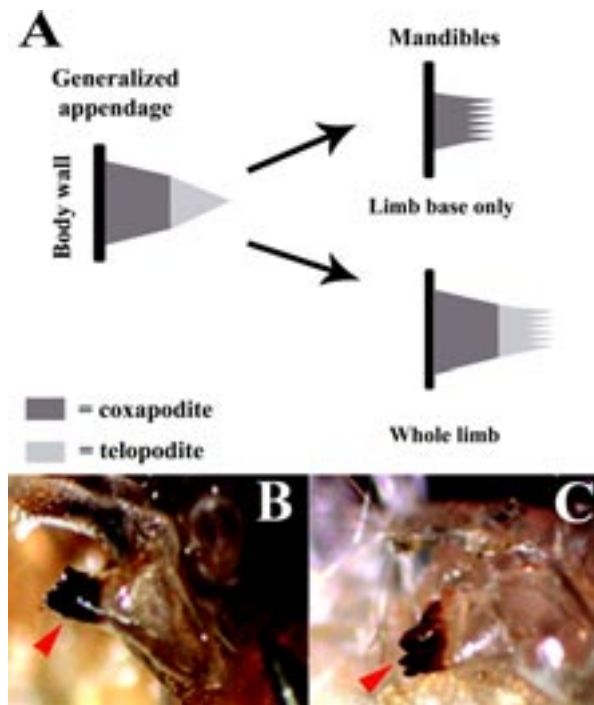


Figure 2. (A) A diagram depicting mandibular composition (whole limb vs. limb base only). (B) Dissected adult mandibles of the pillbug, *Armadillidium vulgare*. (C) Dissected adult mandibles of the firebrat, *Thermobia domestica*. In (B, C), arrowheads point to dissected mandibular appendages.

Because current molecular phylogenies do not support the Atelocerata concept, there is an increasing need for a critical re-examination of traditionally assigned character states (Boore et al. 1995; Cook et al. 2001; Friedrich & Tautz 1995; Kutsche & Burmester 2001; Regier & Shultz 1997). In the case of mandibles, this means to unambiguously determine which arthropods have a mandibular telopodite and which do not. The principal limitation of the traditional approach is that it lacks an in depth understanding of the developmental processes and genetic mechanisms responsible for these differences. This may restrict our ability to fully elucidate the evolution of a particular trait in the arthropod under study. The recent move to integrate the fields of evolutionary and developmental biology holds promise for overcoming this problem. By using a set of novel molecular markers (cross-reacting antibodies to gene products that regulate various developmental processes) and comparing their expression patterns in developing animals, it is now becoming possible to better understand the molecular basis, and hence, the origins of particular morphological features.

In *Drosophila melanogaster* embryos and larval imaginal disks, both expression and functional analyses have revealed that the gene *Distal-less* (*Dll*) is essential for formation and development of the distal portion of all appendages (Gonzalez-Crespo & Morata 1996;

Gorfinkiel et al. 1997). Moreover, subsequent comparative RNA inhibition experiments have indicated that the function of *Dll* is conserved across arthropods, from chelicerates to insects (Prpic et al. 2001; Schoppmeier & Damen 2001). Of equal importance, a cross-specific antibody that recognizes the DISTAL-LESS protein has been used successfully to study the expression of this gene in a variety of protostomes and deuterostomes (Panganiban et al. 1997; Popadic et al. 1998a). Both its specificity to distal portions of the appendages and its broad cross-reactivity make the *Dll* antibody an ideal molecular marker for delineating the true composition of arthropod mandibles. Coupled with its conserved function, an analysis of *Dll* expression provides a straightforward, unambiguous way of determining whether the distal portion of an appendage is present or not. This is critically important if one considers how difficult it is to infer the mandibular origins based solely on morphology. As an illustration, the dissected adult insect and crustacean mandibles are shown in figures 2B and C. Even to a trained eye, these appendages appear very similar, and yet, in the former, the mandible is thought to consist of a whole limb, whereas in the latter it encompasses a limb-base only.

The developmental origins of arthropod mandibles have been the focus of several recent studies (Grenier et al. 1997; Popadic 1996; Popadic et al. 1998b; Scholtz et al. 1998), and a summary of *Dll* expression patterns in representative embryos of major arthropod groups is depicted in figure 3. Among arthropods, chelicerates have a unique body plan that lacks a defined “head” region (Brusca & Brusca 1990). Instead, their bodies consist of an anterior prosoma that bears all walking and feeding appendages, and a limbless posterior opisthosoma. It is now generally accepted that in other arthropod lineages, some of the appendages corresponding to chelicerate walking legs have become incorporated into the head and became transformed into mouthparts. As shown for spider embryos, all of the limbs of the prosoma express *Dll* (Fig. 3A). This pattern persists throughout embryogenesis, and continues even after dorsal closure, to the stage just before hatching, when embryos resemble miniature adults (Popadic et al. 1998b). Thus, based on *Dll* expression, all six pairs of prosomal appendages are whole-limbs, consisting of both a basal coxopodite and a distal telopodite. This finding suggests that the ancestral state of all arthropod appendages (both walking and feeding) is of the whole limb type.

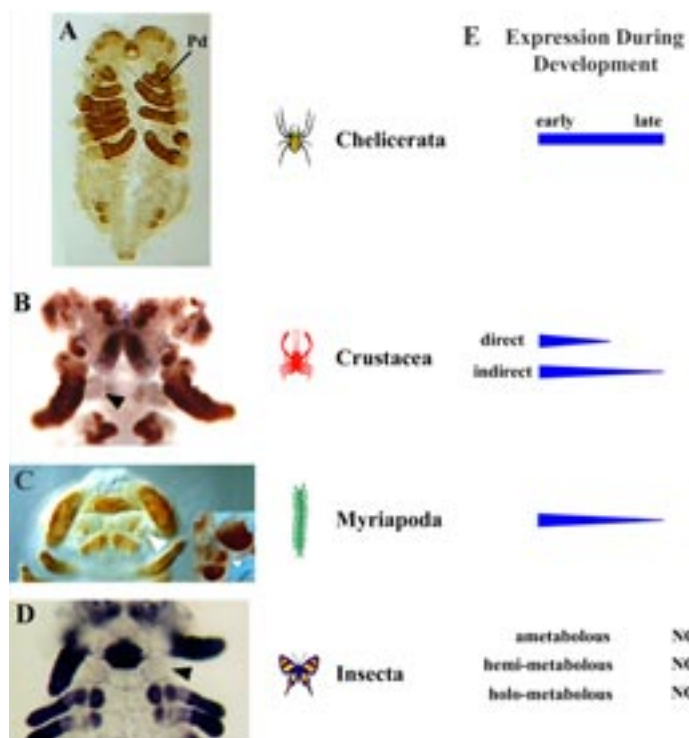


Figure 3. Expression patterns of *Distal-less* (*Dll*) in arthropod embryos. (A) Spider *Achaeranea tepidariorum*; note that although chelicerates do not have mandibles, it is thought that the pedipalpal segment (Pd) corresponds to it in other arthropods (Weygoldt 1979). (B) Pillbug, *Armadillidium vulgare*, a terrestrial isopod. (C) Millipede *Oxidus gracilis*, inset shows an earlier stage of development. (D) Firebrat, *Thermobia domestica*, a representative of a basal insect lineage. Arrowheads point to mandibles in myriapod, crustacean and insect embryos. (E) A diagram of *Dll* expression in the mandibular appendages in representative embryos of major classes of arthropods. *Filled rectangles* depict high, continuous expression of *Dll* from early to late development, whereas *filled arrowheads* indicate reduction of *Dll* expression in later developmental stages. There are no larval stages in chelicerates and myriapods. Modified from (Popadic et al. 1998b).

Among crustaceans, the vast majority of species undergo indirect development, which consists of a succession of larval stages, usually beginning with a nauplius (Schram 1986). These larvae are free living, feeding organisms that can be morphologically quite different from adults. Thus, if one is interested in adult morphology, there is always the question of whether and how much a particular larval feature contributes to adult form. Directly developing crustaceans, on the other hand, hatch from the egg as miniature adults. The nauplius and other larval stages are suppressed or occur sequentially within the egg. For this reason, studying

directly developing terrestrial species such as isopods provides a way to circumvent the potential problem of larval/adult differences. *Dll* expression analysis in early embryos of several directly developing crustaceans has shown a greatly reduced level of mandibular expression when compared to that of the antennal and maxillary segments (Popadic et al. 1998b; Scholtz et al. 1998). This is followed by a complete absence of mandibular *Dll* expression (Fig. 3B) in later stages of development as the limb buds begin to extend (Popadic et al. 1998b). Late nauplius larvae in indirectly developing species also lack mandibular *Dll* expression (Popadic et al. 1998b). Together, these observations provide strong evidence for the gnathobasic nature of mandibles in all crustaceans.

As previously mentioned, it has long been recognized that both myriapods and insects exhibit a mandible composed of both proximal and distal portions (Manton 1964). If this is true, then *Dll* antibody staining in myriapod and insect embryos should reveal *Dll* expression in the distal portion of the mandibular appendage. In two myriapod taxa studied, millipedes and centipedes, *Dll* expression can be observed in mandibular primordia during early embryogenesis (F.g. 3C inset) (Grenier et al. 1997; Popadic et al. 1998a; Popadic et al. 1998b; Scholtz et al. 1998). However, as development progresses, the *Dll* signal gradually decreases (Popadic et al. 1998b). By late embryogenesis, *Dll* cannot be detected in the tips of the mandibles, and with the exception of a few cells in the middle, is essentially absent from the entire limb (figure 3C). The other appendages, however, continue to express *Dll* throughout embryogenesis. This difference in expression pattern suggests that millipede mandibles lack, or have only a vestigial telopodite (distal tip).

Studies of *Dll* expression in insect embryos were equally revealing (Popadic et al. 1998b; Scholtz et al. 1998). From those of basal lineages such as *Thermobia domestica* (firebrats) to those of highly derived flies, there is a complete absence of *Dll* expression in the mandibles (Fig. 3D). Furthermore, this lack of *Dll* expression is observed from very early to late developmental stages, showing that *Dll* is never turned on in these appendages. Coupled with genetic analysis in *Tribolium castaneum* embryos (Prpic et al. 2001), these data show that insect mandibles are missing the distal part of the appendage. Therefore, the analysis of *Dll* patterns in embryos of representative ateloceratan taxa have provided direct evidence that insect and myriapod mandibles are lacking the telopodite and are gnathobasic.

Thus, in direct contrast with the traditional view, these comparative studies show that adult mandibles of insects, crustaceans, and myriapods are gnathobasic (Popadic 1996; Popadic et al. 1998b; Prpic & Tautz 2003; Prpic et al. 2001). Also, the study of expression of another appendage gene, *dachshund* (*dac*), recently provided additional strong evidence for this new view. *dac* is required to establish the medial portion of arthropod appendages (Prpic & Tautz 2003), and consequently, is expressed in the middle of whole-limb appendages such as legs. However, in mandibles *dac* is localized to the tip, exactly where expected if one assumes that the telopodite is absent. Thus, as a direct result of expression analyses of developmental genes such as *Dll* and *dac*, the structure of the mandibles can no longer be used to unite insects and myriapods in Atelocerata to the exclusion of crustaceans.

3 ARTHROPOD APPENDAGES: EMERGING VIEW

Due to individual adaptations to a variety of functions, arthropods exhibit the greatest amount of appendage diversity within the animal kingdom in terms of size, shape, and leg anatomy (Beklemishev 1964; Brusca & Brusca 1990). Traditionally, the existence of shared derived anatomical features has been used to suggest closer relationships within the arthropods. For example, one of the characteristics unifying insects and myriapods into Atelocerata is the presence of unbranched (uniramous) legs, which are anatomically distinct from the branched (multiramous) legs observed in a few chelicerates and most crustaceans (Manton 1964; Telford & Thomas 1995). What are the genetic underpinnings of this diversity? Results of recent molecular studies provide a significant insight into the genetic basis of leg evolution in arthropods.

In the past two decades, through the efforts of numerous research groups, the developmental mechanisms governing leg patterning in *Drosophila* imaginal disks have been determined to a fairly detailed level (Abu-Shaar & Mann 1998; Cohen et al. 1989; Gonzalez-Crespo & Morata 1996; Lecuit & Cohen 1997; Mardon et al. 1994). Secretion of the signal molecules *decapentaplegic* (*dpp*) and *wingless* (*wg*) acting in a combinatorial mode, activate various downstream genes across the proximo-distal (PD) axis of the developing leg disc (Fig. 4A). High levels of *dpp* and *wg* expression in the distal portion of the appendage activate *Distal-less* (*Dll*), thereby causing formation of the distal portion of the tibia, tarsus, and pre-tarsus (Cohen et al. 1989; Gonzalez-Crespo & Morata 1996; Lecuit & Cohen 1997). Moderate levels of *dpp* and *wg* in the medial portion of the leg activate *dachshund* (*dac*), a gene responsible for formation of the femur, and proximal tibia (Lecuit & Cohen 1997; Mardon et al. 1994). The proximal portion of the developing appendage (coxa and trochanter), is regulated through the effects of two other leg patterning genes, *homothorax* (*hth*) and *extradenticle* (*exd*) (Lecuit & Cohen 1997). A comparison between the genes governing appendage development in embryos of the more basal species such as cricket (*Acheta domesticus*) and the highly derived flies (*D. melanogaster*) has revealed expression patterns and dynamics of these genes to be highly similar (Abzhanov & Kaufman 2000). In addition to this general conservation, subtle differences have been observed in embryos of the flour beetle *Tribolium castaneum* in which the *dac/Dll* region of overlap is much smaller than in *Drosophila* (Prpic et al. 2001). However, the overall basic expression patterns of the genes determining the proximal, medial and distal portions of developing appendages (*hth/exd*, *dac* and *Dll*, respectively) are a conserved feature throughout the insects. This finding further indicates that the *Drosophila* PD axis patterning mechanism as a whole (Fig. 4A) is conserved in most insects. Recent studies focused on determining the expression of leg patterning genes in embryos of other arthropods (myriapods, crustaceans, and chelicerates) indicate that PD axis patterning is generally conserved, although some individual species-specific differences occur (Abzhanov & Kaufman 2000; Prpic et al. 2003; Prpic & Tautz 2003; Prpic et al. 2001). It is now recognized that PD axis patterning predates the evolution of arthropods (Prpic et al. 2003).

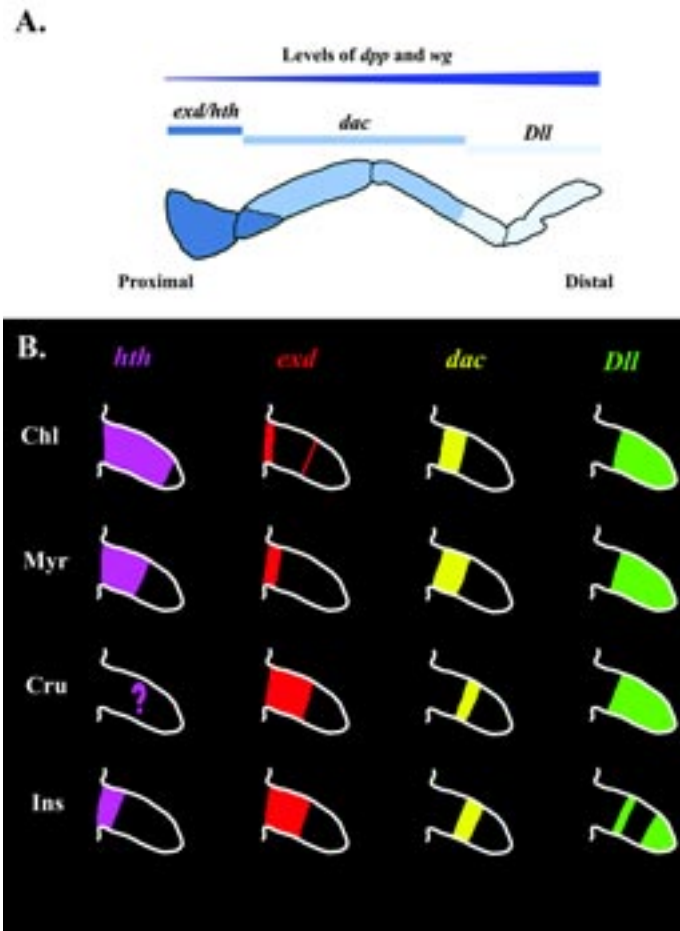


Figure 4. (A) Generalized leg PD axis patterning in *D. melanogaster* (B) Expression patterns of the four leg patterning genes in representatives of major arthropod lineages. Abbreviations: Chl (chelicerates), Myr (myriapods), Cru (crustaceans), Ins (insects), *exd* (*extradenticle*), *hth* (*homothorax*), *dac* (*dachshund*), *Dll* (*Distal-less*). The generated summary of expression patterns is based on the data from the following studies: (Abzhanov & Kaufman 2000; Prpic et al. 2003; Prpic & Tautz 2003; Prpic et al. 2001). The question mark denotes absence of data regarding *hth* expression in crustaceans.

When the expression domains of the major leg patterning genes in the embryos and larvae of various arthropods are compared, a number of similarities and differences are detected. This is evident in figure 4B, which depicts the spatial expression of the four leg patterning genes in representatives of the major arthropod groups during early leg embryogenesis (approximately 30%). A closer examination of *Dll* expression reveals that insects exhibit a unique “ring and

sock” pattern (Abzhanov & Kaufman 2000; Popadic et al. 1998b). More specifically, the proximal domain of *Dll* expression is confined to the proximal portion of the femur (ie. ring), while the distal domain encompasses distal half of the tibia and the entire tarsus with the exception of the pre-tarsus (ie. sock). Consequently, there is a gap in expression between the tibia and femur. *Dll* expression in myriapod embryos begins proximal to that of insects, and encompasses the entire medial and distal portion of the appendage (Prpic & Tautz 2003). Likewise, in chelicerate and crustacean embryos, *Dll* expression is present in all podomeres except the most proximal (coxa in chelicerates; coxa and trochanter in crustaceans) (Abzhanov & Kaufman 2000; Grenier et al. 1997; Popadic et al. 1998b). Collectively, these data suggest that *Dll* patterning is conserved throughout the arthropods with a derived pattern in insects.

As previously mentioned, the *dac* gene specifies development of the medial portion of appendages. As is evident in figure 4B, comparison of the *dac* expression among representative embryos of the major arthropod groups yields two findings: (i) similarity between insects and crustaceans, and (ii) between myriapods and chelicerates (Abzhanov & Kaufman 2000; Prpic et al. 2003; Prpic & Tautz 2003; Prpic et al. 2001). In insect embryos, *dac* is generally expressed in the femur, tibia and first tarsal segment (Prpic et al. 2001). In crustaceans, *dac* expression is confined to a single medial segment known as the merus (Abzhanov & Kaufman 2000). However, in chelicerate and myriapod embryos *dac* expression is restricted to trochanter and femur (Abzhanov & Kaufman 2000). Thus, insects and crustaceans exhibit medial *dac* expression, while myriapods and chelicerates have more proximal patterns. This indicates that *dac* may serve as a good candidate gene for comparative studies of arthropod development and has a potential to resolve further relationships between the major groups. Future comparison of regulatory mechanisms controlling *dac* expression in arthropod embryos could yield significant insights into the evolution of arthropod leg patterning.

Throughout the investigated arthropod embryos, *exd* is one of two genes that are responsible for the formation of the proximal portion of developing appendages. Comparison of *exd* expression patterns among arthropods can be used to contribute further to understanding of phylogenetic relationships. As is evident in figure 4B, the expression boundaries of *exd* in crustaceans and insects are similar to each other, as are those in chelicerates and myriapods. In insects and terrestrial crustaceans (isopods), *exd* expression starts proximally. Later in development, *exd* expression extends significantly more distally than in chelicerate and myriapod embryos (Abzhanov & Kaufman 2000; Prpic et al. 2003). Chelicerate *exd* expression is observed as a single stripe in the medial portion of the developing appendage with a second, more proximal domain (Prpic et al. 2003). Myriapod expression is similar, but lacks the band of medial expression (Prpic & Tautz 2003). However, it is important to recognize that the above data is based on expression at approximately 30% of development, when specific leg segments are still unrecognizable in most arthropod embryos.

More precise data on *exd* expression in later developmental stages in which leg segmentation is distinguishable has recently become available for several species (Fig. 5). Within the insects, two patterns have been detected: (i) in embryos of the more derived, holometabolous insects such as *Tribolium* and *Drosophila*, *exd* expression is ubiquitous, encompassing all podomeres, and; (ii) in those more basal, hemimetabolous insects such as *Acheta domesticus*, *exd* expression is restricted to the coxa and trochanter (Abzhanov & Kaufman 2000). In crustacean embryos, *exd* expression is also restricted to the coxa and basis

(Abzhanov & Kaufman 2000). Contrastingly, in chelicerate embryos, the medial pattern of *exd* is found in a single stripe around the joint separating the tibia and patella, with proximal expression confined to the proximal portion of the coxa (Prpic et al. 2003). The specific podomeres in which *exd* is expressed in myriapod embryos has yet to be determined. Although incomplete, available data of *exd* expression has revealed a shared expression pattern in both basal insects and crustaceans (Fig. 5). However, before any conclusions can be reached, it is important that equally detailed *exd* expression studies be performed on additional species throughout each subphylum, especially in myriapods.

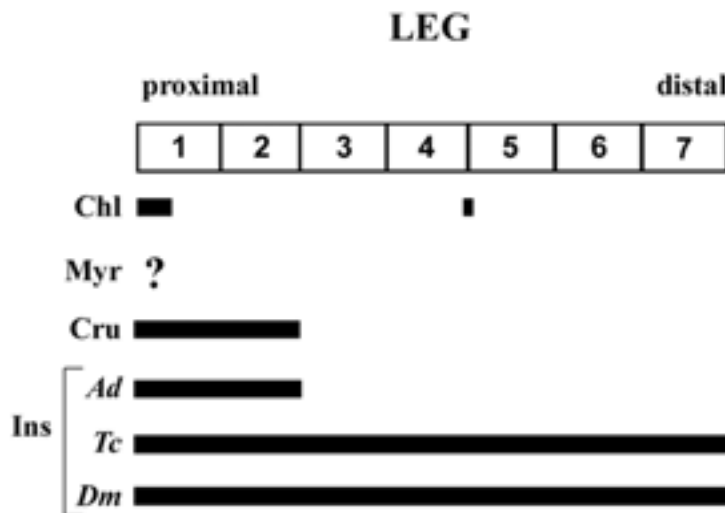


Figure 5. Expression of *extradenticle (exd)* in specific leg podomeres during later stages of development. Abbreviations: Chl (chelicerates), Myr (myriapods), Cru (crustaceans), Ins (insects), *Ad* (*Acheta domestica*), *Tc* (*Tribolium castaneum*), and *Dm* (*Drosophila melanogaster*). Based on the data from (Abzhanov & Kaufman 2000; Prpic et al. 2003; Rauskolb et al. 1995). The question mark denotes the absence of currently available information regarding *exd* expression in distinct leg segments in myriapods.

Homothorax (hth), the last of the four major leg patterning genes to be discussed, has been analyzed in all major arthropod lineages with the exception of the crustaceans (Abzhanov & Kaufman 2000; Prpic et al. 2003; Prpic & Tautz 2003). Within the chelicerates (Fig. 4B), *hth* expression is present throughout the appendage except in the apex (Prpic et al. 2003). In contrast, insect *hth* expression is localized to the proximal-most portion of each developing leg (Prpic et al. 2003). Myriapod expression is intermediate to the other two patterns (Fig. 4B), extending more proximally than in insects, but also retracting more distally than in chelicerates (Prpic & Tautz 2003). As illustrated in figure 4B, comparison of the mechanisms of spatial *hth* regulation between derived insect and the basal chelicerate embryos may provide a novel insight into the evolution of PD axis patterning. Disparities observed between the two groups

could serve as meaningful reference points, with each difference representing two extreme ends of a continuum. This can then be used to determine if crustacean *hth* expression is similar to that of embryos of any other group or whether it is unique. Based on data to date, it seems as if *hth* expression retracts in a proximal direction in the embryos of the more recent arthropod taxa. This would suggest that the crustacean *hth* pattern may be more similar to the situation observed in insects. However, as previously stated, future detailed crustacean *hth* expression analyses will be essential in order to make such an inference.

The emerging view from the above comparative analyses indicates that studies of appendage development have a potential to provide a significant, in-depth understanding of appendage evolution in arthropods. Whereas the genes responsible for PD axis specification are generally conserved, there is also a fair amount of variation in their expression domains, and consequently, their regulation. Some of this variation, as is the case with the “ring and sock” pattern of *Dll* in insects (fig. 4B), is unique to a particular lineage and would have no phylogenetic relevance. At the same time, the evo-devo studies have revealed a presence of shared variation in the observed expression patterns. For example, *exd* is localized in the first two leg podomeres in both basal insects and crustaceans (Fig. 5). *dac* expression patterns are also highly similar between insects and crustaceans, and quite different from that observed in myriapods (Fig. 4B). While highly indicative, these results also require a much better understanding of the origins of variation in gene regulation that exist in nature. To address this question, future evo-devo studies should shift focus from the gene expression analyses to the elucidation of the actual genetic mechanisms that govern the observed changes. Such research endeavors would not be trivial, but if they reveal a common regulatory mechanism, it would add a significant, independent support for a close relationship between insects and crustaceans.

4 NEXT CHALLENGE: ORIGINS OF COMPLEX FEATURES SUCH AS TRACHEAL SYSTEM

Tracheal systems in most arthropods comprise of a branching network of tubules that facilitate gas exchange in a terrestrial environment. The presence of a tracheal system also serves as an important phylogenetic character, as reflected in the name Tracheata which is also used to describe the assembly of insects and myriapods (Brusca & Brusca 1990; Kraus & Kraus 1994). As previously mentioned, recent molecular evidence argues against this concept, suggesting instead that insects are more closely related to crustaceans (Boore et al. 1995; Friedrich & Tautz 1995; Hwang et al. 2001). The former implies the homology of tracheae in insects and myriapods and thus a single origin in a common ancestor, whereas the latter suggests that these systems evolved independently. Distinguishing between these two hypotheses can be greatly facilitated by the understanding of the developmental basis and genetic architecture of arthropod tracheogenesis.

The respiratory system in most insects consists of a network of branched epithelial tubes ramifying throughout the body. The branching structure is organized in three levels: primary, secondary and terminal (Ohshiro et al. 2002; Sutherland et al. 1996). The terminal branches end close to or within tissues, directly delivering oxygen to and removing carbon dioxide from

them (Brusca & Brusca 1990). The system is bilaterally symmetrical, although each individual tube may be of different length and/or diameter. The cellular mechanisms of tube formation have been shown to differ in each of the three stages of branching (Ohshiro et al. 2002; Sutherland et al. 1996). The myriapod tracheal system is morphologically similar to that of insects, although some differences exist. Among the main myriapod lineages, most of Chilopoda (centipedes) have branched trachea, whereas most of Diplopoda (millipedes) have segmental clusters of unbranched respiratory tubules (Hilken 1997; Snodgrass 1935). Insight of tracheal systems in chelicerates is primarily based on studies of spiders. Only advanced spiders have one or two pairs of tubular tracheae which can branch throughout the body (Foelix 1996). Furthermore, some species possess only very short tubes, while others have highly branched tubes that pervade the prosoma and even the extremities. Most crustaceans are marine and use gills for gas exchange. However, terrestrial forms are characterized by the presence of thin-walled, blind ending sacs (pseudotracheae) in which the diffusion of gasses transpires. In summary, the presence of some form of tracheae is not restricted to insects and myriapods, and such structures are found in other terrestrial arthropods (eg. arachnids) and even onychophorans. This fact highlights the need to obtain the detailed understanding of the molecular aspects of tracheogenesis in order to discuss the origins of tracheal systems in various arthropods.

The *Drosophila* tracheal system is the only one within the arthropods with its developmental genetic basis thoroughly studied. In the past decade, these studies have identified key genes essential for tracheogenesis in this insect. As illustrated in figure 6, tracheogenesis is initiated when protein kinase B (PKB) interacts with *trachealess* (*trh*), a gene encoding a bHLH-PAS transcription factor (Jin et al. 2001). Embryos in which *trh* has been knocked out never experience the initial invagination event, resulting in the ectodermal cells of the tracheal placode remaining at the surface and with no formation of a tracheal system (Jin et al. 2001). *Ventral veinless/drifter* (*vvl/dfr*), another gene which encodes the POU-domain transcription factor CF1a, is also required for tracheogenesis (Bradley & Andrew 2001). The transcription factors encoded by both *trh* and *vvl/dfr* then directly regulate the activation of *branchless* (*bnl*), an insect fibroblast growth factor (FGF) homolog (Bradley & Andrew 2001; Sutherland et al. 1996). *Branchless* then directly activates the gene *breathless* (*btl*) whose effects are essential for primary branch formation (Bradley & Andrew 2001; Ohshiro et al. 2002; Sutherland et al. 1996). Mutations of *btl* in embryos result in a fully internalized but undifferentiated sac of ectodermal cells (Ohshiro et al. 2002). Recently, it has been found that the *decapentaplegic* (*dpp*), *epidermal growth factor receptor* (*EGFR*) and *wingless* (*WG/WNT*) signaling pathways also affect primary branching by aiding the specific migration of primary branches (Bradley & Andrew 2001). While expression of additional genes are required for proper tracheogenesis, it is the effects of *trh* and *vvl/dfr* that drive the cascade of events (Fig. 6). Due to the close similarity of tracheal structure and function in the vast majority of other insects, the *Drosophila* respiratory system can likely serve as a model representing tracheal system development in all insects. It is this level of understanding that needs to be achieved for tracheogenesis in other arthropod embryos.

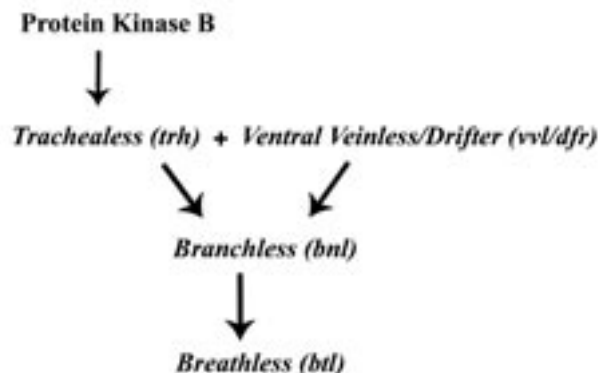


Figure 6. Genetic regulation of tracheogenesis in *D. melanogaster*, based on data from (Bradley & Andrew 2001; Jin et al. 2001; Ohshiro et al. 2002; Sutherland et al. 1996).

The research on tracheal structure and function represents a well studied aspect of arthropod biology which has been treated in great detail by a number of authors (Dohle 1985; Fahlander 1938; Hilken 1997). For the purpose of this article, it is sufficient to say that there is a diversity of views with regard to tracheal origins. These views range from strongly supportive of the single origin in insects and myriapods (Wagele & Stanjek 1995) to ones postulating that tracheae originated independently at least four (Kraus 1998), and perhaps as much as six times (Dohle 1988). We may begin to resolve these conflicting views by assessing the complexity of the molecular regulation used to develop a system of invaginated ectodermal tubes with respiratory function. By this we mean characterizing the genes and their interactions in the pathway, and assessing their presence or absence in diverse taxa. Are all the genes present in all taxa, and hence ancestral? Are parts of the pathway used in other developmental contexts or are they novel? It must be recognized that it is now generally accepted that morphological novelties arise by the tinkering of already existing developmental machinery, and not by generating developmental networks *de novo* (Wilkins 2002). For example, development of both the *Drosophila* tracheal system and mammalian lungs is partly regulated through the same signaling pathway suggesting a common scheme for patterning branching morphogenesis (Metzger & Krasnow 1999). Thus, it is likely that some similarities will be observed when comparing details of insect and myriapod tracheogenesis. Nevertheless, we should still be able to detect an appreciable degree of regulatory differences if these groups are not sister taxa. The opposing view, favoring Atelocerata, could also be further tested by studying genetic mechanisms of tracheogenesis in chelicerates such as spiders. This is because advanced spiders possess a tracheal system that is evolutionarily distinct from the ones present in insects and myriapods. Thus, similarities observed between the chelicerate and insect/myriapod systems can be attributed to developmental canalization. Any remaining similarities between insects and myriapods would then likely reflect common ancestry. Future evo-devo studies of this kind have a potential to elucidate the origins of arthropod tracheal systems.

5 CONCLUSION

In the past decade, primarily by comparing gene expression patterns, the field of evolution of development has offered a new insight into the evolution of arthropod body plans. With regard to arthropod systematics, several highly focused investigations have now revealed that all adult arthropod mandibles are gnathobasic in nature (Grenier et al. 1997; Popadic et al. 1998b; Scholtz et al. 1998). As a consequence, this character (mandibular composition) can no longer be used to group insects and myriapods together to the exclusion of crustaceans. Another recent study comparing brain morphologies has provided new insight into the phylogenetic position of another arthropod, that of remipede crustaceans. Since their discovery in 1979, remipedes were considered a basal, proto-crustacean lineage (Schram 1986). However, it has now been revealed that the remipede brain is highly organized and well differentiated at a level of complexity matched only by the brain of “higher” crustaceans (Malacostraca) and Hexapods (Fanenbruck et al. 2004). This surprising result therefore argues in favor of a remipede-malacostracan-hexapod clade. Collectively, these studies highlight the potential of utilizing novel approaches to help further clarify arthropod relationships.

Our motivation for writing this article was to provide a brief, “evo-devo centric” perspective on how to resolve the issue of homologies of morphological traits that have been traditionally used in arthropod systematics. As more data emerges, the complexity of the developmental mechanisms governing the formation of various arthropod features (such as tracheae and malpighian tubules) has become much more apparent. As exemplified by appendage development, the genetic cascade of events responsible for structural formation is known to include many target genes and numerous regulatory events. It is therefore necessary that future studies of evo-devo reach beyond the comparison of expressional domains of just key genes and instead focus on delineating these developmental pathways in their entirety. Only by determining the genetic mechanisms of structural development down to their lowest levels (encompassing specific regulatory events, target genes, etc.) in key taxa, will we be able to reach a true understanding of the origins of the key morphological features in arthropods.

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REFERENCES

- Abu-Shaar, M. & Mann, R. S. (1998). Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development* 125: 3821-30.
- Abzhanov, A. & Kaufman, T. C. (2000). Homologs of *Drosophila* appendage genes in the patterning of arthropod limbs. *Dev. Biol.* 227: 673-89.

- Beklemishev, W. (1964). *Principles of the comparative anatomy of invertebrates*. Chicago: University of Chicago Press.
- Boore, J. L., Collins, T. M., Stanton, D., Daehler, L. L. & Brown, W. M. (1995). Deducing the pattern of arthropod phylogeny from mitochondrial DNA rearrangements. *Nature* 376: 163-5.
- Boore, J. L., Lavrov, D. V. & Brown, W. M. (1998). Gene translocation links insects and crustaceans. *Nature* 392: 667-8.
- Bradley, P. L. & Andrew, D. J. (2001). *ribbon* encodes a novel BTB/POZ protein required for directed cell migration in *Drosophila melanogaster*. *Development* 128: 3001-15.
- Brusca, R. & Brusca, G. (1990). *Invertebrates*. Sunderland: Sinauer.
- Cohen, S. M., Bronner, G., Kuttner, F., Jurgens, G. & Jackle, H. (1989). *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* 338: 432-4.
- Cook, C. E., Smith, M. L., Telford, M. J., Bastianello, A. & Akam, M. (2001). Hox genes and the phylogeny of the arthropods. *Curr. Biol.* 11: 759-63.
- Dohle, W. (1985). Phylogenetic pathways in the Chilopoda. *Bijdragen tot de Dierkunde* 55: 55-66.
- Dohle, W. (1988). *Myriapoda and the Ancestry of insects*. Manchester, UK: The Manchester Polytechnic.
- Fahlander, K. (1938). Beitrage zur Anatomie und systematischen Einteilung der Chilopoden. *Zoologische Bidrag fran Uppsala* 17: 1-148.
- Fanenbruck, M., Harzsch, S. & Wagele, J. W. (2004). The brain of the Remipedia (Crustacea) and an alternative hypothesis on their phylogenetic relationships. *Proc. Natl. Acad. Sci. U S A* 101: 3868-73.
- Foelix, R. (1996). *Biology of Spiders*. New York: Oxford University Press.
- Friedrich, M. & Tautz, D. (1995). Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. *Nature* 376: 165-7.
- Gonzalez-Crespo, S. & Morata, G. (1996). Genetic evidence for the subdivision of the arthropod limb into coxopodite and telopodite. *Development* 122: 3921-8.
- Gorfinkiel, N., Morata, G. & Guerrero, I. (1997). The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*. *Genes. Dev.* 11: 2259-71.
- Grenier, J. K., Garber, T. L., Warren, R., Whittington, P. M. & Carroll, S. (1997). Evolution of the entire arthropod Hox gene set predated the origin and radiation of the onychophoran/arthropod clade. *Curr. Biol.* 7: 547-53.
- Hilken, G. (1997). Vergleich von Tracheensystemen unter phylogenetischem Aspekt. *Verhandlungen des naturwissenschaftlichen Vereins in Hamburg* NF,37.
- Hwang, U. W., Friedrich, M., Tautz, D., Park, C. J. & Kim, W. (2001). Mitochondrial protein phylogeny joins myriapods with chelicerates. *Nature* 413: 154-7.
- Jin, J., Anthopoulos, N., Wetsch, B., Binari, R. C., Isaac, D. D., Andrew, D. J., Woodgett, J. R. & Manoukian, A. S. (2001). Regulation of *Drosophila* tracheal system development by protein kinase B. *Dev. Cell* 1: 817-27.
- Kraus, O. & Kraus, M. (1994). Phylogenetic system of the Tracheata (Mandibulata): on 'Myriapoda'-Insecta interrelationships, phylogenetic age and primary ecological niches. *Verhandlungen des naturwissenschaftlichen Vereins in Hamburg* 34: 5-31.
- Kukalova-Peck, J. (1992). The "Uniramia" do not exist: The ground plan of the Pterygota as revealed by Permian Diaphanopteroidea from Russia. *Can. J. Zool.* 70: 236-255.
- Kusche, K. & Burmester, T. (2001). Diplopod hemocyanin sequence and the phylogenetic position of the Myriapoda. *Mol. Biol. Evol.* 18: 1566-73.
- Lecuit, T. & Cohen, S. M. (1997). Proximal-distal axis formation in the *Drosophila* leg. *Nature* 388: 139-45.
- Manton, S. (1964). Mandibular mechanisms and the evolution of arthropods. *Philos. Trans. R. Soc. London Ser. B* 247: 1-183.

- Mardon, G., Solomon, N. M. & Rubin, G. M. (1994). *dachshund* encodes a nuclear protein required for normal eye and leg development in *Drosophila*. *Development* 120: 3473-86.
- Metzger, R. J. & Krasnow, M. A. (1999). Genetic control of branching morphogenesis. *Science* 284: 1635-9.
- Ohshiro, T., Emori, Y. & Saigo, K. (2002). Ligand-dependent activation of *breathless* FGF receptor gene in *Drosophila* developing trachea. *Mech. Dev.* 114: 3-11.
- Panganiban, G., Irvine, S. M., Lowe, C., Roehl, H., Corley, L. S., Sherbon, B., Grenier, J. K., Fallon, J. F., Kimble, J., Walker, M., Wray, G. A., Swalla, B. J., Martindale, M. Q. & Carroll, S. B. (1997). The origin and evolution of animal appendages. *Proc. Natl. Acad. Sci. U S A* 94: 5162-6.
- Pisani, D., Poling, L. L., Lyons-Weiler, M. & Hedges, S. B. (2004). The colonization of land by animals: molecular phylogeny and divergence times among arthropods. *BMC Biol* 2: 1.
- Popadić, A. (1996). Origin of the arthropod mandible. *Nature* 380: 395.
- Popadić, A., Abzhanov, A., Rusch, D. & Kaufman, T. C. (1998a). Understanding the genetic basis of morphological evolution: the role of homeotic genes in the diversification of the arthropod bauplan. *Int. J. Dev. Biol.* 42: 453-61.
- Popadić, A., Panganiban, G., Rusch, D., Shear, W. A. & Kaufman, T. C. (1998b). Molecular evidence for the gnathobasic derivation of arthropod mandibles and for the appendicular origin of the labrum and other structures. *Dev. Genes Evol.* 208: 142-50.
- Prpic, N. M., Janssen, R., Wigand, B., Klingler, M. & Damen, W. G. (2003). Gene expression in spider appendages reveals reversal of *exd/hth* spatial specificity, altered leg gap gene dynamics, and suggests divergent distal morphogen signaling. *Dev. Biol.* 264: 119-40.
- Prpic, N. M. & Tautz, D. (2003). The expression of the proximodistal axis patterning genes *Distal-less* and *dachshund* in the appendages of *Glomeris marginata* (Myriapoda: Diplopoda) suggests a special role of these genes in patterning the head appendages. *Dev. Biol.* 260: 97-112.
- Prpic, N. M., Wigand, B., Damen, W. G. & Klingler, M. (2001). Expression of *dachshund* in wild-type and *Distal-less* mutant *Tribolium* corroborates serial homologies in insect appendages. *Dev. Genes Evol.* 211: 467-77.
- Rauskolb, C., Smith, K. M., Peifer, M. & Wieschaus, E. (1995). *extradenticle* determines segmental identities throughout *Drosophila* development. *Development* 121: 3663-73.
- Regier, J. C. & Shultz, J. W. (1997). Molecular phylogeny of the major arthropod groups indicates polyphyly of crustaceans and a new hypothesis for the origin of hexapods. *Mol. Biol. Evol.* 14: 902-13.
- Scholtz, G., Mittmann, B. & Gerberding, M. (1998). The pattern of *Distal-less* expression in the mouthparts of crustaceans, myriapods and insects: new evidence for a gnathobasic mandible and the common origin of Mandibulata. *Int. J. Dev. Biol.* 42: 801-10.
- Schoppmeier, M. & Damen, W. G. (2001). Double-stranded RNA interference in the spider *Cupiennius salei*: the role of *Distal-less* is evolutionarily conserved in arthropod appendage formation. *Dev. Genes Evol.* 211: 76-82.
- Schram, F. R. (1986). *Crustacea*. New York: Oxford University Press.
- Sharov, A. G. (1966). *Basic Arthropodan Stock*. New York: Pergamon.
- Snodgrass, R. E. (1935). *Principles of Insect Morphology*. New York: McGraw-Hill Book Company, Inc.
- Sutherland, D., Samakovlis, C. & Krasnow, M. A. (1996). *branchless* encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* 87: 1091-101.
- Telford, M. & Thomas, R. (1995). Demise of Atelocerata? *Nature* 376: 123-4.
- Wagele, J. W. & Stanjek, G. (1995). Arthropod phylogeny inferred from 12S rRNA revisited: monophyly of Tracheata depends on the sequence alignment. *J. Zool. Syst. Evol. Res.* 33: 75-80.
- Wilkins, A. S. (2002). *The Evolution of Developmental Pathways*. Sunderland: Sinauer Associates, Inc.