Onychophoran head segmentation

ABSTRACT The arthropod head problem has been a long lasting conundrum which has puzzled arthropodists for more than a century. Onychophorans are the sister group of the arthropods and are a phylum that has for a long time been regarded as the link between a simple worm-like arthropod ancestor and the crown-group arthropods. The arthropod head is a complicated structure with cryptic segment borders because of fusion and migration of segments; hence the long standing debate of the different parts segmental origin. The onychophorans on the other hand have a rather simple head comprising three well defined segments during development, which gives rise to an adult head with three appendages that are specialised for sensory and food capture/manipulative purposes. Based on the expression pattern of the anterior hox-genes; *labial*, *proboscipaedia*, *hox3* and *Deformed*, as well as the head patterning genes *otd* and *six3*, we show that these three segments and their appendages can be correlated to the segments of the arthropod proto- deuto- and trito-cerebrum and that the onychophoran antenna is an appendage associated with the most anterior territory of the onychophoran head, it is the frontal appendage or primary antenna of stem-group arthropods and its affinities to the arthropod labrum is discussed.

Introduction

The arthropods have a segmented body that shows a tendency to fuse segments into functional units termed tagmata, e.g. the head, thorax and abdomen of an insect. The arthropod head is the tagma were fusion of segments is so extreme that it is now very difficult to tell different segments apart, and this is especially true for the anterior part that lies in front of the mouth, and indeed, to tell if it is segmental at all. This problematic pre-oral area has generated a multitude of hypothesises of arthropod head segmentation (Rempel, 1975). Recent advances in molecular biology have generated additional contributions to the issue (Abzhanov and Kaufman, 1999; Damen et al., 1998; Rogers and Kaufman, 1996; Schmidt-Ott et al., 1994; Schmidt-Ott and Technau, 1992; Scholtz and Edgecombe, 2005; Telford and Thomas, 1998). The number of preoral segments varies between three and four in these later proposals.

Another issue concerning the arthropod head is the nature of the labrum or upper lip. Is it an appendage or structure of a pre ocular segment (Posnien et al., 2009; Schmidt-Ott et al., 1994; Schmidt-Ott and Technau, 1992), the ocular segment (Budd, 2002; Eriksson and Budd, 2000; Eriksson et al., 2003) and homologous to the great appendage or primary antenna of certain fossil stem-group arthropods (Budd, 2002; Scholtz and Edgecombe, 2005) or a limb belonging to a segment placed further posterior (Haas et al., 2001a; Haas et al., 2001b). There have been suggestions that the antenna of the onychophorans corresponds to the labrum of arthropods (Budd, 2002; Eriksson and Budd, 2000; Eriksson et al., 2003).

It has been shown that the anterior expression borders of some anterior *Hox*-genes are conserved among arthropods and workers have aligned head segments between different arthropod groups (Abzhanov and Kaufman, 1999; Damen et al., 1998; Jager et al., 2006; Mittmann and Scholtz, 2003; Telford and Thomas, 1998) and the head patterning genes *six3* and *otd* have been used as markers for the most anterior region of bilaterians (Li et al., 1996; Lowe et al., 2003; Posnien et al., 2009; Schinko et al., 2008; Schröder, 2003; Seo et al., 1999).

The Onychophora is probably a sister-group to the Arthropoda (Dunn et al., 2008; Zantke et al., 2008). Both phyla, together with the related tardigrades, are segmented and therefore probably share a segmented ancestor. Since onychophorans have a less complicated head made up of fewer appendage bearing segments than arthropods (Eriksson and Budd, 2000; Eriksson et al., 2003; Mayer and Koch, 2005; Strausfeld et al., 2006), and hence, easier to identify, we decided to investigate the expression pattern of anterior *Hox*-genes together with the head patterning genes *six3* and *otd* in order to align homologous segments and other areas between onychophorans and arthropods with the aim to try and clarify the nature of the pre oral region of onychophorans and arthropods.

MATERIALS AND METHODS

Collection, animal husbandry and staging

Female *Euperipatoides kanangrensis* Reid, 1996 were collected in Kanangra Boyd National Park, NSW, Australia 33º 59'S 150º 08'E. Females were kept in containers with dampened sphagnum moss at 13°C and were fed first instar locusts or crickets once every second week. Dissected females were found to contain developing embryos for at least 12 months after collection, with individual females harbouring 20-150 embryos at various stages of development.

(Walker and Tait, 2004) describe the development of a number of onychophoran species closely related to *E. kanangrensis*. We staged embryos according to the criteria suggested by (Walker and Tait, 2004).

Fixation of embryos for in situ hybridisation and Light Microscopy

Embryos were dissected from the females and, after removal of the egg membranes, fixed in 4% formaldehyde in PBS overnight at 4°C. Fixed embryos were dehydrated in a graded series of methanol (25, 50, 75% in PBS with 0.1 % Tween-20 for 10 mins. each) and stored in 100 % methanol at -20° C.

Isolation and sequencing of *E. kanangrensis* genes

We isolated fragments of genes from embryonic cDNA libraries and genomic DNA. These fragments were extended by doing nested PCR on cDNA library with gene specific primers and vector specific primers. The consensus of these gene sequences has Genbank accession nos.: *Eka-otd*, EU347401, *Eka-six3*, EU347400, *Eka-labial*, NNNNNNN, *Eka-proboscipaedia*, NNNNNNNN, *Eka-hox3*, NNNNNNN, *Eka-Deformed*, NNNNNNN,. See supplementary data 1 for a more detailed description of the procedures.

In situ hybridisation

In situ hybridisation was carried out as described by Eriksson et al. (2009). See supplementary data 1 for detailed protocol of the methods.

Sequence analysis

See supplementary data 1 for detailed protocol of the methods.

Results

Gene identification

Working on this. Phylogenetic analyses of hox, six3 and otd with trees to put in supplement.

Blastopore and germband formation

Early development is similar with what has been described by Manton (1949) for some of the South African species. The blastopore forms as a pit in the blastodisc and becomes elongated (Fig. 2 a). Mesoderm is forming after the appearance of the slit like blastopore at the posterior end of the blastopore (Fig. 4 a). The mesoderm migrates as one band of cells on each side of

the posterior blastopore and soon the first somite is formed on each side of the slit-like anterior blastopore (see Figs. 33, 114 in Manton, 1949). The slit-like blastopore was termed mouth-anus by Manton (1949). We have chosen to call it blastopore because it is continuous in time and space with the original pit-like blastopore opening (Figs. 2a, 4a).

Expression of *labial*, *proboscipaedia*, *hox3* and *deformed*

The expression of *labial* and *proposcipaedia* is basically identical, with the anterior expression border at the anterior of the slime papilla segment and the expression are seen in mesoderm and ectoderm of limb-buds as well as neuroectoderm (Fig. 1 a-b and supp. Fig. 1 a-d). *Labial* is expressed in the surface layer of the ectoderm as well, but *proboscipaedia* and also *hox3* is restricted to the deeper layer of ectoderm (Fig. 1 a-d and supp. Fig. 1 a-f). The expression of *labial*, *proboscipaedia*, *hox3* and *deformed* extend all the way to the proctodeum (see Fig. 1 e-f for *hox3*). The anterior expression border of *Hox3* is in the slime papilla segment as well, but expression is lacking in the most anterior part (Fig. 1 c, e-f) and in the later stage it is even more posterior starting in the second half of the slime papilla segment (Fig. 1 c) and expression is lacking in the slime papilla limb itself (Fig. 1 c). *Deformed* has its anterior expression border at the anterior of the segment of the first walking leg, the fourth segment (Fig. 1 d).

Early expression of *otd*

The first observed expression of the head patterning gene *otd* is during the first phase of mesoderm formation on both sides of the blastopore slit (Fig 2. a-b). *Otd* is expressed diffusely in the blastodisc centered on the blastopore slit. The expression is lacking in the posterior area around the posterior part of the blastopore. The expression is also asymmetrical with a more extended expression pattern on one side of the slit-like blastopore.

Early expression of *six3*

The first sign of *six3* expression coincides with the early expression of *otd*. The expression is restricted to a thin band around the most anterior part of the slit-like blastopore (Fig. 4 a-b).

Expression of *otd* and *six3* during germ band extension

At stage II both *otd* and *six3* are expressed in the developing brain anlage. *Otd* is initially restricted to the posterior margin, extending lateral from dorsal to ventral (Fig. 2 c-d) and *six3* at the anterior of the brain anlage (Fig. 4 c-d). It appears as if the two genes to a large extent are expressed in mutually exclusive zones but we cannot rule out some limited overlap. *Otd* is then expressed in a field perpendicular to the original marginal expression, extending towards the anterior but not reaching the most anterior quarter of the anterior-posterior distance (Fig. 2 e-f). *Otd* is also expressed around the stomodeum when it has been separated from the posterior blastopore-slit. In the stage IV embryo *otd* is expressed in a relatively larger area of the brain anlage but expression is lacking in the absolute anterior and in a wedge shaped field pointing towards the eye (Fig. 3 a-d). There is no expression of *otd* in the antenna. Expression in the future trunk nervous system is also appearing during the stage IV embryo (Fig. 3 a-b, and supp. Fig). The expression of *six3* during the later stages continues to be in the anterior of the brain anlage and is including the anterior part of the antenna (Fig. e-f). There is no expression of *six3* in the developing eye (Fig.

Discussion

Early *otd* expression and relationship with other *otd/otx* homologues

In the crustacean *Parhyale hawaiensis* there are two paralogues of *otd* with differing expression patterns and difference in onset of expression. The paralogue that contains the WSP motif is starting to be expressed late and is more similar in amino acid sequence to the vertebrates (Browne et al., 2006). In *E. Kanangrensis* we found one gene homologous to *otd* and it contained the WSP motif that is included in the SIWSPASI motif found in vertebrates (Li et al., 1996). The *otd2* genes are also in the *Tribolium* expressed much later than *otd1*. The single *otd* gene found in *E. Kanangrensis* is expressed early in development and continues to be expressed in the brain neuromere as well as in a cluster of cells in the neuroectoderm in each segment in the VNC. Considering the fact that the *Eka-otd* contains the conserved SIWSPASI as the *otd2* genes of *Parhyale* and *Tribolium* and its early expression in *E. kanagrensis*, which contrasts with that of *Parhyale* and *Tribolium*, it is

possible to draw the conclusion that the single *E. kanangrensis otd* has the combined function of *otd1* and *otd2* in *Parhyale* and *Tribolium* and a duplication even occurred after the split of the arthropod and onychophoran lineages. This partly supports the views that a single *otd/otx* gene was present in the common ancestor to vertebrates and arthropods, and that this gene was duplicated in the lineages leading to chordates and arthropods respectively (Browne et al., 2006; Li et al., 1996). Li et al. (1996) suggested on basis of the expression pattern of *Tribolium* otd2 that the ancestral function might have been to specify certain regions in the pre antennal brain, however, our data suggest that this ancestral gene would have combined the functions of *otd1* and *otd2*.

In the early *E. kanangrensis* embryo the *otd* gene is expressed in a broad field in the blastodisc surrounding the slit-like blastopore, it is missing from the most posterior part around the posterior blastopore. This expression pattern is similar to the expression pattern seen in *Drosophila otd* and for the *otd2* expression in *Tribolium* and *Parhyale* (Browne et al., 2006; Li et al., 1996) The expression of *Eka-otd* is sometimes, however, asymmetrical around the blastopore (Fig. 2 a-b). This asymmetry might reflect the fact that the early development of the germ band is also sometimes developing asymmetrical, i.e. the germ band extends with different speed, on each side of the blastopore (see e.g. Fig. 5 in Manton 1949).

Eka-otd and head segmentation

Head expression of otd in *E. Kanangrensis* is clearly restricted to the first neuromere. In arthropods the expression is less clear with some examples of expression in more posterior structures but it appear as if the original expression in arthropods is restricted to the protocerebrum (Browne et al., 2006; Finkelstein and Perrimon, 1991; Hirth et al., 2003; Li et al., 1996; Telford and Thomas, 1998), which is the first neuromere of arthropods. The present data thus support earlier claims that the onychophoran antenna is situated on the most anterior neuromere (Eriksson et al., 2009; Eriksson and Budd, 2000; Eriksson et al., 2003; Mayer and Koch, 2005; Sedgwick, 1887), and hence, the onychophoran antenna is not homologous to the insect antenna or its equivalent structures on other arthropods.

Early *Eka-six3* expression

The early expression of six3 is around the anterior margin of the slit-like blastopore. This looks very similar to what has been described in the Medaka fish (Loosli et al., 1998), were the early *six3* expression is seen around the anterior margin of the embryonic shield. This, together with the general resemblance between onychophoran and vertebrate segmentation tempts to do further investigation on the molecular aspects of onychophoran axis formation and segmentation, e.g. do the onychophoran slit-like blastopore have an organising function like the fish embryonic shield or amphibian organizer? The *Drosophila Dsix3* gene is expressed in the anterior of the stage 5 or blastoderm stage but is lacking from the extreme terminal areas (Seo et al., 1999). However, from a *Drosophila* fate map (Campos-Ortega and Hartenstein, 1985) one can see that the area of *Dsix3* expression corresponds to the prospective areas of the most anterior neurogenic area, which would correspond to the early expression pattern seen in *E. kanangrensis*.

Eka-six3 and head segmentation

From the expression studies so far it is clear that *six3* marks the most anterior neurogenic territory in animals (Li et al., 1996; Lowe et al., 2003; Posnien et al., 2009; Schinko et al., 2008; Schröder, 2003; Seo et al., 1999). This investigation corroborates the earlier ones in that *six3* is expressed in the extreme anterior region of the neuroectoderm of the brain anlage. Thus *Eka-six3* and *Eka-otd* demarcates the anterior and posterior borders of the first neuromere or brain anlage in onychophorans. The onychophoran antenna is situated in the *six3* expressing area and this is supporting earlier investigations that claim that the onychophoran antenna is an appendage of the most anterior neuromere (Eriksson and Budd, 2000; Eriksson et al., 2003).

In *Drosophila* and *Tribolium, six3* is expressed in and essential for the development of the clypeolabrum and labrum respectively (Posnien et al., 2009; Seo et al., 1999). Indeed, the labrum has been much debated and it appear to posses appendicular characters homologous to segmental appendages but on the other hand displays unique features that have lead researchers attribute it to an unsegmental region of the arthropod head (Haas et al., 2001b; Posnien et al., 2009; Rempel, 1975; Scholtz and Edgecombe, 2005). It is certainly premature to homologize the onychophoran antenna with the arthropod labrum on the grounds of the common expression of one gene and on them being positioned on the homologous, *otd-six3*

defined zone on the most anterior neuromere, territory, but future molecular characterization might prove enlightening.

The onychophoran head segments, the segmental nature of the *otd-six3* territory and their implication for understanding arthropod head segmentation

The onychophoran head incorporates three paired neuromeres, each with an associated coelomic cavity and appendage. A segment is normally defined as a repetitive unit with structures such as neuromeres, coelomic cavities, appendages, annuli, set of muscles and nephridia (Scholtz, 2002). Apart from annuli and muscle sets, all of these characters are associated with each of the three onychophoran head neuromeres (Eriksson and Budd, 2000; Eriksson et al., 2003; Mayer and Koch, 2005; Sedgwick, 1887; Storch and Ruhberg, 1993) and Eriksson et al. (2009) reported *engrailed* expression in each of these units, and therefore, it seems correct to apply the term segment to these three units. However, we know that the most anterior segment, the antennal segment, carries unique characters that distinguishes it from the two other head segments, which apart from morphological differences appearing relatively late in development, are very similar to the trunk segments. Some characters defining the unique nature of the antennal segment are; presence of an eye (Storch and Ruhberg, 1993), ventral or infracerebral organ remaining in adult (Anderson, 1973; Dakin, 1922; Eriksson et al., 2005), lack of *engrailed* and *wingless* expression in neuroectoderm (Eriksson et al., 2009), nerve tracts initially not part of or connected to ventral nerve cord (Eriksson et al., 2003; Mayer and Whitington, 2009). Hence, based on morphological and molecular characters the onychophoran body can be divided into two units, the trunk and trunk-like head segments segments and the *otd-six3* defined anterior head region (Fig. 5). The expression pattern of the four anterior hox-genes presented herein correlates the anterior segments of the onychophorans with the scheme presented for arthropods (Damen et al., 1998; Telford and Thomas, 1998) (Fig. 5); The *otd-six3* region is equivalent to the protocerebral or ocular region of arthropods and the following segments match up according to the scheme in figure 5 so that the onychophoran jaw segment corresponds to the arthropod first antenna, onychopghoran slime papilla to arthropod second antenna, onychophoran first walking leg to arthropod mandible and so forth.

We propose the following scenario for the evolution of the arthropod head. The onychophoran arthropod ancestor was an animal with a head made up of one unit, we will refer to it as a head segment to distinguish it from the other segments, and a trunk with homonomous segments (fig. 6). Based on the fact that onychophoran mouth position was most likely terminal (Eriksson and Budd, 2000; Eriksson et al., 2003) and stem group onychophorans and arthropods also show a terminal mouth (Ma et al., 2009) we can conclude that also the last common ancestor of onychophorans and arthropods were equipped with a terminal mouth as well. The head then developed independently as the lineage split leading to the two different heads seen today in onychophorans and arthropods. This separate head

evolution in the two clades has led to some interesting points like e.g. the functional antenna in onychphorans is evolved from an appendage that has been lost in arthropods and that the food prosessing appendage in onychophorans, the jaw, is made up of the appendage that in arthropods developed to become the functional antenna. Another effect from the evolution of the head is the convergent ventral position of the mouth (Eriksson and Budd, 2000).

Earlier workers have often included an unsegmented anterior unit referred to as the acron (Scholtz, 2002; Scholtz and Edgecombe, 2005). However, in the present scenario there is no clearly unsegmental part in the basal panarthropod ancestor, instead it had a unique anterior segment-like unit, the head segment and the rest of the head were sequentially during evolution made up of modified trunk segments. It is tempting to go one step further back in evolution and suggest that the urbilateria was an organism made up of a head and an unsegmented trunk, it seems most logical to assume the presence of a head before segments and not vice versa. Segmentation then arose by dividing up the trunk into units (segments) while still retaining the head. This would explain the unique characters of the onychophoran antennal segment as compared to the trunk and the two posterior head segments. It is till debateable if one should call this anterior region a segment or an acron. In onychophora this regieon is very segment like indeed, see above, and an acron has seldom, if at all, been described (Anderson, 1973). To just call the *otd/six3* region a segment would also be misleading and hides the fact that it is truly different from the other segments.

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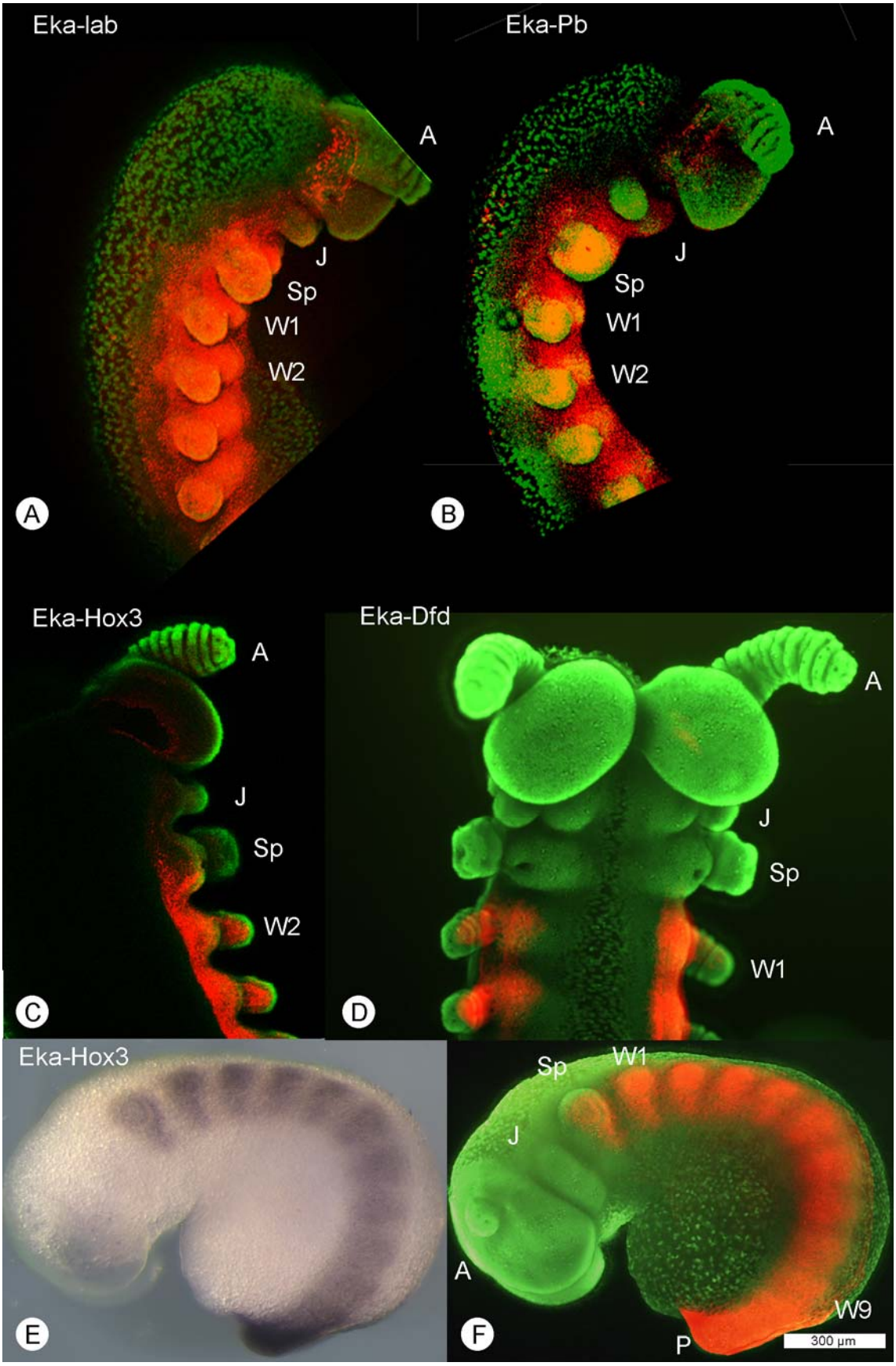


Figure 1. Expression of *labial*, *proboscipaedia*, *Hox3* and *deformed* in an *Euperipatoides kanangrensis* stage IV embryo. A, *labial* expression in the third segment which bears the slime papilla appendage (Sp), lateral view anterior is up. B, proboscipaedia expression in the third segment, lateral view anterior is up. C, Hox3 expression in the second half of the third segment, ventral view anterior is up. D, deformed expression starting in the fourth segment bearing the first walkin leg (W), ventral view anterior is up. E-F hox3 expression in a stage II embryo showing that the expression extends all the way to the proctodeum (P). A-C are maximum projections from confocal microscopy stacks, D and F are false red colour images from a colorimetric stain imposed onto the same object photographed with UV-excited nuclear stain. A = antenna, J = jaw, scale bar = 300 µm.

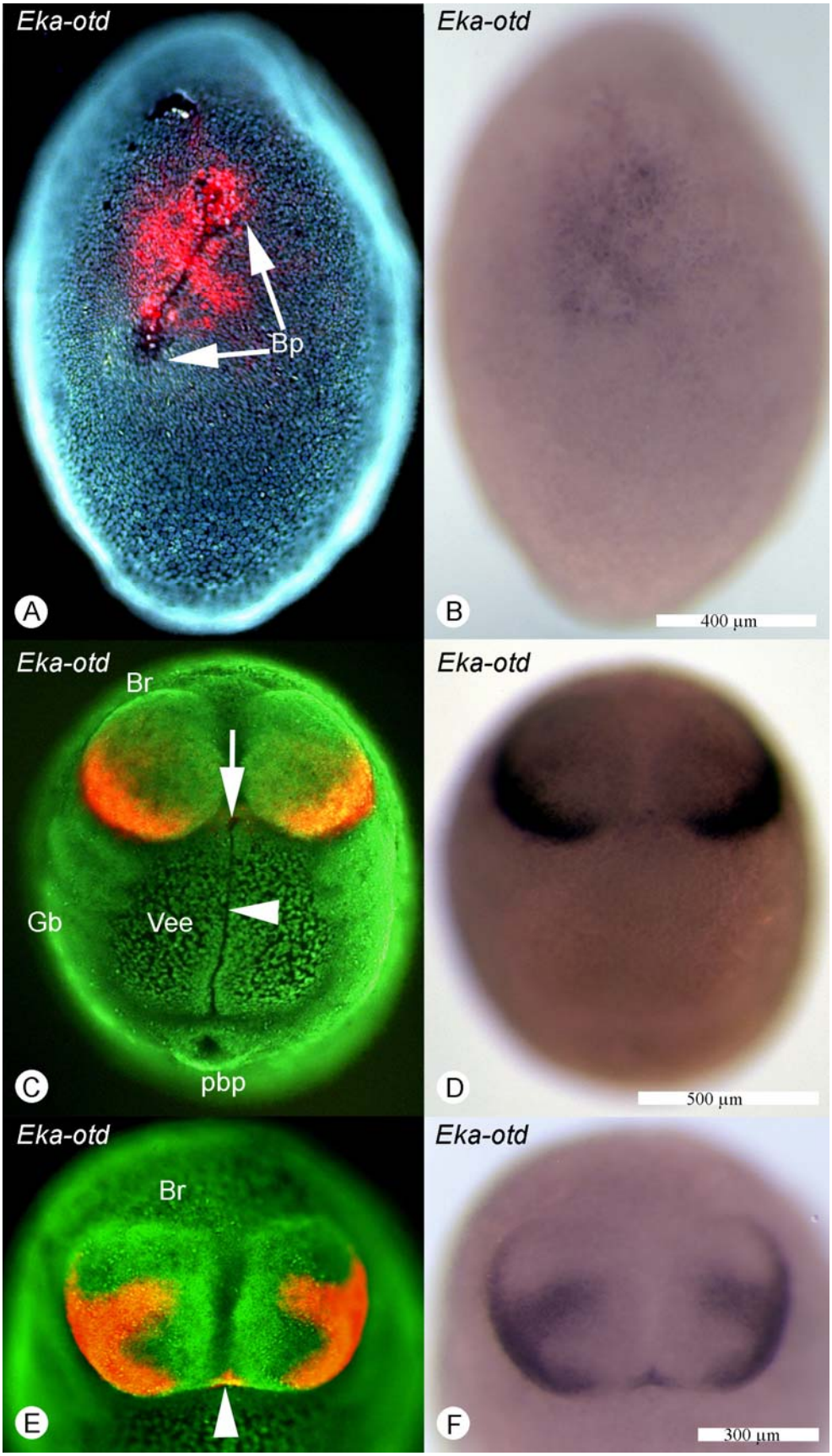


Figure 2. Expression of *Eka-otd* in *Euperipatoides kanangrensis* embryos. A-B Stage I embryo with elongated blastopore (Bp), but before segment formation. *Eka-otd* is expressed diffusely in the area around the slit-like blastopore with the exception of the posterior part. C-D Stage II embryo with expression in the posterior area of the first somite, the brain rudiment (Br). E-F Stage II embryo slightly later than the one shown in C-D. Expression is now also detected in a zone perpendicular to the previous embryo as well as around the stomodeum (arrowhead). Gb = germ band, Pbp = posterior blastopore, Vee = ventral extraembryonic ectoderm, scale bar A-B = 400 µm, scale bar C-D = 500 µm, scale bar E-F = 300 µm.

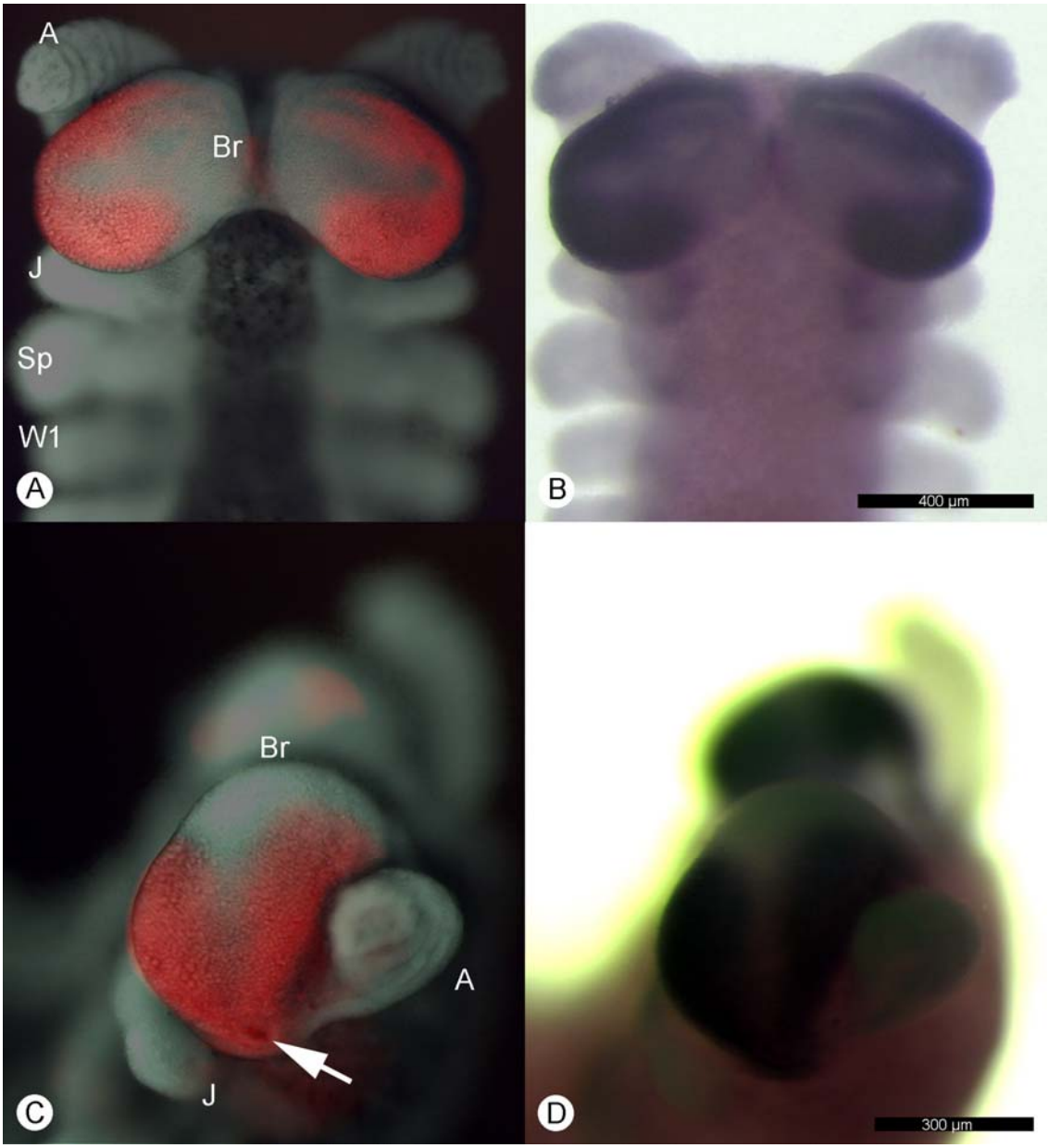


Figure 3. Expression of *Eka-otd* in *Euperipatoides kanangrensis* embryos of stage IV. A-B expression is in the dorsal and ventral area of the posterior part of the brain anlage (Br) with wedge shaped area lacking expression, ventral view anterior is up. C-D the areas of ventral and dorsal expression meet in the dorsal area were the eye rudiment is situated (arrowhead), lateral view anterior is up. A = antenna, J = jaw, W = walking leg, scale bar A-B = 400 µm, scale bar C-D = 300 µm.

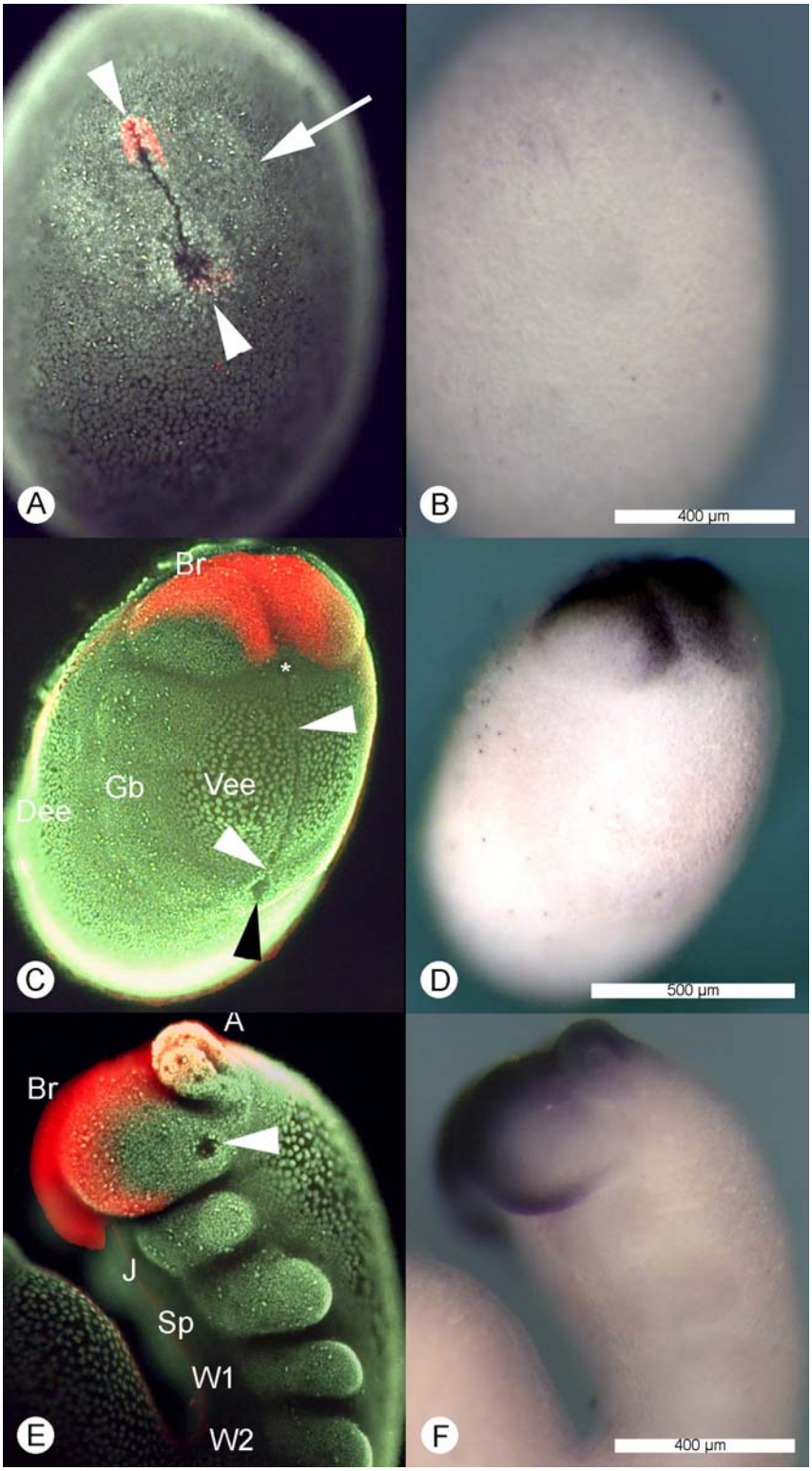


Figure 4. Expression of *Eka-six3* in *Euperipatoides kanangrensis* embryos. A-B stage II embryo with elongated blastopore (between arrowheads) and with a developing germband (arrow) but before segment formation. Expression of six3 is restricted to the area bordering the anterior blastopore (upper arrowhead). C-D a later stage II embryo with a segmenting germ band (Gb). The slit-like blastopore has been divided up into three parts; proctodeum (black arrowhead), a middle part and the stomodeum (star), areas thet separate these blastopore regions are marked with white arrowheads. The expression of *Eka-six3* is restricted to the anterior part of the brain rudiment (Br) and extend ventrally and dorsally to the edge of the ventral and dorsal extra embryonic ectoderm (Vee and Dee respectively). E-F later stage II embryo with developing antenna (A). The expression of *Eka-six3* can now be seen in the antenna. J = jaw, Dee = dorsal extra-embryonic ectoderm, Sp = slime papilla, Vee = ventral extra-embryonic ectoderm, W= walking leg, scale bar A-B, E-F = 400 µm, C-D = 500 µm.

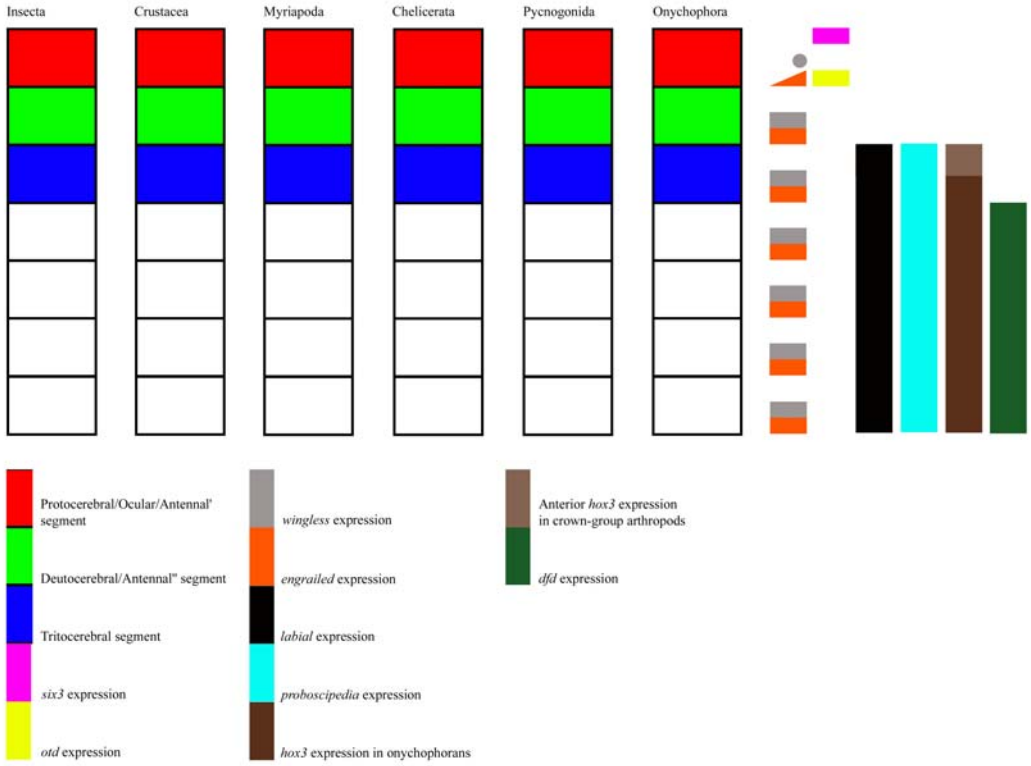


Figure 5. Scheme showing the segments correlated between different panarthropod groups derived from the expression pattern of homologues of the four anterior hox genes, wingless, engrailed, six3 and otd in different panarthropd groups. The expression of engrailed and wingless in the onychophoran first segment differs slightly from their expression in the more posterior segment, the filled circle marking wingless expression indicates that wingless is only expressed in the distal tip of the antenna, just like in the posterior segments but the stripy expression in the neuroectoderm is lacking. The triangle of engrailed expression indicates that engrailed expression is lacking in the neuroectoderm but present in the posterior of the segment.

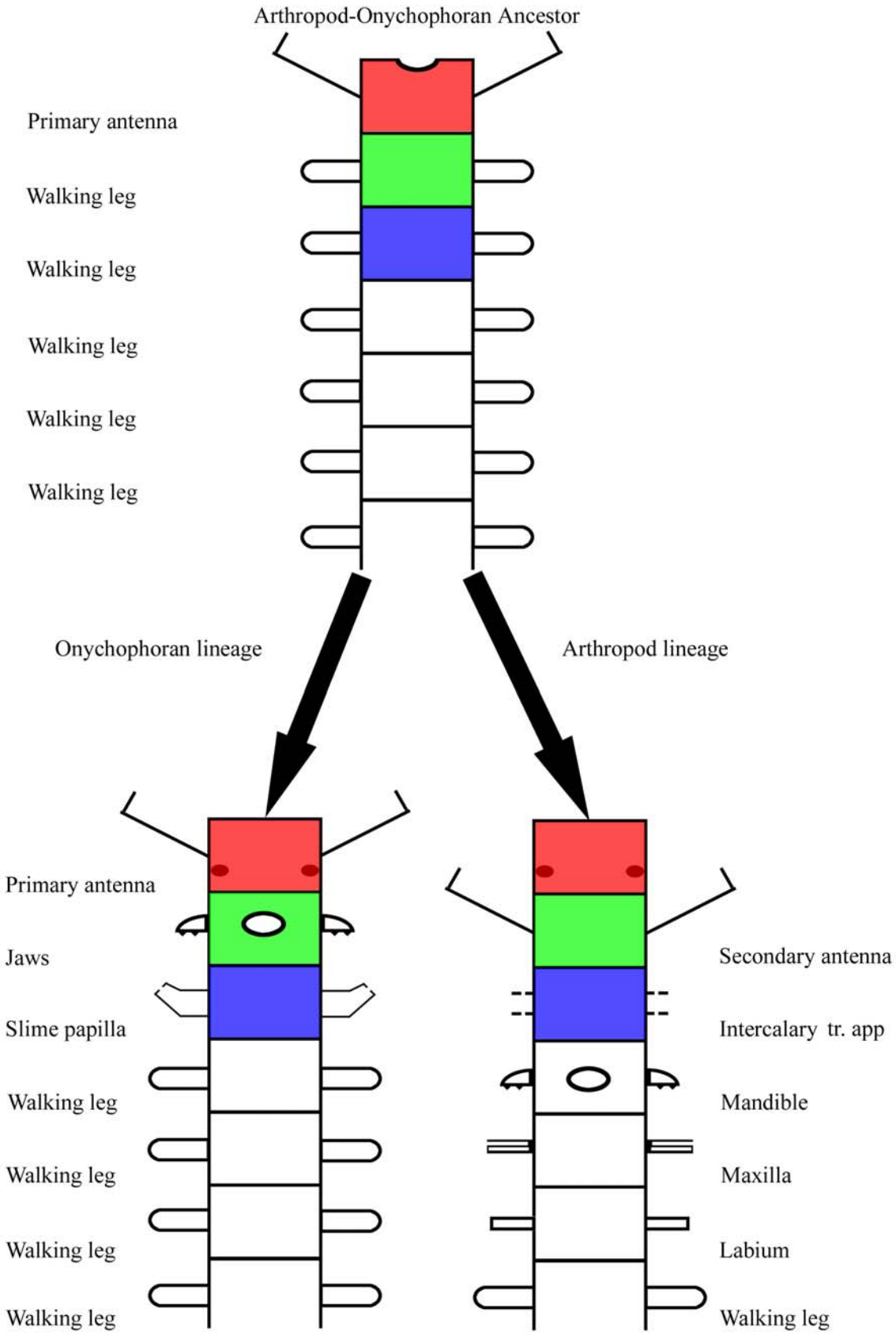
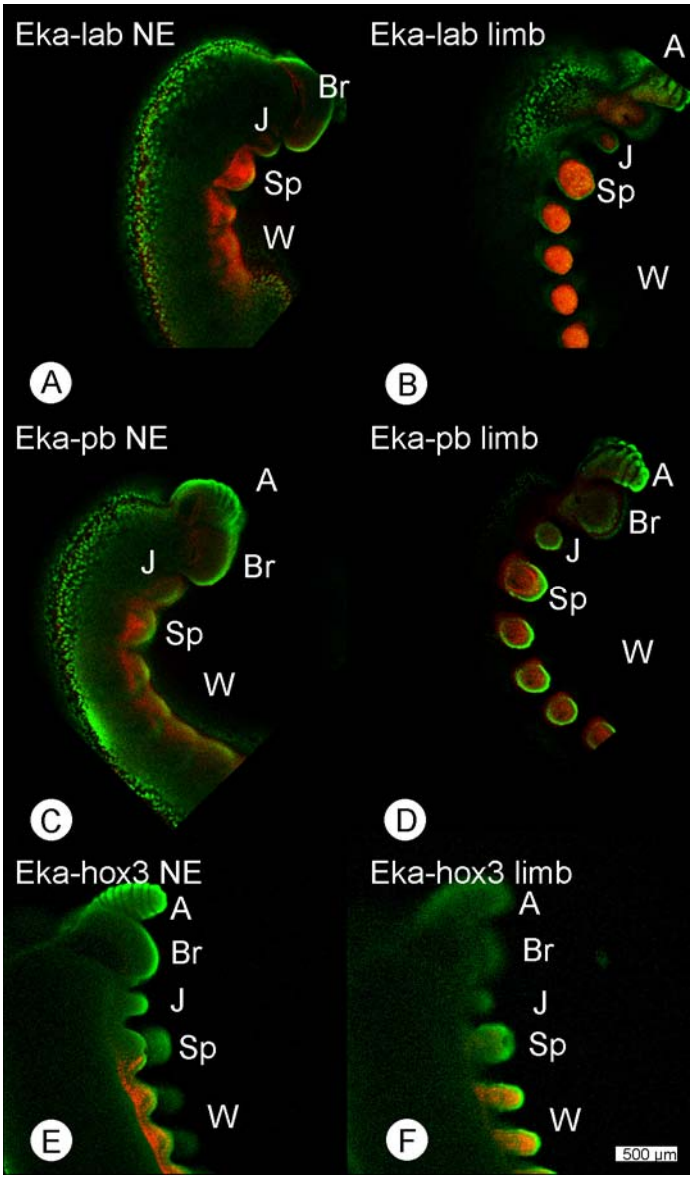


Figure 6. Scheme showing a possible evolutionare scenario of the arthropod/onychophoran lineage from a common ancestor. The correlated segments are colour coded and the onychophoran/arthropod ancestor had a head composed of one segment with an modified appendage. The two lines acquired a head tagma independently, the line leading to the onychophorans retained the primary antenna whereas this function was taken over by the appendage of the deutocerebral/second segment in the arthropod linage. Filled ellipses in the first segment (red) indicate eyes and open ellipses indicate the functional position in the adult animals.



Supplementary figure 1. Optical sections from CLSM showing embryos of *Euperipatoides kanangrensis* stained for: *lab*, *pb* and *hox3*. A, Section through the neuroectoderm showing *lab* expression in the central as well as the surface of segments from the slime papilla (Sp) and continuing posteriorly. B, Same embryo as in A with a section through the limbs showing expression of *lab* in the central as well as the surface of segments from the slime papilla and continuing posteriorly. C, Section through the neuroectoderm showing expression of *pb* in the interior but lacking in the surface layer of segments from the slime papilla and continuing posteriorly. D, Same embryo as in C with a section through the limbs showing expression of *pb* in the interior but lacking in the surface layer of segments from the