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Original Article

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

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ABSTRACT We examined embryos of representatives of crustaceans, myriapods and insects with respect to *Dll* expression in the mouthparts. In order to examine the relationships between mandibular *Dll* expression and the occurrence of a mandibular palp we compared amphipod, isopod and decapod crustacean species. In species with mandibular palps, *Dll* expression is maintained throughout development and is restricted to the palps. The species lacking a palp as an adult show only transient *Dll* expression in early embryonic stages. Furthermore, we studied mandibular *Dll* expression in the myriapod *Glomeris marginata* that lacks like all myriapods mandibular palps as an adult. The expression pattern is similar to that in crustaceans lacking a palp as an adult. We examined entognathous and ectognathous insects. No sign of mandibular expression could be detected. It is shown that the distal parts of the mandibular appendage were reduced in several steps and lineages independently up to a total loss. Furthermore, we studied *Dll* expression in the first and second maxillae. Except for *Glomeris* and the collembolans, the first maxillae of all species show a similar pattern of three lobes expressing *Dll*: the outer expression marks the maxillary palp and the inner two mark the outgrowing endites (galea and lacinia of insects). In the first maxillae of collembolans only two expression areas could be detected. In palpless adult first maxillae of isopod crustaceans a transitory embryonic palp occurs which is also *Dll* positive. In the second maxillae of insects, isopod and amphipod crustaceans only two *Dll*-positive lobes occur. Our data suggest a gnathobasic character of the mandibles of crustaceans, myriapods and insects supporting the monophyly of Mandibulata *sensu* Snodgrass. The interpretation of *Dll* expression patterns and its limits are critically evaluated.

KEY WORDS: *mandibles, maxillae, arthropods, limb development, evolution*

Introduction

The nature, evolutionary origin and homology of arthropod appendages have always been a matter of controversy. Some long standing questions concern the relationship of annelid parapodia and arthropod legs, the question of the ground-pattern of the arthropod leg, or the origin of the labrum and other non leg-like appendages from segmental arthropodia (Lauterbach, 1978; Walossek, 1993; Scholtz, 1995,1997; Fryer, 1997; Kukalova-Peck, 1997). In some cases the specific characteristics of appendages have been used to establish monophyletic groups, for example the chelicera for the Chelicerata (Xiphosura, Pantopoda, Arachnida) (Heymons, 1901) or the mandible and maxillae for the Mandibulata (crustaceans, insects, myriapods) (Snodgrass, 1938). These views are based on the claim of homology for these particular appendage types. In the case of the mandibles the specific similarities are seen in the segmental position in the head and in the overall morphology (molar, incisivus). Furthermore, mandibles are interpreted as being enlarged proximal parts (coxopodite) with the distal parts (telopodite) being either reduced to a palp (some crustaceans) or completely absent (some crustaceans, all myriapods and insects). According to this view, biting is generally done with the inner margin of the enlarged coxopodite (gnathobasic mandible) (Snodgrass, 1938; Lauterbach, 1972; Boudreaux, 1987; Wägele, 1993). However, authors such as

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Abbreviations used in this paper: Dll , Distal-less.

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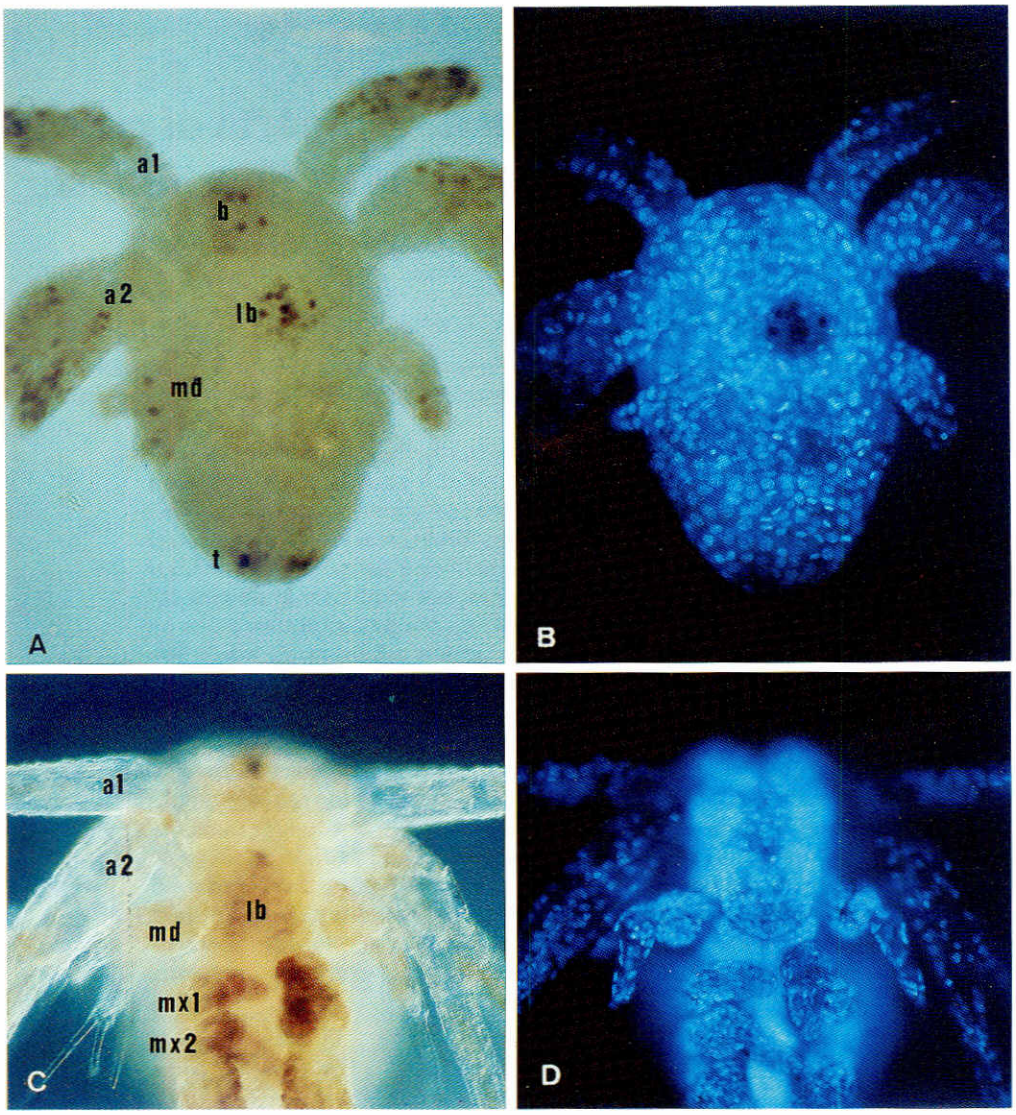

Fig. 1. *Dll* expression in the nauplius larvae of the decapod crustacean *Penaeus monodon*. (A) *First nauplius with Dll expression in the distal parts of first and second antennae (a1, a2) and in the branches of the biramous mandibles (md). In addition Dll expression is seen in the brain anlage (b), in the forming labrum (lb), and the telson (t).* **(B)** *Same preparation as in A, counterstained to show the morphology. No structures for feeding are formed in the mandibles.* **(C)** *Last metanauplius stage. The proximal parts of the mandibles are enlarged forming the prospective gnathal elements. The first and second maxillae (mx1, mx2) express Dll in the distal parts as well as in the inner lobes.* **(D)** *Same preparation as in C, counterstained to show the morphology.*

Manton (1964,1973) and Fryer (1996) doubt mandible homology. They agree that crustaceans mandibles are gnathobasic but they claim that the mandibles of myriapods and insects represent whole limbs and biting is done with the tips (whole-limb mandible). In conclusion, these authors do not accept Mandibulata as a valid taxon but suggest an independent origin of myriapods and insects (Uniramia) on the one hand, and crustaceans on the other hand. More recently, similar ideas have been stressed by Brusca and Brusca (1990) and Kraus and Kraus (1994). These authors claim that the whole-limb mandible is a synapomorphy of myriapods and insects but they do not deny the monophyly of the Mandibulata, including the Crustacea. Kraus (1997) modified this view suggesting that insect and myriapod mandibles are not composed of the whole limb but of three basal limb segments with the most distal one forming the biting and chewing parts (telognathic mandible). The question of homology between crustacean, insect and myriapod mandibles has also been raised by arthropod phylogenies based on molecular characters or paleontological data because in some cases the mandibulates are not monophyletic (e.g., Friedrich and Tautz, 1995; Wills *et al.*, 1995).

Recently, the expression patterns of the gene *Distal-less (Dll)* have provoked a new discussion of the long standing questions of arthropod limb homologies. The expression pattern of *Dll* has been used to analyze leg formation and evolution in representatives of crustaceans, insects, myriapods, and chelicerates (Panganiban *et al.*, 1994,1995; Popadic *et al.*, 1996; Williams and Nagy, 1996; Niwa *et al.*, 1997; Rogers and Kaufman, 1997; Scholtz and Gerberding, 1997; Williams, 1998). From experiments with *Drosophila* embryos and larvae it has been shown that *Dll* plays a key role in the differentiation of appendages (Cohen and Jürgens, 1989a,b). Moreover, it has been suggested that *Dll* expression marks the telopodite of arthropod legs (González-Crespo and Morata, 1996; Niwa *et al.* 1997). If this assumption is correct one would expect that in a gnathobasic mandible no *Dll* expression occurs whereas the tip of a whole-limb mandible should express *Dll*. A striking result of many of these investigations is that there is no *Dll* expression in the mandibles of any insect examined. From this absence of mandibular *Dll* expression it was concluded that insects possess a gnathobasic mandible (Panganiban *et al.*, 1994,1995; Popadic *et al.* 1996; Niwa *et al.*, 1997; Rogers and

Kaufman, 1997). Popadic *et al.* (1996) went so far as to suggest that the absence of mandibular *Dll* expression they found in an isopod crustacean is indicative of a close crustacean-insect relationship. However, there are some inconsistencies with either classical model (Snodgrass or Manton). Some crustacean larvae and embryos show mandibular *Dll* expression while others do not (Panganiban *et al.*, 1995; Popadic *et al.*, 1996). Furthermore, *Dll* expression is seen in the mandibles of myriapods (Popadic *et al.*, 1996; Grenier *et al.*, 1997). Also *Dll* expression is not restricted to distal leg structures in all cases. *Dll* expression was found in clearly proximal elements such as the endites (galea, lacinia) of insect maxillae (Williams and Nagy, 1996; Niwa *et al.*, 1997; Rogers and Kaufman, 1997) and in the basal parts of the phyllopodous legs of crustaceans (Panganiban *et al.*, 1995; Williams, 1998). Therefore, conclusions concerning phylogenetic relationships among mandibulates or the interpretation of the occurrence of gnathobasic or whole limb mandibles based on the available data of *Dll* expression seem to be precocious.

Against this background we undertook a comparative analysis of *Dll* expression patterns in the mouthparts of various crustaceans, insects including entognathous collembolans and a myriapod representative. In particular, we address the following questions:

How and to what extent can the expression pattern of *Dll* in relation to morphogenesis help to solve the question of whether crustaceans, myriapods and insects share a gnathobasic mandible or whether myriapods and/or insects possess a whole-limb mandible? What is the nature of the mandibular palp in crustaceans – does it represent the distal parts of the mandibular limb? What is the spatial and temporal pattern of *Dll* expression in the mandibles of crustaceans which clearly lost the palps in evolution? Can these species provide us with a model for a putative loss of distal structures (palps?) in myriapod and insect mandibles? Do all insects, even entognathans, share the total absence of mandibular *Dll* expression? In addition we analyze the *Dll* expression pattern in the first and second maxillae of the species investigated. In the discussion we draw conclusions concerning mandibulate monophyly and evolution. Furthermore, we discuss a putative twofold function of the *Dll* gene.

Results

Crustaceans

The development of the decapod crustacean *Penaeus monodon* is characterized by several larval stages (Fig. 1). During late embryonic development *Dll* is expressed in the buds of the two pairs of antennae and the mandibles. The earliest post-embryonic stage is the nauplius larva comprising the three anteriormost segments and their appendages, the first antennal segment, the second antennal segment and the mandibular segment. The gnathal parts of the mandibles are not yet developed because the first naupliar stages of penaeids do not feed. *Dll* is expressed in the distal parts of all appendages. In the second antennae and the mandibles, expression occurs in both branches, exopodite and endopodite (Fig. 1A). The gnathal parts of the mandibles develop in the late metanauplius stages (Fig. 1C,D). They do not express *Dll*. In the distal branches of the mandibles *Dll* expression ceases and after the moult to the protozoea stage, the distal branches are gone and only the gnathal part remains. However, a new palp is formed during further development. The two pairs of maxillae begin as simple buds with a terminal *Dll* expression. During the late metanaupliar stages they are leg-like, somewhat flattened and

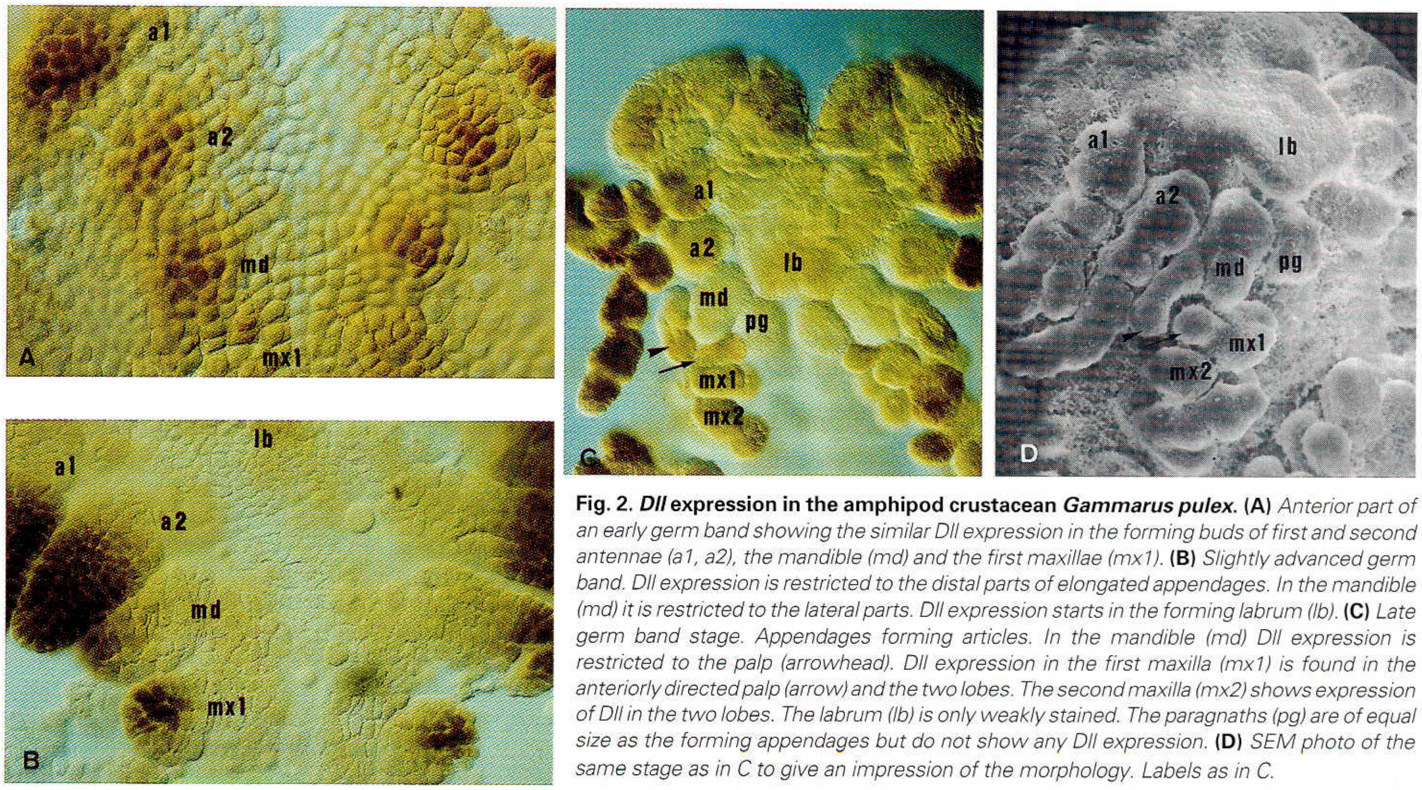

Fig. 2. *Dll* expression in the amphipod crustacean *Gammarus pulex*. (A) *Anterior part of an early germ band showing the similar Dll expression in the forming buds of first and second antennae (a1, a2), the mandible (md) and the first maxillae (mx1).* **(B)** *Slightly advanced germ band. Dll expression is restricted to the distal parts of elongated appendages. In the mandible (md) it is restricted to the lateral parts. Dll expression starts in the forming labrum (lb).* **(C)** *Late germ band stage. Appendages forming articles. In the mandible (md) Dll expression is restricted to the palp (arrowhead). Dll expression in the first maxilla (mx1) is found in the anteriorly directed palp (arrow) and the two lobes. The second maxilla (mx2) shows expression of Dll in the two lobes. The labrum (lb) is only weakly stained. The paragnaths (pg) are of equal size as the forming appendages but do not show any Dll expression.* **(D)** *SEM photo of the same stage as in C to give an impression of the morphology. Labels as in C.*

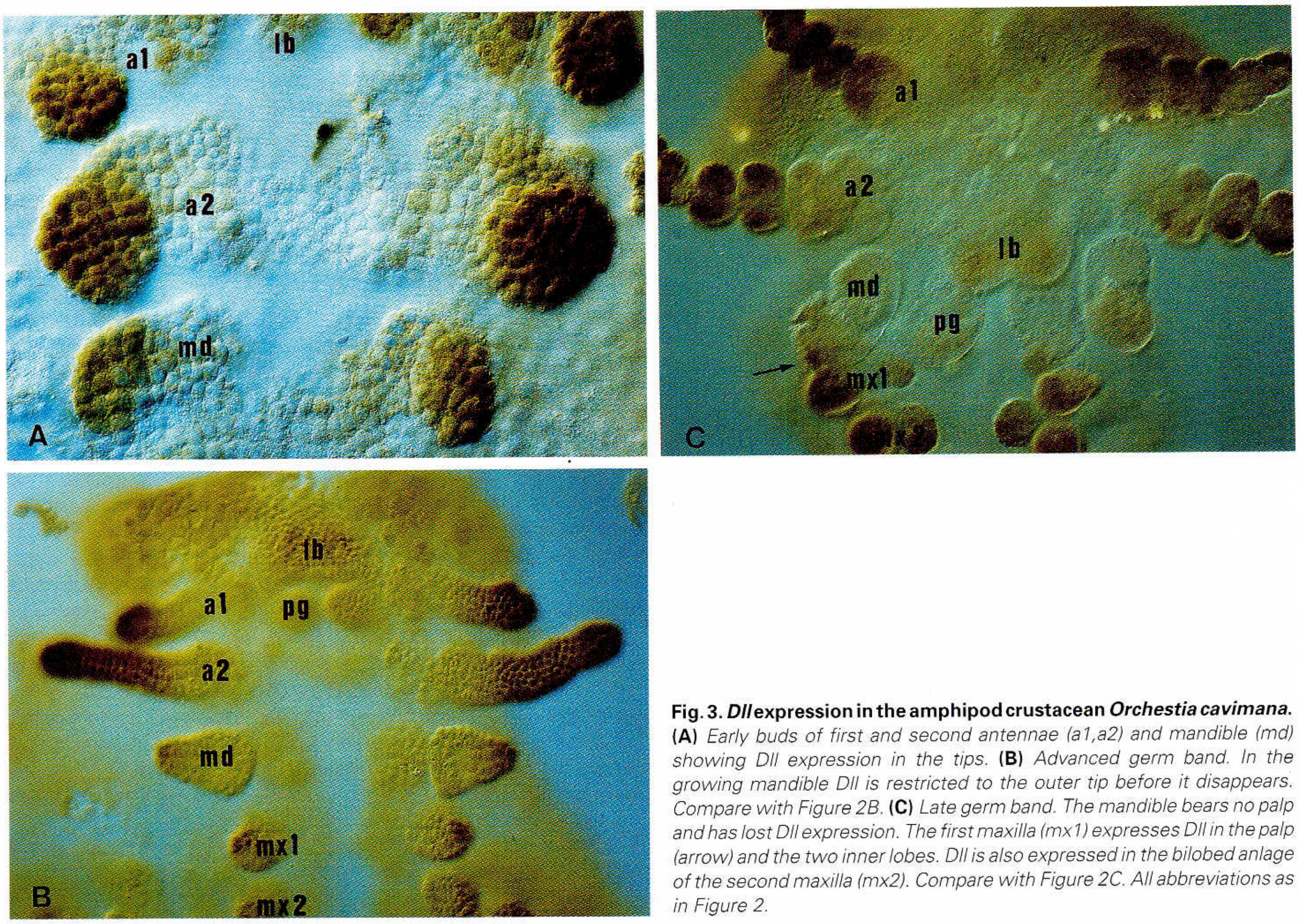

Fig. 3. ***Dll* expression in the amphipod crustacean *Orchestia cavimana*.** **(A)** *Early buds of first and second antennae (a1,a2) and mandible (md) showing Dll expression in the tips.* **(B)** *Advanced germ band. In the growing mandible Dll is restricted to the outer tip before it disappears. Compare with Figure 2B.* **(C)** *Late germ band. The mandible bears no palp and has lost Dll expression. The first maxilla (mx1) expresses Dll in the palp (arrow) and the two inner lobes. Dll is also expressed in the bilobed anlage of the second maxilla (mx2). Compare with Figure 2C. All abbreviations as in Figure 2.*

equipped with lobate endites. Expression of *Dll* is seen throughout the appendage including the endites (Fig. 1C).

All other crustaceans examined undergo direct development. In the amphipod *Gammarus pulex*, we find another crustacean species which possesses a mandibular palp in the adult stage. As in *Penaeus* this palp has the 3-segmented uniramous structure which is characteristic for adult malacostracan crustaceans (Schminke, 1996). *Dll* expression is visible before the mandibles can be recognized morphologically. It forms a round spot which corresponds to the circular shape of the early limb bud (Fig. 2A). The initial buds of the mandibular anlagen and the corresponding *Dll* expression do not differ from the early buds of other appendages. In more advanced stages *Dll* expression is restricted to the laterally projecting tips of the mandibular buds (Fig. 2B). Morphologically, the initially round mandibular bud elongates in transverse direction resulting in a rectangular shape when seen ventrally (Fig. 2C,D). This structure is later on subdivided into two parts by a slight groove (Fig. 2D). The *Dll* positive area marks the region of the outgrowth of the palp. During growth the mandibular palp continuously expresses *Dll* (Fig. 2C). Other parts of the embryonic mandibles remain without *Dll* expression.

Orchestia cavimana, the other amphipod representative investigated is a species without a mandibular palp. Within amphipods and malacostracans in general, this is certainly the derived condition. This species is thus particularly well suited to studying mandible morphogenesis and *Dll* expression in comparison to the closely related amphipod *Gammarus pulex*. Initially the round mandibular bud expresses *Dll* in a pattern similar to *Gammarus* (Fig. 3A). With lateral widening of the mandibular anlage *Dll* expression is restricted to an outer lateral spot as is the case in *Gammarus* (Fig. 3B). However, there is no outgrowing palp and in advanced stages *Dll* expression is lost (Fig. 3C).

The isopod *Porcellio scaber* belongs to the terrestrial isopods, the Oniscidea, which are characterized by a palpless mandible. As in the case of the amphipod *Orchestia* this is a derived character. *Dll* expression starts in the mandible anlagen of *Porcellio* before they are morphologically visible (Fig. 4A). From the onset of morphological growth the expression is restricted to a relatively small spot at the outer margin of the mandible bud. During further development *Dll* expression is restricted to this lateral position until its decay (Fig. 4B). A bud for the palp is never visible.

Early morphogenesis and *Dll* expression patterns of the first maxillae of the isopod and amphipods examined is quite similar. The initial buds are subdivided into three lobes which each express *Dll* in their tips (Figs. 2,3,4). The outer lobe is the anlage of the palp. Adult *Orchestia* possess only a reduced first maxillary palp, while

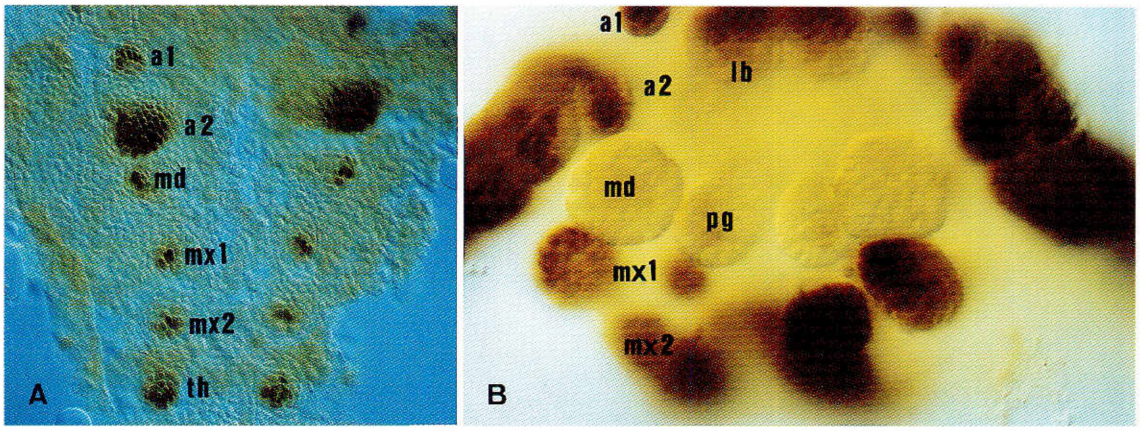

Fig. 4. *Dll* expression in the isopod crustacean *Porcellio scaber*. (A) *Early anterior germ band. The Dll expression in the mandible (md) is restricted to a small lateral part in the outgrowing bud.* **(B)** *Late germ band. The mandible lost Dll expression (compare with Figs. 2C,3C). th, thoracic leg. All other abbreviations as in Figure 2.*

in adult *Porcellio* a first maxillary palp is absent. Nevertheless, a vestigial palp anlage with *Dll* expression is present during embryonic development. The second maxillae of all three species are reduced. Embryologically a bilobed anlage follows the early bud. Both lobes express *Dll* (Figs. 2,3,4).

Myriapods

The diplopod *Glomeris marginata* lacks a mandibular palp in the adult. The mandible is uniramous as in all myriapod representatives. Early germ band stages of *Glomeris marginata* show a distinct *Dll* expression pattern in the area of the mandible primordium. Expression is visible before the mandibular buds can be detected morphologically (Fig. 5A). The *Dll*-positive region is circular and does not differ significantly from those of other early appendage anlagen such as maxillae, thoracic legs or antennae (Fig. 5B). In slightly more advanced stages, mandibular *Dll*-expression weakens and it shifts from the centre towards the external lateral side of the mandibular buds (Fig. 5B,C). These are expanding in their transverse axis resulting in a shape different from other limbs. In more advanced stages, mandibular expression of *Dll* disappears entirely. This pattern is unique for the mandibular *Dll* expression and cannot be found in any other head or trunk appendage. Expression in the first maxilla occurs in the early bud similar to that of the mandible (Fig. 5A,B). Later it is restricted to the tip (Fig. 5C). The palpless first maxillae of adult *Glomeris* contribute to a plate-like structure, the gnathochilarium. This is a derived character shared by diplopods and pauropods (Dohle, 1997b). The second maxillary segment does not show any *Dll* expression and morphologically no limb bud is formed (Fig. 5B).

Insects

We investigated three species of the Collembola, *Tetrodontophora bielanensis*, *Heteromurus nitidus* and *Tomocerus vulgaris*, which belong to the Entognatha and the silverfish *Lepisma saccharina* a member of the ectognathous insects. From the comparison of representatives of the two large monophyletic taxa amongst insects we hope to reconstruct the ancestral situation for insects as a whole. Previously data were only available on *Dll* expression in ectognathan insects, mostly from pterygotes. All insects, entognathans and ectognathans, lack mandibular palps and there is no report on embryonic anlagen of such a structure. In all insect species examined by us, the mandible buds are the only ones among head and thoracic segments that do not express *Dll* at any stage (Fig. 6). When the mandible buds are first detectable they are of similar size and shape to the maxillary buds. Nevertheless, no *Dll* expression occurs in the mandible anlagen whereas in the maxillary and labial anlagen (and other segmental appendages) the tips are intensely stained by the antibody (Fig. 6C,D). The mandibular bud starts as a round outgrowth which is followed by transverse widening as in the crustacean species examined (Fig. 6B). Later the bud is slightly subdivided in two parts. During development of *Lepisma* the outgrowths of the two pairs of maxillae are subdivided into three lobes. The initial *Dll* expression of the early bud continues into the outermost lobe. In the first maxillae the inner lobe starts to express *Dll* as outgrowth occurs followed by the middle lobe (Fig. 6B,E). In the second maxillae (labium) we found *Dll* expression in the inner and outer lobes but we could not detect it in the middle lobe. The outer lobe eventually forms the palp, and the inner two lobes become the galea and lacinia of the maxilla or the glossa and paraglossa of the labium (second maxillae). In collembolans early maxillary *Dll* expression is similar to that in *Lepisma*. Due to the differentiation of the entognathous condition, the later development of maxillae and labium could not be analyzed. The morphogenesis of these appendages is somewhat different from those of other insects (Hoffmann, 1911).

Discussion

Dll expression in mouthparts of crustaceans, myriapods and insects

All crustaceans examined by us show mandibular *Dll* expression. The characteristics of the patterns of *Dll* expression in crustacean mandibles, however, are correlated with the presence or absence of a mandibular palp. In crustaceans with a mandibular palp *Dll* is expressed in the palp and the area where the palp forms. This is also the case when the palp is only present in the larva or the embryo as the example of *Artemia* shows (Panganiban *et al.*, 1995). The relationship between the biramous mandibular palp in the nauplius larva and the uniramous palp of the adult of *Penaeus* is not clear since the naupliar palp is lost during the moult to the protozoea (Schminke, 1996). However, *Dll* is expressed in the naupliar mandibular palp of *Penaeus* as well as in the palps of isopods and amphipods. This suggests a homology between these structures. In crustaceans without a palp in the adult stage there is only an early and transient *Dll* expression as is shown in the amphipod *Orchestia* and the terrestrial isopod *Porcellio*. This

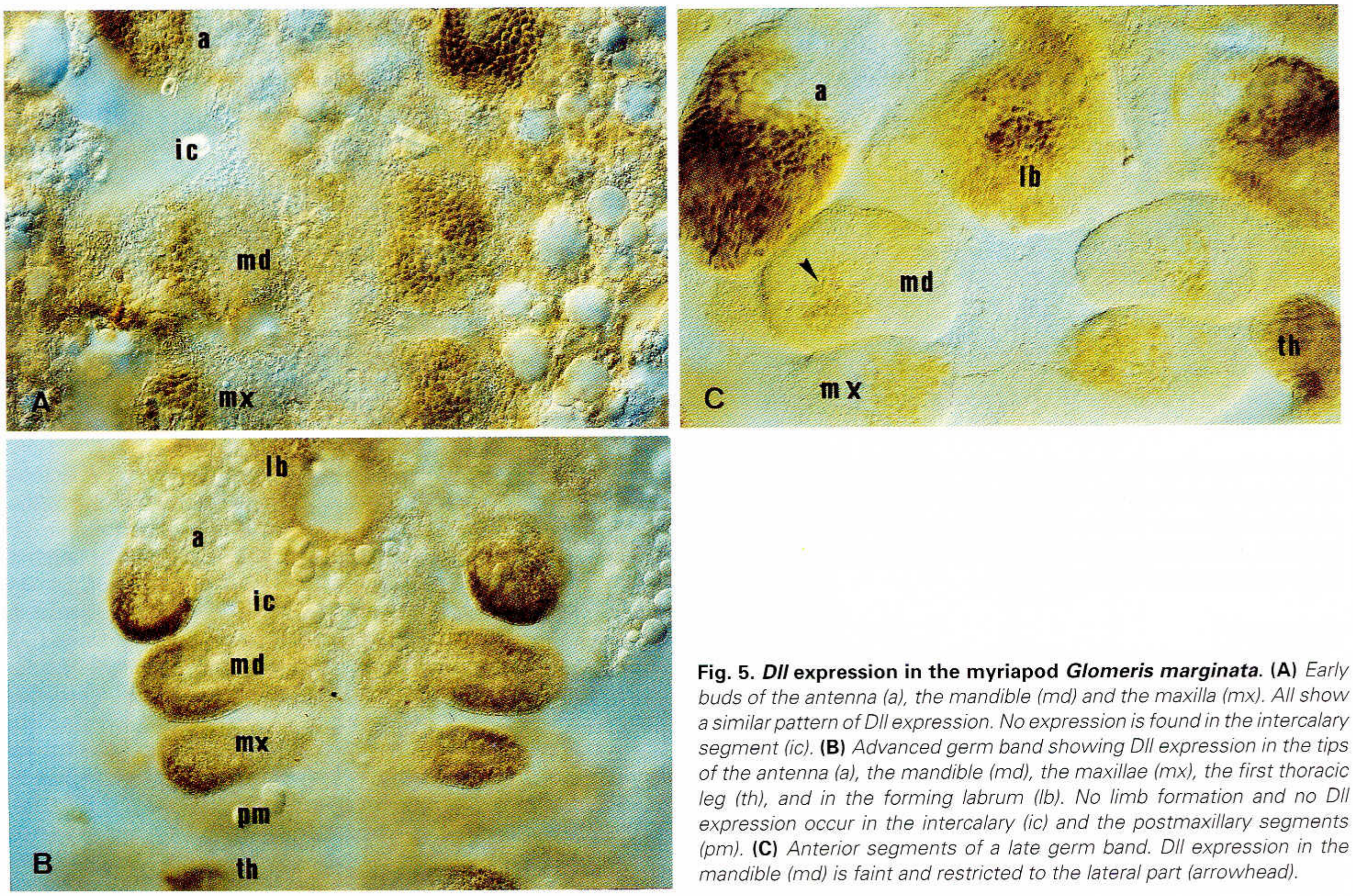

Fig. 5. *Dll* expression in the myriapod *Glomeris marginata*. (A) *Early buds of the antenna (a), the mandible (md) and the maxilla (mx). All show a similar pattern of Dll expression. No expression is found in the intercalary segment (ic).* **(B)** *Advanced germ band showing Dll expression in the tips of the antenna (a), the mandible (md), the maxillae (mx), the first thoracic leg (th), and in the forming labrum (lb). No limb formation and no Dll expression occur in the intercalary (ic) and the postmaxillary segments (pm).* **(C)** *Anterior segments of a late germ band. Dll expression in the mandible (md) is faint and restricted to the lateral part (arrowhead).*

stands in contrast to the findings of Popadic *et al.* (1996) who did not detect *Dll* expression in the mandibles of another terrestrial isopod species, *Armadillidium vulgare*. *Armadillidium* and *Porcellio* both belong to the monophyletic Oniscidea, the terrestrial isopods, which are characterized by the apomorphic loss of mandibular palps (Wägele, 1989; Brusca and Wilson, 1991). Since both isopod species are closely related and since Popadic *et al.* (1996) show a relatively mature embryo, we would expect that earlier stages of *Armadillidium* also express *Dll* in their mandibles. Furthermore, our results contradict the suggestion of Popadic *et al.* (1996) that *Dll* is not expressed in the mandibles of directly developing crustaceans. All amphipods and isopods undergo direct development of a similar type (Dohle and Scholtz, 1988).

The myriapod *Glomeris* shows a temporal and spatial pattern of mandibular *Dll* expression comparable to that of crustaceans without mandibular palps. There is a transient *Dll* expression which starts centrally in the mandibular bud. Later on, it becomes restricted to the outer margin of the widening mandible before it eventually disappears. The previous reports on mandibular *Dll* expression in myriapod representatives, the millipede *Oxidus gracilis* (Popadic *et al.*, 1996) and the centipede *Ethmostigmus rubripes* (Grenier *et al.*, 1997) show the early expression but do not mention the loss of it in advanced embryonic stages. Based on the similarity of morphogenesis of mandibles among myriapods (Heymons, 1901; Tiegs, 1940; Dohle, 1997b), we tentatively conclude that the pattern of *Dll* expression found in the mandibles of *Glomeris* can be generalized for myriapods.

Our data on the development of the mandibles in the entognathous Collembola add new examples to the observation that in insect mandibles there is no detectable *Dll* expression in any stage. This surprising phenomenon is obviously not restricted to *Drosophila* and other ectognathan insects such as orthopterans, lepidopterans, hemipterans, zygentomans and coleopterans (Panganiban *et al.*, 1994; Popadic et al., 1996; Williams and Nagy, 1996; Niwa *et al.*, 1997; Rogers and Kaufman, 1997). Thus the total absence of *Dll* expression seems to be a general character of mandible ontogeny in insects.

In the first and second maxillae of the insects and crustaceans investigated, *Dll* expression is not restricted to distal elements. The outer *Dll* expression clearly corresponds to the maxillary palp which as in the mandibles is seen as the distal parts of the appendages. The two inner lobes of the maxillae of insects and crustaceans are classically interpreted as representing endites of the two most proximal leg elements (Boudreaux, 1987; Walossek, 1993; Boxshall, 1997). *Dll* expression in these endites has been reported in a number of insects (Williams and Nagy, 1996; Niwa *et al.*, 1997; Rogers and Kaufman, 1997). But not in all cases each endite reacts *Dll* positive. This might be due to the stages examined. At least in the first maxillae of ectognathous insects a characteristic sequence in the expression pattern can be stated.

The expression in the palp is followed by expression in the innermost lobe. The median lobe is the last one to start expressing *Dll*. Remarkably, this pattern corresponds with that found in thoracic phyllopodous legs of some crustaceans (Panganiban *et al.*, 1995; Williams, 1998). Therefore it might represent the ancient expression pattern of *Dll* in arthropod limbs. And the absence of *Dll* expression in proximal structures could be a convergent loss in several arthropod lineages.

Gnathobasic versus whole-limb mandibles

The gnathobasic character of the crustacean mandible is confirmed by the data presented here – in particular the ontogenetic transformation of the mandible of *Penaeus* with the early *Dll* expression in both mandibular branches which are serially homologous to those of the second antennae supports this. The absence of *Dll* expression in the mandibles of insects has also been interpreted as being indicative of a gnathobasic mandible lacking distal elements. Our comparative data suggest that the absence of *Dll* expression in insect mandibles is the endpoint of a transformation series, starting with mandibles which express *Dll* in the palps as in several crustaceans. The intermediate state is the transient expression in palpless mandibles found in some crustaceans and myriapods. This transformation series can be hypothesized no matter whether a close relationship between myriapods and insects is favored (e.g., Kraus and Kraus, 1994) or a crustacean/insect sister group relationship (e.g., Dohle, 1997a,b). In thoracopods and antennae of insects *Dll* expression is restricted to distal elements (e.g., Panganiban *et al.*, 1994). Furthermore, in genetic mosaics of *Drosophila* only the coxa, the most proximal leg element, develops independently of *Dll* activity (Cohen and Jürgens, 1989a). Against this background we conclude that crustaceans, myriapods and insects share a mandible of the gnathobasic type. This is consistent with the comparative analysis of mandibular muscle patterns (Boudreaux, 1987). There is neither evidence for the so called whole-limb mandible in myriapods alone (Popadic *et al.*, 1996) or in myriapods and insects together (Manton, 1964; Brusca and Brusca, 1990; Kraus and Kraus, 1994). We furthermore conclude that there is no evidence for the assumption of a 3-segmented telognathic mandible in myriapods and insects (Kraus, 1997). Otherwise the lateral position of the vestigial *Dll* expression in *Glomeris* cannot be explained.

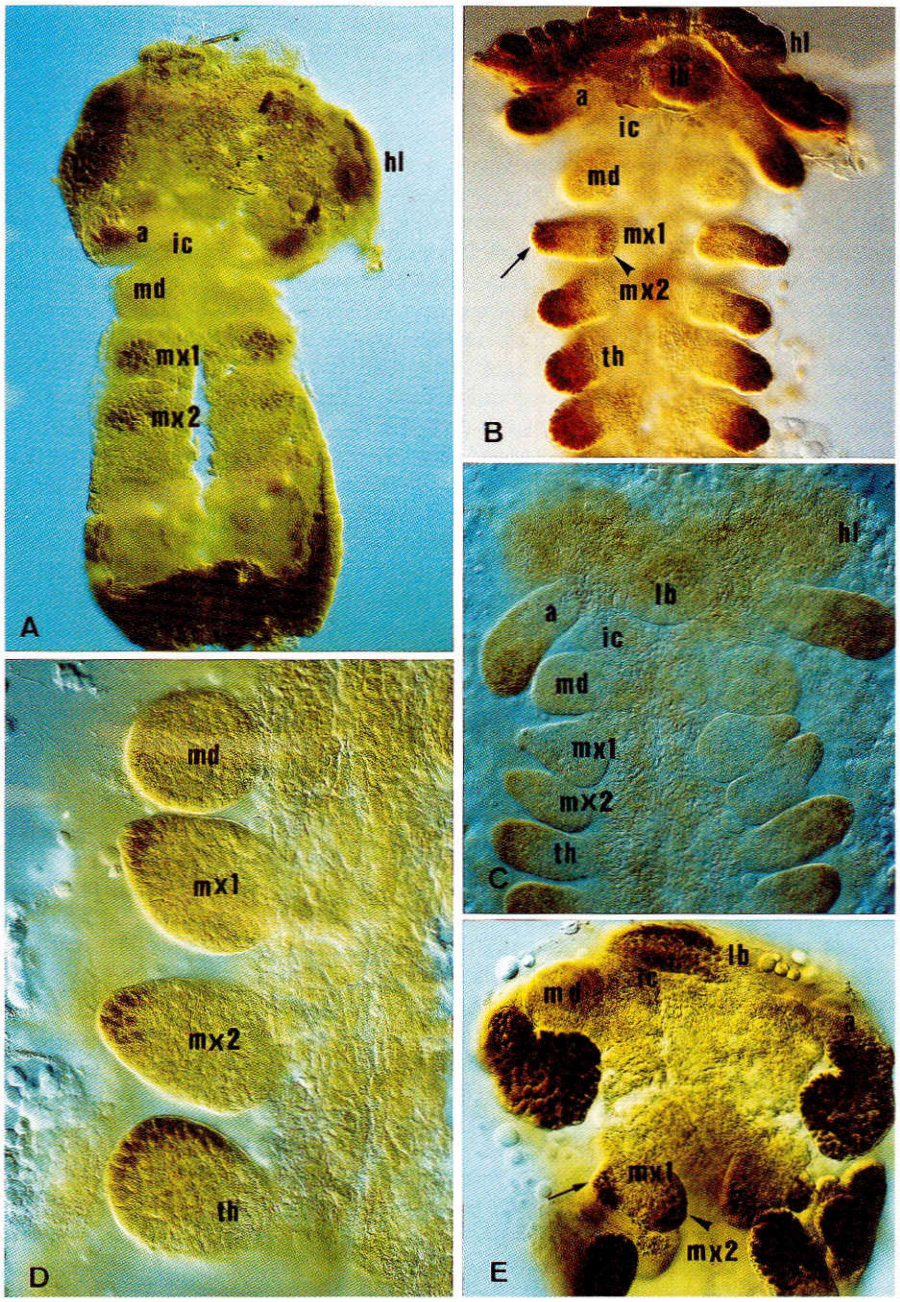

Fig 6. *Dll* expression in the anterior segments of insects. (A) *An early germ band of the silverfish Lepisma saccharina. Dll expression is seen in the forming antenna (a) and the first and second maxillae (mx1,mx2). In addition Dll is expressed in the head lobes and the posterior end of the germ band. Compare with Figure 1. No expression occurs in the mandible region and in the intercalary segment.* **(B)** *Lepisma in a later stage. The mandible (md) is still devoid of Dll expression. In the first maxilla (mx1) expression starts in the inner lobe (arrowhead). The arrow marks the forming maxillary palp.* **(C)** *Dll expression in the collembolan Tetrodontophora bielanensis. Again the mandible lacks Dll expression.* **(D)** *Enlarged part of appendages of the right side of an embryo of Tetrodontophora bielanensis. In the two maxillae and the thoracic leg Dll is found in the tips. The mandible, although of comparable size, is Dll negative.* **(E)** *Late embryo of another collembolan species, Tomocerus vulgaris, showing the staining of the inner lobe (arrowhead) of the first maxilla (mx1). The arrow points to the palp.*

The gnathobasic mandible is interpreted as an enlarged endite (Lauterbach, 1972). With the *Dll* expression patterns in the maxillae in mind, one would expect that the mandible body would also express *Dll*. This is not the case and the reasons can only be speculated on. Perhaps the mandible is not a real endite but the result of transverse growth of the proximal appendage parts.

Alternatively the evolutionary transformations are so great that a different mechanism is involved in mandibular endite formation.

Phylogenetic considerations

Our results support a common origin of crustaceans, myriapods and insects from a stem species which already possessed a gnathobasic mandible. This original gnathobasic mandible was not devoid of distal elements but these were reduced to a palp. The palpate character of the original mandible is deduced from the similarity in the pattern of *Dll* expression in myriapod and palpless crustacean mandibles. In both, relative size and the lateral position of *Dll* expression correspond to the *Dll* expression in palpate crustacean mandibles before the palp is formed. Thus late *Dll* expression patterns in palpless crustacean and myriapod mandibles can be interpreted as vestigial palp anlagen. The occurrence of a gnathobasic mandible in the second segment posterior to the antennae is a strong argument in support of the Mandibulata as a monophyletic group. Nothing similar exists in any other arthropod group. Phylogenetic scenarios with an independent evolution of mandibles in several arthropod lineages are less likely (e.g., Manton, 1973; Friedrich and Tautz, 1995). However, even if the conclusion about the gnathobasic character of the mandibles proves not to be correct, the total absence of *Dll* expression in the body of late embryonic mandibles is an apomorphic character for mandibulates. There are not sufficient data concerning *Dll* in chelicerates but we anticipate that *Dll* expression is maintained in the corresponding appendage (first post-chelicera appendage) in that group.

In contrast to the suggestions of Popadic *et al.* (1996), no sister group relationships can be based on the data of mandibular *Dll* expression patterns within the Mandibulata. Neither a close relationship between myriapods and insects nor a sister group relationship between crustaceans and insects is supported by our findings. It has been suggested that the loss of mandibular palps is a shared derived character (synapomorphy) of myriapods and insects (Wägele and Stanjek, 1995). This is not convincing since the loss of characters is only a weak or even invalid argument for establishing monophyletic groups (Dohle, 1997a). As the crustaceans show, a loss of the mandibular palp has occurred independently several times and there is no way to homologize the loss of the palp in myriapods and insects via a specific developmental pattern of reduction. In particular, when the *Dll* expression patterns of these two groups differ to the degree found in our study (transient expression *vs* no expression). The only phylogenetic conclusion that can be drawn within the mandibulates on the basis of our findings is the following. The formation of mandibles without any *Dll* expression described for all entognathan and ectognathan insects examined so far is an apomorphy of the Insecta (Hexapoda) confirming its monophyletic status.

The data concerning *Dll* expression in the maxillae are not sufficient to draw phylogenetic conclusions for mandibulates. In particular, the situation in *Glomeris* as a representative of diplopod millipedes is derived due to the total absence of a second maxillae and the formation of a plate-like gnathochilarium by elements of the first maxillae. The total absence of second maxillae deduced from morphological data (see Dohle, 1997b) is well supported by the absence of any *Dll* expression in the corresponding segment. Thus, hypotheses that the second maxillae contribute to the gnathochilarium (see Kraus and Kraus, 1994) cannot be substantiated. Whether the tripartite morphogenesis and *Dll* expression of the first maxillae found in crustaceans and insects is a derived character for all mandibulates or a subgroup of it remains unclear. To discuss this further, data on maxillary *Dll* expression in chilopod myriapods are required.

Limits of conclusions based on Dll expression patterns

The complete absence of *Dll* expression in the insect mandible can be shown to be an evolutionary loss only by comparison with crustaceans and myriapods. However, it also shows that *Dll* expression alone is not sufficient to indicate the limb character of an outgrowth. The absence of *Dll* does not prove that the insect mandible is not derived from a limb. It rather presents a final point of an evolutionary transformation resulting in a total reduction of distal regions of the original mandibular limb. Thus caution must be applied to any claim that the lack of *Dll* expression contradicts the assumption of the appendiculate origin of an arthropod outgrowth if there is not additional evidence. Likewise, the presence of *Dll* expression does not necessarily show that an outgrowth is a limb derivative as not all areas of *Dll* expression can be related to appendages. This is evident for the expression pattern in the embryonic brain of insects (Panganiban *et al.*, 1994) and crustaceans (Panganiban *et al.*, 1995; present study). Furthermore, this has also been suggested for the labrum and the telson of arthropods (Rogers and Kaufmann 1997; Scholtz 1997) and in a wider sense for *Dll* expression in appendages of different higher animal taxa in general (Lowe and Wray, 1997; Panganiban *et al.*, 1997).

Functional aspects

It has been suggested that *Dll* plays a crucial role in the formation of the proximal/distal (P/D) axis of arthropod appendages. This has been inferred from expression patterns and the analysis of *Dll* mutants (Cohen and Jürgens, 1989b; Diaz-Benjumea *et al.*, 1994; Panganiban *et al.*, 1995). In this context both the total absence of *Dll* expression in insect mandibles and the transient expression in palpless mandibles of crustaceans and myriapods are surprising. The pattern in insects has been (e.g., Panganiban *et al.*, 1994) and can be explained by a total loss of distal elements of the mandibular appendage. But how can the early mandible anlagen grow without a defined P/D axis? Morphologically the early mandibular buds do not differ from those of other appendages which initially express *Dll*. Moreover, the distal parts are also absent in palpless crustacean and myriapod mandibles and yet they initially express *Dll*. Nothing is known about gene interactions during appendage formation of crustaceans and myriapods. However, using the comparative approach (Scholtz and Dohle, 1996) for the analysis of *Dll* expression patterns in relation to morphogenesis, some tentative conclusions can be drawn concerning *Dll* functions. The main conclusion is that distalization of arthropod appendages includes two distinct steps – the early initiation of the P/D axis and later the maintenance and differentiation of distal fate. *Dll* seems to be required for both. Therefore the initial proximal/distal patterning by *Dll* is independent of the second role in defining and maintaining distal structures of appendages. This independence can be seen in the independent loss of either or both of these functions in various limbs and different mandibles. It appears as if *Dll* expression in the early mandibular buds of crustaceans and myriapods is necessary to establish the proximodistal axis as in other appendages. With the transverse growth of the proximal parts, which eventually form the mandible this function is no longer required since the distal parts do not further differentiate – hence expression decays. In insects the initial function of *Dll* must have been replaced by a different mechanism. Since *Dll* is also not required for the later differentiation of distal parts no expression of *Dll* occurs at all. The loss of the initial *Dll* role can also be found in branchiopod crustaceans where the outgrowth of limb buds precedes *Dll* expression (Williams, 1998). However, in this case the second function of *Dll* is maintained and distal structures are formed. Although it cannot be excluded that in insect mandibles the role of *Dll* is replaced by some other genetic mechanism there is still evidence that distal parts of the mandibular appendage are absent. The proximal border of *Dll* expression in appendages varies between the species investigated (Williams, 1998). The view that *Dll* only marks the distal telopodite as opposed to the proximal coxopodite (González-Crespo and Morata, 1996) seems to be too simplistic. However, there is no exception to the observation that distal parts of true appendages are *Dll* positive even in the highly branched legs of branchiopod crustaceans (Panganiban *et al.*, 1995; Williams, 1998). Thus it is appropriate to infer that in the absence of *Dll* expression these distal parts are not formed.

Materials and Methods

We studied the crustacean species *Penaeus monodon* (Decapoda), *Gammarus pulex* and *Orchestia cavimana* (Amphipoda), and *Porcellio scaber* (Isopoda). The myriapod species *Glomeris marginata* (Diplopoda) and the insects *Tomocerus vulgaris*, *Tetrodontophora bielanensis*, and *Heteromurus nitidus* (Collembola) and *Lepisma saccharina* (Zygentoma). The specimens of the decapod *Penaeus monodon* stem from the culture at the Australian Institute of Marine Science in Townsville/Australia. The other species were collected in different habitats in Germany: *Orchestia* near Oldenburg, *Gammarus* from the river Oder near Schwedt, *Porcellio* and *Tomocerus* in Berlin, *Tetrodontophora* in the Zittau Mountains, *Heteromurus* from the breeding boxes of Amblypygi from P. Weygoldt (Freiburg), *Lepisma* was obtained from a culture of the Institut für Wasser-, Boden-, Lufthygiene (Berlin), *Glomeris* was collected in the Weserbergland. All terrestrial species were kept in containers for breeding. Except for *Penaeus* whose whole nauplii were treated, the eggs were isolated and the chorion and the yolk were removed with insect pins and tweezers. Immunostaining followed the description of Panganiban (personal communication). The embryos were transferred to the PEM-FA fixative (0.1M PIPES (pH 6.95), 2.0 mM EGTA, 0.1 mM $MgSO_4$, 3.7% formaldehyde) for 30 to 60 min. After fixation the embryos were washed two times for 5 min in PBS, two times for 5 min in PBT (PBS, 2% BSA, 0.1% Triton X-100) and then kept for 30 min in PBT. After that the embryos were incubated in PBT and polyclonal anti-*Dll* (dilution 200:1) overnight at 4°C. After incubation they were washed three times for 5 min and four times for 30 min in PBT and again incubated overnight at 4 °C in PBT and goat antirabbit IgG (Jackson Immunoresearch) which was added at a dilution of about 800:1. After incubation the embryos were washed three times for 5 min and four times for 30 min in PBT and then transferred to a solution of 1 mg/ml DAB (diaminobenzidine) in PBT (dilution 1:2) for 10 min. H_2O_2 (3%) was added to a dilution of 100:1 and the reaction was allowed to proceed for about 10 min. The stained embryos were transferred to PBS and counterstained with fluorescent dye (0.1% solution of bisbenzimid H33258) for 10 min. Then they were washed in PBS for 10 min and mounted in glycerol on a slide. Further analysis and photography were done with brightfield, differential interference contrast (Nomarski optics) and fluorescence microscopy. SEM investigations were carried out according to the methods used in Scholtz (1990).

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Addendum: During the reviewing process of the present publication Popadic *et al.* (1998, *Dev. Genes. Evol. 208:* 142-150) published a paper in which they present corresponding data and in which they came to similar conclusions concerning the gnathobasic character of mandibles.