

The clonal composition of biramous and uniramous arthropod limbs

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We present the first comparative cell lineage analysis of uniramous and biramous limbs of an arthropod, the crustacean *Orchestia cavimana*. Via single cell labelling of the cells that are involved in limb development, we are able to present the first complete clonal composition of an arthropod limb. We show that the two main branches of crustacean limbs, exopod and endopod, are formed by a secondary subdivision of the growth zone of the main limb axis. Additional limb outgrowths such as exites result from the establishment of new axes. In contrast to general belief, uniramous limbs in *Orchestia* are not formed by the loss of the exopod but by suppression of the split into exopod and endopod. Our results offer a developmental approach to discriminate between the different kinds of branches of arthropod appendages. This leads to the conclusion that a 'true' biramous limb comprising an endopod and an exopod might have occurred much later in euarthropod evolution than has previously been thought, probably either in the lineage of the Mandibulata or that of the Tetraconata.

Keywords: limb development; limb evolution; *Orchestia cavimana*; Crustacea

1. INTRODUCTION

The incredible functional and structural diversity among arthropod limbs is commonly interpreted as a variation of two or three basic limb types (figure 1) that are identified by the number of branches and axes (Brusca & Brusca 2002; Williams 2004). Despite some recent gene expression studies (e.g. Panganiban *et al.* 1995; Abzhanov & Kaufman 2000; Prpic *et al.* 2003; Angelini & Kaufman 2005), it is still not clear how the various limb branches are formed in terms of hierarchies of axis differentiation and how arthropod limbs evolved. The detailed knowledge of comparable issues in vertebrate limbs is based on the powerful synthesis of cell lineage, morphogenetic and molecular studies (Wolpert *et al.* 2006; Sato *et al.* 2007). In contrast to this, there are only few studies on cell lineage and cell proliferation of legs in insects, but a detailed cell-by-cell analysis and clonal composition of a complete arthropod limb and a broader approach are still lacking (Dohle & Scholtz 1988; Lawrence 1992; Weigmann & Cohen 1999; Kojima 2004; Tanaka & Truman 2005).

The various branches of crustacean limbs are often classified into two major parts: exopod and endopod, and a number of outgrowths called exites and endites, depending on their orientation towards the ventral body midline (figure 1; Hansen 1925; Boxshall 2004). However, whether this classification is justified based on ontogenetic or evolutionary processes is far from clear: we have no unambiguous concept of how the axes and branches of uniramous and biramous limbs are hierarchically related to each other. Accordingly, some authors suggest a different view in that only the endopod forms the primary axis of the limb and the exopod, and all endites and exites are just secondary side branches (Thiele 1905; Borradaile 1926; Snodgrass 1958).

Phylogenetic analyses of arthropod relationships and the fossil record reveal that a limb with more than one branch is plesiomorphic for crustaceans and euarthropods and that uniramous limbs evolved independently in several lineages (Walossek & Müller 1997; Budd 2002). In addition, there is a general agreement that uniramous limbs evolved by a loss of exopods (Hansen 1925; Olesen *et al.* 2001).

To address these questions and to complement molecular studies on arthropod limb development, we pursued a cell lineage approach to study the clonal composition of the limbs of the amphipod crustacean *Orchestia cavimana* by using the lipophilic dye DiI in combination with confocal laser scanning microscopy and computer-aided three-dimensional reconstruction (Wolff & Scholtz 2006). We chose a representative of malacostracan crustaceans because they exhibit a stereotypic cell division pattern during germ band formation and differentiation that allows the study of morphogenesis at the single cell level. Furthermore, many malacostracans, including *Orchestia*, show uniramous limbs in the thorax and biramous limbs in the pleon, which allows a direct comparison of the formation of two limb types within one embryo.

2. MATERIAL AND METHODS

(a) Animals

Adult specimens of *O. cavimana* were collected from the beaches of the Tegeler See (Berlin, Germany) and maintained in terrariums at approximately 20°C and fed with vegetables and oatmeal. To receive embryos in relevant stages, females with eggs in their marsupium were carefully anaesthetized in mineral water containing CO₂. The eggs were flushed out of the marsupium from the anterior with a Pasteur pipette and transferred to a saline solution, mimicking the liquid of the marsupium (Wolff & Scholtz 2002).

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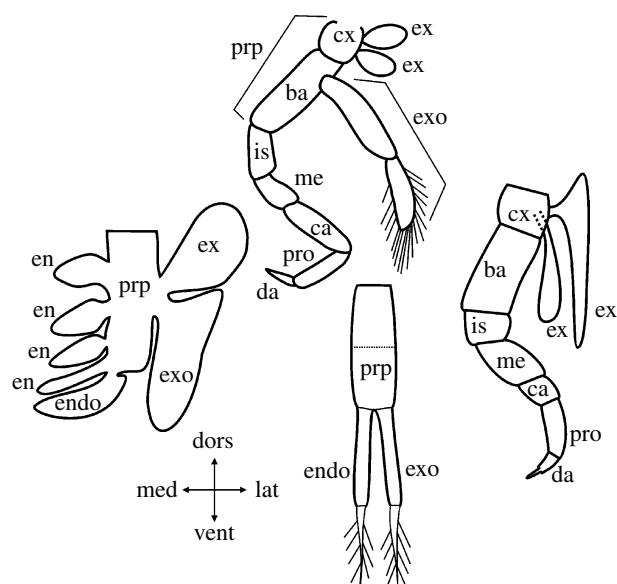


Figure 1. Crustacean limb types. Schematic of the three main types of crustacean limb morphology which also represent the extremes of euarthropod limb morphology in general (left: polyramous/phyllopodous limb, middle: two biramous limbs of a different complexity, right: uniramous limb). The biramous limb consists of a proximal protopod (prp), which is mostly subdivided into coxa (cx) and basis (ba), an often segmented inner endopod (endo) and an outer exopod (exo). The uniramous leg of crustaceans is typically seven segments consisting of coxa and basis—together forming the protopod, the ischium (is), merus (me), carpus (ca), propodus (pro) and the dactylus (da). Biramous and uniramous limbs may possess additional branches forming inner endites (en) and outer exites (ex). The latter are often differentiated as gills. For instance, in the amphipod crustacean, *Orchestia cavimana*, being used for this study, the biramous pleopods (top) possess an undivided protopod and lack any exites and endites, whereas most of the uniramous thoracopods show two exites differentiated as gill and coxal plate. The uniramous limb (right) is commonly understood as the result of an exopod loss (Boxshall 2004). The polyramous/phyllopodous limb is often interpreted as a special case of the biramous limb type with a low degree of segmentation, many endites and one to several exites (Wolossek 1993; Olesen *et al.* 2001).

(b) *In vivo* labelling

The cells were labelled using an inverted microscope (Leica DMIRB) equipped with a micromanipulator (Eppendorf). The fluorescent marker used was DiI (Molecular Probes)—a lipophilic fluorescence dye that binds to the cell membrane and guarantees that the dye is exclusively restricted to the daughter cells. For a detailed description of *in vivo* labelling, see Wolff & Scholtz (2006). We adopt the common nomenclature for malacostracan crustacean cell lineages of the post-naupliar ectoderm as modified for amphipods by Scholtz (1990).

In thoracic and pleonic regions, all cells of a genealogical row abcd (e.g. abcd3) as well as its sister cells (e.g. ab3 and cd3) and its daughter cells (e.g. a3, b3, c3 and d3) were marked and their contribution on limb composition was analysed.

(c) *Fluorescence microscopy, confocal laser scanning microscopy and three-dimensional reconstruction*

To get a complete developmental sequence, labelled embryos at different developmental stages were fixed in 3.7%

paraformaldehyde in phosphate-buffered saline (1×PBS; 1.86 mM NaH₂PO₄, 8.41 mM Na₂HPO₄, 175 mM NaCl, pH 7.2) for 15–30 min at room temperature. The animals were washed in 1×PBS several times and counterstained with the nuclear staining dye Hoechst (100 µg ml⁻¹ bisbenzimidazole, H33258 in 1×PBS) and mounted in DABCO-glycerol (25 mg DABCO (1,4-diazabicyclo[2,2,2]-octane, Merck) in 1 ml 1×PBS to 9 ml glycerol). The documentation took place either on a fluorescence microscope (Zeiss Axiophot1) using blue light or green light (strongest stimulation of DiI) or on a laser scanning microscope (Leica SP2). Image stacks produced by the laser scanning microscope were analysed with the software IMARIS 5.0.1 (Bitplane AG). A three-dimensional reconstruction of the counter staining (Hoechst) and the clones of the *in vivo* labelled cell have the advantage of very high resolution with respect to morphological data. The feature 'Volume' in the program module 'Surpass' created a three-dimensional object, which can be magnified and oriented in all directions.

3. RESULTS AND DISCUSSION

The ectoderm in the post-naupliar part of malacostracan crustaceans is initially arranged in regular transverse cell rows that form genealogical units (figure 2a,b). A genealogical unit is comparable to a *Drosophila* parasegment in that its anterior region (row a and anterior parts of row b) constitutes the posterior of a segment and the posterior region (posterior parts of rows b, c and d) forms the anterior part of the adjacent posterior segment (figure 2a; Dohle & Scholtz 1988). Each of the initial ectodermal rows of a genealogical unit undergoes two rounds of medio-laterally propagating division waves with longitudinally oriented mitotic spindles. Each genealogical unit is thus composed of rows and columns of regularly arranged cells (figure 2a,b). We labelled individual cells of the columns 1–10 after an ectodermal row has been established or after each mitotic wave. Accordingly, the resulting clones are of different sizes in the differentiated limbs.

To test whether differentiated limbs are composite structures of two genealogical units, we labelled the founder cells (cell abcd) of entire columns of a genealogical unit, in the transverse ectodermal row stage of a genealogical unit (figure 2a,b). We found that indeed, one genealogical unit contributes to two limbs and in turn each limb is composed of cells originating from two genealogical units. In a second step, we marked individual cells after the transverse rows of a genealogical unit had undergone two mitotic waves resulting in four descendant rows (figure 2a). This reveals the contribution of the descendant rows to the limb parts and it confirms the previous result that the segmental boundary is formed in the region of descendant row b. The distribution of several clones, in particular the descendants of columns 3–6 along a large part of the proximal-distal (PD) axis of the limbs, indicates that the limb is formed from a growth zone of relatively few cells. Moreover, this growth zone is situated at the tip of a limb bud and it is characterized by cell divisions with spindle directions orientated along the longitudinal axis of the limb.

The pleonic appendages are biramous with a similar-sized inner endopod and outer exopod connected to a common basal limb segment, thus representing what is considered the ancestral state for crustacean limbs

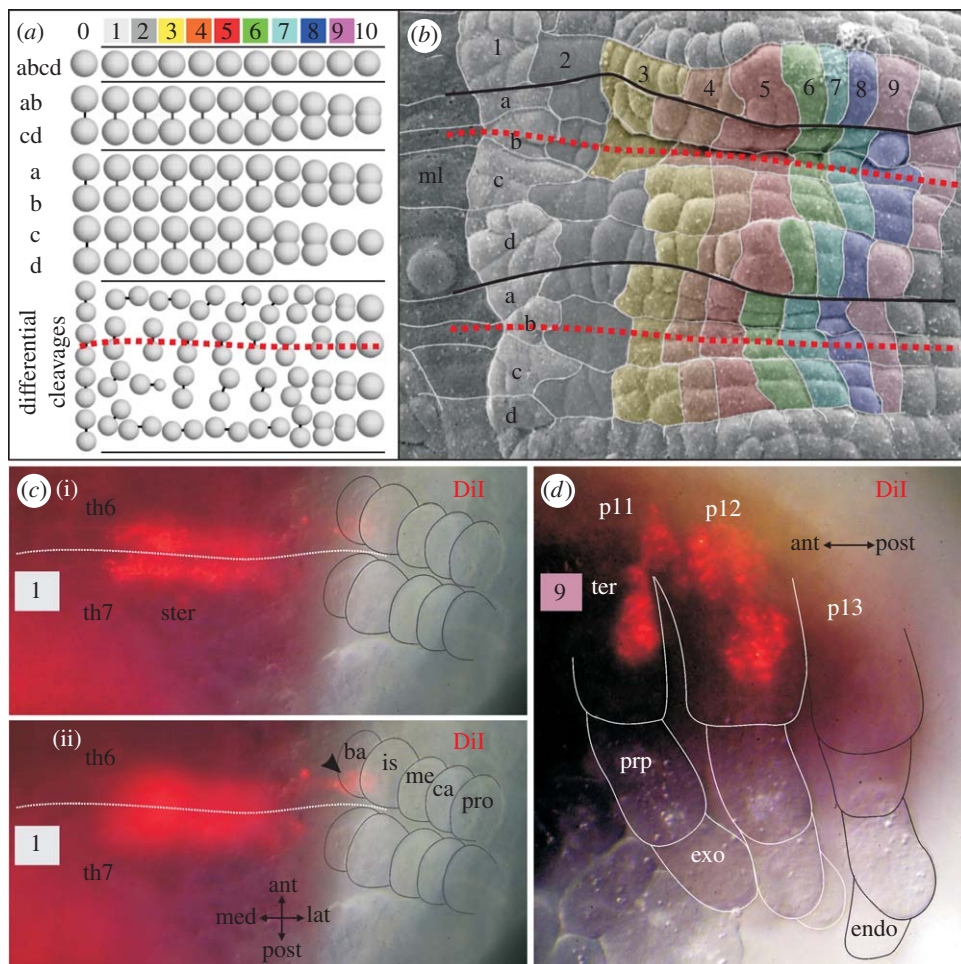


Figure 2. Cell lineage of the ventral ectoderm in *Orchestia*. (a) Scheme of the cell division pattern in the post-naupliar ectoderm of malacostracan crustaceans. Top: Each of the consecutively numbered (E(1) to E(17)) ectodermal post-naupliar genealogical units (row abcd) shows the same development. First, it undergoes two mitotic waves with longitudinal spindle directions. Middle: the result is a grid-like pattern of four rows (from anterior to posterior: a, b, c and d). Bottom: with the first differential cleavages (bars mark sister cells), morphogenesis (limb buds, ganglia and intersegmental furrows) begins. The morphological segments do not match the genealogical units. The segmental border (red dotted line) runs between the descendants of row b and does not match the genealogical border (black lines). Accordingly, limb buds, ganglia and other segmental organs are composed structures formed by the descendants of two adjacent ectoderm rows. (b) SEM-picture of left thoracic segments during differential cleavages. The start of segment formation is indicated by intersegmental furrows and limb buds. To visualize clones of each genealogical cell row (a, b, c and d) and columns, one to nine are colour coded (see (a)). The dotted red lines mark the border of two consecutive morphological segments; the black lines indicate genealogical borders. To the left the midline (ml) is visible. (c) Column 1 (DiI labelling of cell abcd1 of the left half of genealogical unit E(9)). (i) Ventral view (double exposure) focused on the sternite. The descendants of cell abcd1 form parts of the sternal plates (ster) of the thoracic segments 6 and 7 (th6–7). (ii) The same section focused on proximal leg segments. Only a small number of cells (arrowhead) are involved in the formation of the proximal limb segments. (ba, basis; is, ischium; me, merus; ca, carpus; pro, propodus). (d) Column 9 (DiI labelling of cell abcd9 of the left half of genealogical unit E(12)) in the pleonic region. Lateral view (double exposure) of the left body side showing the first three pleonic segments with pleopods. The descendants of abcd9 are not involved in limb formation; they form lateral parts of the tergite (ter) of the first and second pleonic segments (p11–2).

(Hansen 1925). The early limb buds appear in the area of columns 3–6 with the crest of the early bud being formed progressively by cells 3d–6d. Later, the limb bud is subdivided into two tips with two areas of growth. These are the anlagen of endopod and exopod. The endopod is generated by the clones of columns 3 and 4, and the exopod by columns 5 and 6 (figure 3a–c). Column 3 forms the medial (inner) part of the exopod from proximal to distal (figure 3a). The clones of columns 7 and 8 are found in the basal part of the limb at the dorsal margin (figure 3d). Columns 1 and 2 give rise to the sternites and the nervous system and do not directly contribute to limb formation (figure 2c). Column 9 and laterally adjacent columns are not involved in limb formation

(figure 2d). If cells 3d, 4d or 5d, and 6d are marked, the resulting clones are spread along the entire length of the endopod or the exopod (figure 3e).

The thoracic limbs of amphipods are uniramous and mostly equipped with a coxal plate and a gill. As is the case with the pleopods, the crest of early limb buds is formed by cells 3d–6d. Hence, the early limb buds of biramous and uniramous limbs are quite similar at the morphological and cellular levels (Hejnol & Scholtz 2004). In contrast to the pleopods, however, the thoracic limbs remain undivided throughout development. Columns 3 and 4 form the tip and most of the inner side of the limb (figure 4a,b). One exception is a short cell band derived from cell d4, which forms an outer part of the thoracopod (figure 4b). Cells of

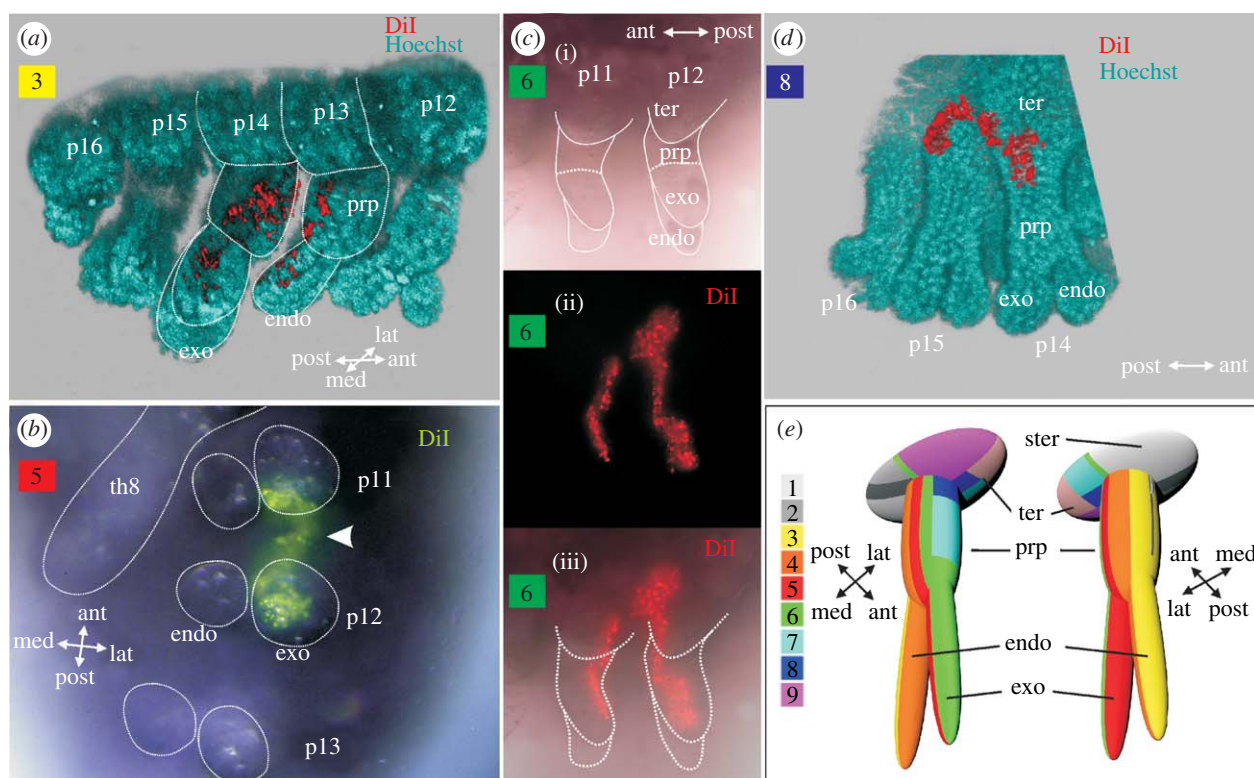


Figure 3. Clonal composition of biramous limbs (pleopods) in *Orchestia*. (a) Column 3 (DiI labelling of cell abcd3 of the right half of genealogical unit E(14)). Three-dimensional reconstruction (medial view) of the right pleopods 2–6 (p12–6). The nuclei are counterstained with Hoechst. The descendants of cell abcd3 (red) form inner parts of the endopod of pleopods 3 and 4 (endo) and parts of the corresponding protopods (prp). (b) Column 5 (DiI labelling of cell abcd5 of the left half of genealogical unit E(12)). Double exposure of a ventral view of the pleonic segments 1–3 (p11–3). The clone of cell abcd5 (green) forms the inner parts of the exopod (exo) of pleopods 1 and 2 and additionally the inner parts of their protopods. The clone crosses the segmental border (arrowhead). The endopod (endo) is not formed by the descendants of column 5. (c) Column 6 (DiI labelling of cell abcd6 of the left half of genealogical unit E (12)). (i) Lateral view of pleopods 1 and 2. The shape of the typical architecture in proximal protopod (prp), and distal an inner endopod (endo) and an outer exopod (exo) are traced by a white line. (ii) The same detail with fluorescence light shows exclusively descending cells from abcd6. (iii) Double exposure to demonstrate that the descendants of column 6 form only the outer part of the exopod and part of the protopod. In addition, an adjacent part of the tergite (ter) is formed by these cells. (d) Column 8 (DiI labelling of cell abcd8 of the right half of genealogical unit E(15)). Lateral view of a three-dimensional reconstruction of the right pleonic appendages 4–6 (p14–6). The nuclei are counterstained with Hoechst. The descendants of cell abcd8 form the proximal part of the protopods and a part of the tergites (ter) of the fourth and fifth pleonic segments. Neither the exopod (exo) nor the endopod (endo) is formed by these cells. (e) Colour-coded scheme of the clonal composition of pleopods in *Orchestia*. A combination of two genealogical units (figure 2). Each colour of the bar represents a column of the ventral ectoderm and its contribution to a pleopod. Endo, endopod; exo, exopod; prp, protopod; ster, sternite; ter, tergite.

columns 5 and 6 which form the exopod in biramous pleopods contribute to the outer parts over most of the PD axis but not to the tip (figure 4c). Columns 2, 7, 8 and 9 give rise to basal limb parts, the latter three contributing to the gill and the coxal plate (figure 4d,e).

Our data indicate a clear hierarchy of limb axes in time and space. Endopod and exopod together form the main PD limb axis that is only secondarily subdivided into the two branches. This process is also reflected by the transformation of the initially undivided DiI expression into two unconnected domains representing the tips of exopod and endopod (Hejnal & Scholtz 2004; Williams 2004). Two testable scenarios are likely to explain the split of the main PD axis—either gene regulation is taking place that suppresses DiI expression in the area between forming exopods and endopods, or apoptosis is involved. Since exopod and endopod can be interpreted as an outer and an inner branch along the main limb axis, we expect a corresponding patterning mechanism in these two branches. One indication for this is the widespread

similarity between exopod and endopod in crustaceans such as remipedes, copepods, or in the malacostracan pleon (Gruner 1993).

The contribution of serially homologous cells that form the exopod in biramous pleonic limbs to large parts of uniramous thoracic limbs is an unexpected result of our study. It reveals that the uniramous limbs in the thorax of the amphipod crustacean *Orchestia* are formed by a suppression of the subdivision of the main limb axis into endopod and exopod. This stands in contrast to the generally held view that uniramous limbs in crustaceans are generated by a reduction or loss of exopods (Hansen 1925; Bitsch 2001). Since it is also thought that biramous limbs represent the ancestral condition in arthropods, the question arises whether all uniramous arthropod limbs are the result of a suppressed split into endopod and exopod. Some data from cladocerans support this view (Olesen *et al.* 2001).

As our results show, other outgrowths such as the gill and the coxal plate, which are both interpreted here as exites, and probably the endites are formed with separate

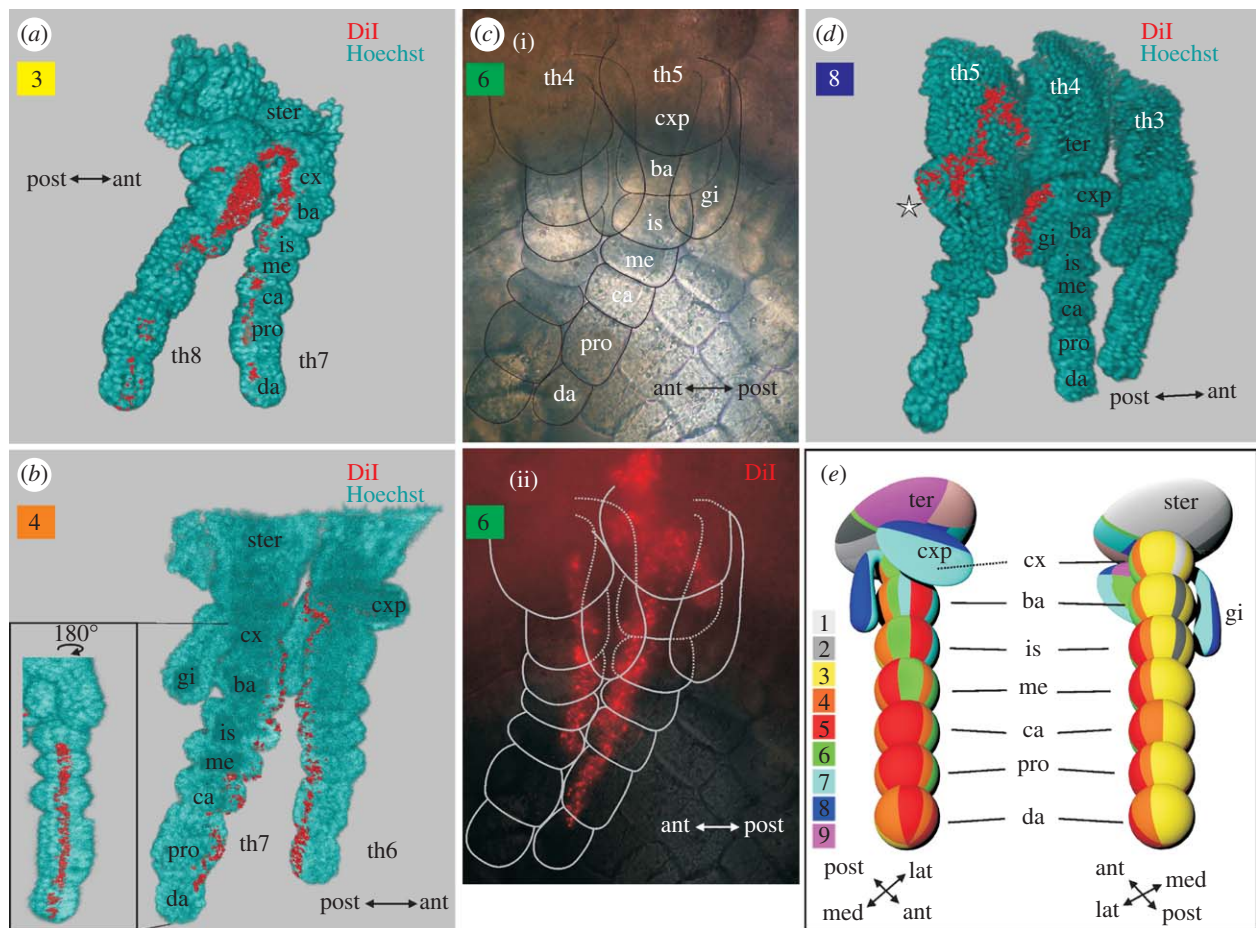


Figure 4. Clonal composition of uniramous limbs (thoracopods) in *Orchestia*. (a) Column 3 (DiI labelling of cell abcd 3 of the left half of genealogical unit E(10)). Three-dimensional reconstruction (median view) of left thoracopods 7 and 8 (th7–8). The nuclei are counterstained with Hoechst. The descendants of cell abcd3 (red) form the inner parts of both limbs along the entire proximal–distal axis. (b) Column 4 (DiI labelling of cell abcd 4 of the right half of genealogical unit E(9)). Three-dimensional reconstruction (lateral view) of right thoracopods 6 and 7 (th6–7). The nuclei are counterstained with Hoechst. The descendants of cell abcd4 (red) form the inner part of both limbs along the entire proximal–distal axis. The insert shows thoracopod 7 rotated at approximately 180° to show an ectodermal cell band (red) formed by the descendants of cell d4. (c) Column 6 (DiI labelling of cell abcd6 of the left half of genealogical segment E(6)). (i) Lateral view of thoracopods 4 and 5. The shape of the typical seven-segmented uniramous limb with coxal plate (cxp), gill (gi), coxa (covered by coxal plate), basis (ba), ischium (is), merus (me), carpus (ca), propodus (pro) and dactylus (da) is marked by black lines. (ii) The same detail (double exposure) shows the descendants of column 6 which form an outer (lateral) part of the limb and a part of the gill and coxal plate. (d) Column 8 (DiI labelling of cell abcd8 of the right half of genealogical unit E(7)). Three-dimensional reconstruction (lateral view) of the right thoracopods 3–5 (th3–5). The nuclei are counterstained with Hoechst. The star indicates a part of the gill that was destroyed during dissection. Most of the descendants of cell abcd8 form parts of the tergite (ter) of thoracic segments 4 and 5. In addition, they form a lateral part of the gill (gi) and the coxal plate (cxp). (e) Colour-coded scheme of the combination of all single results (shown in a–d) to show the clonal composition of a thoracopod in *Orchestia*. A combination of two genealogical units (see figure 2). Each colour of the bar represents a column of the ventral ectoderm and its contribution in the formation of a thoracopod. (ba, basis; ca, carpus; cx, coxal; cxp, coxal plate; da, dactylus; gi, gill; is, ischium; pro, propodus; ster, sternite; ter, tergite).

axes. This is shown by the clonal contribution. The gill and the coxal plate of *Orchestia* are only formed by basal clones that originate from the dorsal-most cell columns 7–9. Moreover, different molecular mechanisms are likely to be involved in the formation and patterning of these additional limb axes. Some results supporting this view have been obtained from studies of endites of insect mouthparts and crustacean exites (Averof & Cohen 1997; Olesen *et al.* 2001; Giorgianni & Patel 2004).

According to our findings, we propose a new approach for the discrimination between exopods and exites based on ontogeny. If a limb outgrowth is a part of the main PD limb axis, as indicated by its morphogenesis and the expression of axis-patterning genes, it can be considered as an endopod or exopod. If it is formed with an additional

axis, it is an exite or endite. For instance, the outer branch of the last walking leg of xiphosurans, the flabellum, is considered to be an exopod by some authors (Walossek 1993; Walossek & Müller 1997). However, the ontogeny of the flabellum as a separate outgrowth long after the normal limb axis has been established (Mittmann & Scholtz 2001) speaks against this idea but rather confirm the older view of an exite nature of this structure. Our findings shed new light on the suggestion that the biramous limb of the stem lineage euarthropods evolved by fusion of two independent structures such as ventral lobopods and lateral gill flaps, as has been speculated based on the situation found in the Cambrian *Opabinia* (Budd 1996). If this scenario is true, then it is difficult to conceive why endopod and exopod in modern arthropods

are formed by a subdivision of the initial PD axis. Hence, it is more reasonable to interpret the two branches in many Cambrian arthropod limbs as a uniramous appendage with an exite. From our data we conclude that a 'true' biramous limb comprising an endopod and an exopod evolved as a result of a split of the initial limb bud within euarthropods, probably either in the lineage of the Mandibulata or that of the Tetraconata. The result is comparable to what has been found in experiments carried out in *Drosophila*, where ectopic outgrowths were initiated by a misexpression of *dpp* or *wg* (Campbell & Tomlinson 1995).

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