Velvet worm development links myriapods with chelicerates

Georg Mayer* and Paul M. Whittington

Department of Anatomy and Cell Biology, University of Melbourne, Melbourne, Victoria 3010, Australia

Despite the advent of modern molecular and computational methods, the phylogeny of the four major arthropod groups (Chelicera, Myriapoda, Crustacea and Hexapoda, including the insects) remains enigmatic. One particular challenge is the position of myriapods as either the closest relatives to chelicerates (Paradoxopoda/Myriochelata hypothesis), or to crustaceans and hexapods (Mandibulata hypothesis). While neither hypothesis receives conclusive support from molecular analyses, most morphological studies favour the Mandibulata concept, with the mandible being the most prominent feature of this group. Although no morphological evidence was initially available to support the Paradoxopoda hypothesis, a putative synapomorphy of chelicerates and myriapods has recently been put forward based on studies of neurogenesis. However, this and other morphological characters remain of limited use for phylogenetic systematics owing to the lack of data from an appropriate outgroup. Here, we show that several embryonic characters are synapomorphies uniting the chelicerates and myriapods, as revealed by an outgroup comparison with the Onychophora or velvet worms. Our findings, thus provide, to our knowledge, first morphological/embryological support for the monophyly of the Paradoxopoda and suggest that the mandible might have evolved twice within the arthropods.

Keywords: arthropods; Onychophora; neurogenesis; neuroblast; synapomorphy; phylogeny

1. INTRODUCTION

Historically, myriapods have been affiliated with virtually every major arthropod group, but only two competing hypotheses for their phylogenetic position currently remain in favour (figure 1). Most morphological, combined, and some molecular, studies unite myriapods with crustaceans and hexapods within the Mandibulata (Scholtz et al. 1998; Edgecombe et al. 2000; Giribet et al. 2001, 2005; Nielsen 2001; Kusche et al. 2002, 2003; Edgecombe 2004; Harzsch et al. 2005; Scholtz & Edgecombe 2005, 2006; Bourat et al. 2008; Regier et al. 2008; Rota-Stabelli & Telford 2008). However, there is also strong molecular evidence for joining myriapods with chelicerates in a group dubbed Myriochelata or Paradoxopoda, the latter name reflecting the presumed absence of morphological evidence supporting this clade (Friedrich & Tautz 1995; Giribet et al. 1996; Cook et al. 2001; Hwang et al. 2001; Kusche & Burmester 2001; Mallatt et al. 2004; Negrisolò et al. 2004; Pisani et al. 2004; Hassain 2006; Mallatt & Giribet 2006; Dunn et al. 2008; Gai et al. 2008; Podsiedlnowski et al. 2008; Breugelmans et al. 2009). In fact, similarities have been recorded in chelicerate and myriapod development (Dove & Stollewerk 2003; Kadner & Stollewerk 2004; Stollewerk & Chipman 2006; McGregor et al. 2008). However, these similarities do not by themselves clarify the controversial position of Myriapoda because they might be synapomorphies (shared ancestral traits) rather than synapomorphies (shared derived characters). Hence, studies of an outgroup are necessary to polarize derived versus ancestral character states among the arthropods.

Onychophora are the most promising candidates because they are the closest relatives of arthropods (Kusche et al. 2002; Mallatt & Giribet 2006; Dunn et al. 2008) and have changed little since the Early Cambrian, as evidenced by their resemblance to the fossil lobopodians (Budd 2001; Maas et al. 2007). We therefore analysed the development in representatives from two distantly related onychophoran groups that diverged prior to the break-up of Gondwana 175–140 Myr ago (Upchurch 2008) and display two different modes of embryogenesis. The embryo of Euperipatoides rowelli (Peripatopsidae), an ovoviviparous Australian species is surrounded by egg membranes and contains yolk, whereas that of Epiperipatus isthmicola (Peripatidae), a viviparous Costa Rican species, lacks embryonic envelopes and has placental structures instead. The study of development in both groups is likely to reveal the basic features for Onychophora.

2. MATERIAL AND METHODS

(a) Obtaining specimens

Specimens of the onychophoran species E. rowelli Reid, 1996 and Ep. isthmicola (Bouvier, 1905) were collected from rotten logs in the Tallaganda State Forest (New South Wales, Australia) and the Reserva Biologica Hitorycerer (Costa Rica) as described previously (Mayer & Tait in press). Cocoons of the spider Steatoda grossa (Koch, 1838) were obtained from the Department of Genetics (University of Melbourne, Australia). Specimens of the symphytan Hanseniella agilis Tieg, 1939 were collected in the Dandenong Ranges (Melbourne) and maintained in the laboratory. The eggs were collected at regular intervals...
Figure 1. Competing hypotheses on the relationship of panarthropods. The tree on the left represents the traditional Mandibulata hypothesis, with myriapods as sister group to crustaceans and hexapods. The tree on the right illustrates the Paradoxopoda/Myriochelata hypothesis uniting myriapods with chelicerates. The double lines for Crustacea indicate that this group might not be monophyletic.

(Tiegs 1940). Prior to fixation, the spider and symphylan eggs were dechorionated by using household bleach according to standard protocols (e.g. Kadner & Stollewerk 2004).

(b) Scanning electron microscopy
Specimens were fixed in 4 per cent formaldehyde. After dehydration in an ethanol series, the specimens were dried in a CPD 030 Critical Point Dryer (BAL-TEC AG, Balzers, Liechtenstein), coated with gold in a SCD 040 Sputter Coater (BALZERS UNION, Balzers, Liechtenstein), and examined in a Quanta 200 Scanning Electron Microscope (FEI, Hillsboro, Oregon, USA).

(c) Immunohistochemistry
Embryos were fixed overnight in 4 per cent paraformaldehyde in phosphate-buffered saline (PBS, 0.1 M, pH 7.4), rinsed in PBS and pre-incubated in 1 per cent bovine serum albumin and 0.3 per cent Triton X-100 in PBS for 3 h at room temperature. Incubations with antibodies were carried out overnight at 4°C. The following primary antibodies were used either separately or in combination diluted in PBS: (i) mouse monoclonal anti-acetylated α-tubulin IgG2b isotype (catalogue no. T6793, Sigma-Aldrich, St. Louis, MO, USA; diluted 1:500); (ii) rabbit polyclonal anti-phospho-histone H3 mitosis marker (α-PH3, catalogue no. 06-570, Upstate, Temecula, CA, USA; diluted 1:500); (iii) mouse monoclonal anti-actin, JLA20 (Developmental Studies Hybridoma Bank, Iowa, USA; diluted 1:500); (iv) Cy5-conjugated goat anti-Horseradish Peroxidase (anti-HRP, catalogue no. 123-175-021, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; diluted 1:10); and (v) Cy5-conjugated goat anti-Horseradish Peroxidase (anti-HRP, catalogue no. 123-175-021, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; diluted 1:100). Specimens were rinsed in several changes of PBS and incubated with secondary antibodies (Invitrogen, Carlsbad, CA, USA; each diluted 1:500 in PBS): (i) Alexa Fluor 488 goat anti-mouse IgG (catalogue no. A11001); or (ii) Alexa Fluor 488 goat anti-rabbit IgG (catalogue no. A11034) and/or (iii) Alexa Fluor 594 goat anti-rabbit IgG (catalogue no. A11037). Embryos were then incubated for 1 h in a solution containing phalloidin-rhodamine (catalogue no. R-415300, Molecular Probes (Invitrogen)); 10 units per 100 μl PBS), after which the DNA-selective fluorescent dye Hoechst (Bisbenzimide, H33258, catalogue no. 861405, Sigma-Aldrich; 1 μg ml⁻¹ in PBS) was applied for 15 min.

Embryos were rinsed in PBS and either mounted directly on glass slides in Vectashield Mounting Medium (catalogue no. H-1000, Vector Laboratories Inc., Burlingame, CA, USA), or dehydrated through an isopropanol series and mounted between two cover slips in a 2 : 1 mixture of benzyl benzoate and benzyl alcohol. Omission of the primary antibodies abolished staining.

(d) Confocal microscopy and image processing
Embryos were analysed with confocal laser-scanning microscopes LSM 510 META and LSM 5 PASCAL (Carl Zeiss MicroImaging GmbH, Jena, Germany). Optical sections were taken at intervals ranging from 0.18 to 11.1 μm, and the resulting image stacks were merged digitally into maximum projection micrographs with the Zeiss LSM IMAGE Browser software (v. 4.0.0.241). Image intensity histograms were adjusted by using the Adobe (San Jose, CA, USA) PHOTOSHOP CS2. The adjustment was kept at a minimum to allow the micrographs of the same plate to have similar intensity. Final panels and diagrams were designed with an Adobe ILLUSTRATOR CS2.

3. RESULTS AND DISCUSSION

(a) Comparison of early development in onychophorans and arthropods
In embryos of chelicerates, the so-called cumulus (figure 2a), a conspicuous group of mesenchymal cells, initiates the breakdown of radial symmetry and leads to a dorsal split of the embryonic germ disc (Sekiguchi 1973; Chaw et al. 2007; McGregor et al. 2008). Transplantation or removal of the cumulus results in duplication or complete loss of the dorsal embryonic region (Holm 1952), while a gene expression study has revealed that the cumulus functions as an organizer that determines the dorsal region via decapentaplegic signalling (Akiyama-Oda & Oda 2003). A group of mesenchymal cells forms in an equivalent position in myriapod embryos and shows the same spatio-temporal pattern of movements as in chelicerates (Sakuma & Machida 2002, 2004; Janssen et al. 2004). However, a cumulus-like structure has never been reported in crustaceans or hexapods.

To establish whether a cumulus-like structure occurs in onychophoran development, or rather, is a derived feature of chelicerates and myriapods, we focused on early embryonic stages. In E. rowelli, we obtained embryos in the first six months of development at a blastula stage (figure 2b,c). After this stage, the embryo displays a condensed field of cells, within which the blastopore arises and transforms into a slit (figure 2d,e). The paired germ band develops at the posterior end of this slit, grows in the anterior direction and fuses in front of the future mouth, where the antennal somite is formed (figure 2b,f). The embryo elongates further and acquires a flexed posture. During this phase of development, the extra-embryonic ectoderm occupies most of the ventral and dorsal surface, but is later reduced in extent. In contrast to E. rowelli, the extra-embryonic ectoderm is hardly visible in the embryo of Ep. isthmica, which is attached to the maternal uterus via a placental stalk, with the blastopore originating on the opposite side (figure 3a,b). While the embryo elongates, the mouth and anus separate and the
embryonic segments arise in a rapid anterior to posterior progression (figure 3c, d). We did not observe a cumulus-like structure or a dorsal split of the germ disc in either of the two onychophoran species.

Recruitment of single cells versus cell clusters during neurogenesis

Additional similarities between chelicerates (Stollewerk et al. 2001; Mittmann 2002) and myriapods (Dove & Stollewerk 2003; Kadner & Stollewerk 2004) occur during neurogenesis. In embryos from both groups, the neuroectoderm generates numerous cell clusters with basally located cell bodies and apical cell processes that converge at the epithelial surface (figure 4). The clusters arise in a stereotypic pattern during development and finally dissociate from the ectodermal surface to give rise to neurons (Stollewerk et al. 2001; Dove & Stollewerk 2003; Kadner & Stollewerk 2004). Phalloidin-rhodamine staining of embryos of the spider S. grossa carried out in the current study reveals the same pattern of cell clusters previously reported in other chelicerates (see electronic supplementary material, figure S1a, b). In addition, we have observed similar cell clusters in the neuroectoderm of the symphylan H. agilis (see electronic supplementary material, figure S2a–c), showing that this is a widespread developmental phenomenon in the Myriapoda. In contrast to chelicerates and myriapods, cell clusters with convergent processes do not occur in the neuroectoderm of crustaceans and hexapods (Stollewerk & Simpson 2005; Ungerer & Scholtz 2008). Instead, individual cells called neuroblasts are segregated from the neuroectoderm in these animals (figure 4).

To determine whether neuroectodermal cell clusters are an ancestral character state for the arthropods, we studied neurogenesis in Onychophora. Since cell lineage analyses cannot be performed in onychophorans, we have used a full complement of embryonic stages in two distantly related onychophoran species to obtain a detailed picture of neurogenesis. We found that despite considerable differences in development (yolky eggs versus a placenta), neurogenesis is remarkably similar in E. rowelli and Ep. isthmicola. In neither species are cell clusters evident in the neuroectoderm at any stage (figure 5a–g, see electronic supplementary material, figure S3a, b). Instead, single cells with a bottle-like shape migrate basally from the neuroectoderm, where they form a conspicuous layer of cells (figures 4, 5d, S5b, electronic supplementary material).
Mitotic cell divisions are highly abundant in this cell layer, whereas cell divisions are virtually absent from the presumptive nervous tissue, i.e. the future nerve cords (figures 5e–g, see electronic supplementary material, figures S3c,d, S4a–i). Nevertheless, the nervous tissue rapidly increases in thickness and in the number of cells, which suggests that the neurons within each nerve cord originate from the conspicuous layer of dividing cells (figures 4, 5e–g). In addition, there is no apparent morphological structure apart from the ventral nerve cords, to which these dividing cells could give rise.

(c) Involvement of cell clusters in sensory structure formation

Despite the fact that cell clusters are absent from the onychophoran neuroectoderm, groups of cells morphologically resembling those in chelicerates (see electronic supplementary material, figure S1a,b; Stollewerk et al. 2001; Mittmann 2002) and myriapods (see electronic supplementary material, figure S2a–c; Kadner & Stollewerk 2004) do occur in the onychophoran embryos (figure 6a–c). The cell groups or clusters initially arise on the antennal ectoderm, but later in development cover the entire body surface, including the legs. Although the onychophoran cell clusters display the anti-HRP immunoreactivity characteristic of neurons (figure 6c), they do not generate neurons of the ventral cord, but rather, give rise to sensory structures on the body surface (figure 6d). It is of interest that in chelicerates, sensory organs also arise from cell clusters with convergent cell processes, whereas in hexapods these organs originate from single precursor cells (Stollewerk & Seyfarth 2008). Given that sensory structures originating from clusters of cells occur in embryos of chelicerates, myriapods and onychophorans, we suggest that this is an ancestral character of arthropods and, as such, should not be used to link the myriapods with chelicerates.

(d) Lack of segmental invaginations in onychophoran neurogenesis

A conspicuous phenomenon in the neurogenesis of myriapods is the invagination of the neuroectoderm in each hemisegment, once a large number of neurons has been generated. The invagination leads to the formation of epithelial vesicles (ventral organs), which enclose the remaining cell clusters in each hemisegment. This process has previously been documented in the Chilopoda and Diplopoda (Heymons 1901; Dohle 1964; Whittington et al. 1991; Dove & Stollewerk 2003; Kadner & Stollewerk 2004) and we have observed the same process in the symphylan H. agilis (see electronic supplementary material, figure S2c,d; see also Tiegs 1940). Reports of an apparently similar process of ventral organ formation can be found in the literature for a number of chelicerates, including pseudoscorpions and pycnogonids (e.g. Weygoldt 1964: figs 53 and 54; Winter 1980: fig. 1). We have observed a process of neuroectodermal invagination in the spider S. grossa (see electronic supplementary material, figure S1c), which mirrors closely that seen in the myriapods. Stollewerk (2004) reports an apparently different phenomenon in the spider Cupiennius salei, of epidermal cells ‘overgrowing’ the ventral neurones, although the same author also makes reference to ‘epithelial vesicles’ and ‘secondary invaginations’ in this species. Leaving aside the question of whether neuroectodermal invagination is a universal feature of spider development, it is clear that this process does not take place during the formation of the central nervous system in either crustaceans or hexapods (Stollewerk & Simpson 2005; Ungerer & Scholtz 2008).

Our data exclude the possibility that ectodermal invaginations, as seen in chelicerate and myriapod embryos, play a role in onychophoran neurogenesis, because such invaginations occur only in the antennal segment. These
invaginations, which give rise to the hypocerebral organs/glands (Eriksson et al. 2005; Mayer et al. 2005), only appear when the brain is at a relatively advanced developmental stage (figure 7a–d) and so cannot play a role in neurogenesis. The absence of segmental invaginations from the onychophoran neuroectoderm thus contrasts with the occurrence of the 'ventral organs' or 'epithelial vesicles' in chelicerates and myriapods, which are incorporated into developing ganglia (Dohle 1964; Weygoldt 1964; Anderson 1973; Whitington et al. 1991; Kadner & Stollewerk 2004; Stollewerk 2004; Whitington 2007).

**Figure 5. Neurogenesis in the onychophoran E. rowelli.** Confocal micrographs; anterior is up in (a–c), median is left in (c–g). (a) Embryo in ventral view showing an antero-posterior progression in neural development. At the anterior end, axonogenesis gives rise to two longitudinal tracts (=future nerve cord neuropils; arrowheads) whereas axons are not seen at the posterior end. Double-labelling with Hoechst (nuclei; blue) and an antibody raised against acetylated α-tubulin (neurotubules; white). (b) Embryo at the same developmental stage as in (a), labelled with phalloidin-rhodamine (f-actin; red). Note the absence of brightly stained dots in the neuroectoderm (NE). (c) Ventral view of the neuroectoderm from the same embryo as in (b), which does not show brightly stained dots representing convergent apical processes of immigrating cell clusters. (d) Cross-section of neuroectoderm with immigrating neuronal precursors, some of which still have a bottle-like shape (arrowheads). Note the basally displaced nuclei of neuronal precursors forming one layer (asterisks) and the columnar shape of large remaining ectodermal nuclei (EC). Double-labelling with phalloidin-rhodamine (red) and Hoechst (blue). Dorsal is up. (e–g) Further subsequent stages of neurogenesis. Optical cross-sections. Dorsal is up. After immigration, neuronal precursors divide mitotically (arrowheads) and the internal nervous tissue (NC) increases in thickness. Note the absence of cell divisions in the developing nervous tissue. Double-labelling with phalloidin-rhodamine (red) and α-PH3 antibody (prophase cells; green). AT, presumptive antenna; CO, coelomic cavity; EC, ectoderm; JW, presumptive jaw; L1, anlage of the first walking leg; LB, limb bud; ME, mesoderm; NC, presumptive nerve cord; NE, neuroectoderm; SP, presumptive slime papilla; VE, ventral extra-embryonic ectoderm. Scale bars, 300 μm (a,b), 50 μm (c), 10 μm (d) and 20 μm (e–g).

### (e) Bipotential fate of the onychophoran neuroectoderm

The fate of the neuroectoderm is another important issue when considering relationships between the arthropods. We have observed that the onychophoran neuroectoderm contributes cells to both nervous tissue and epidermis. During their basal migration within the neuroectoderm, neuronal progenitors are intermingled with other cells with large columnar nuclei (figure 5d), which become even more conspicuous later in development (figure S5a,b, electronic supplementary material). These specialized cells retain their positions within the epidermis and give rise to the so-called ventral organs (Mayer & Whitington in press). Even though the onychophoran ventral organs have been homologized with the homonymous structures in chelicerates and myriapods (Tiegs 1940; Pflugfelder 1948; Anderson 1973), their homology is unsubstantiated as there are no correspondences in their composition, role and embryonic fate (Whitington 2007; Mayer & Whitington in press). Furthermore, there is no embryological evidence that the ventral organs contribute cells to the central nervous system in Onychophora (Sedgwick 1887; von Kennel 1888; Mayer & Whitington in press; but see Eriksson et al. 2003). They, rather, have a different fate and persist as epidermal structures in adults (von Kennel 1888; Mayer 2007). Since the onychophoran neuroectoderm gives rise to both neurons of the nerve cords and epidermal cells (ventral organs), the onychophorans share with crustaceans and hexapods this bipotential fate of the neuroectoderm. This contrasts with the exclusively neural fate of the central area of the neuroectoderm in chelicerates and myriapods (Dove & Stollewerk 2003; Stollewerk 2004).
4. CONCLUSIONS AND PERSPECTIVES

To determine the ancestral versus derived states among different embryological characters, we have used Onychophora as an outgroup. Our data show that a cumulus-like structure does not occur in onychophoran development, which suggests that the cumulus is not an ancestral arthropod character, as proposed previously (McGregor et al. 2008), but rather a unique feature uniting the chelicerates and myriapods (figure 8). In order to further substantiate this hypothesis, expression studies of decapentaplegic (dpp), a gene specifying the dorsal body region, might be helpful. If dpp is expressed along the dorsal body axis in Onychophora, as for example in hexapods (Carroll et al. 2005), this pattern would support the monophyly of the Paradoxopoda, whereas an expression in a cumulus-like group of cells would suggest that this feature is a ground pattern character of Panarthropoda.

Our data revealed that the mode of neurogenesis in onychophorans is more similar to that found in hexapods and crustaceans than that in chelicerates and myriapods as the onychophoran neuroectoderm shows neither post-mitotic cell clusters nor segmental invaginations. In Onychophora, instead, single precursors are recruited for neuronal fate and migrate internally as bottle-like cells, which is similar to the mode found in hexapods (figure 4). These immigrated cells are mitotically active, and in this respect resemble the neuronal stem cells (neuroblasts) of both crustaceans and hexapods (Harzsch 2001; Stollewerk & Simpson 2005; Ungerer & Scholtz 2008), even though they do not show asymmetric cell divisions. Our findings thus suggest that immigration of single cells, followed by their mitotic activity, is an ancestral feature of arthropod neurogenesis, while asymmetric cell divisions are a synapomorphy of crustaceans and hexapods (figure 8). The absence of the following three characters in onychophorans, crustaceans and hexapods suggests that they are derived features uniting the chelicerates and myriapods as sister groups: (i) clusters of immigrating post-mitotic cells; (ii) paired segmental invaginations; and (iii) exclusively neural fate of the central area of the neuroectoderm (figure 8). These findings, in addition to the occurrence of a cumulus in chelicerates and myriapods, argue against the proposed sister-group relationship of onychophorans and chelicerates (Strausfeld et al. 2006).

Our study, based on various lines of evidence and an outgroup comparison, provides, to our knowledge, the first morphological/embryological evidence for joining myriapods with chelicerates as sister taxa and thus challenges the traditional Mandibulata concept. However, since only few morphological data are currently available for either hypothesis (for Mandibulata, see reviews Edgecombe 2004; Scholtz & Edgecombe 2006), it is premature to perform a phylogenetic analysis including these characters. We predict, however, that a targeted search for similarities...
Figure 8. Summary of major findings from study of onychophoran development and polarized characters supporting the monophyly of Paradoxopoda. 1–3, ground pattern of Panarthropoda; 1, segregation and immigration of single cells during neurogenesis; 2, mitotic activity of neuronal precursors; 3, dual nature of neuroectoderm giving rise to both neurons and epidermis; 4, autapomorphy of Pancrustacea: neuronal stem cells, which divide asymmetrically (see Harzsch 2001); 5–8, autapomorphies of Paradoxopoda: 5, immigrating clusters of post-mitotic cells during neurogenesis; 6, segmental invaginations of neuroectoderm; 7, exclusive generation of neurons in central neuroectoderm of each hemisegment; 8, cumulus determining dorsal body region (function of the cumulus-like structure remains to be clarified in myriapods).

between chelicerates and myriapods might reveal additional synapomorphies in future studies, which could well be used for cladistic analyses.

The proposed monophyly of the Paradoxopoda gives a new view of the evolution of the arthropod 'head', as it suggests that the mandible has evolved independently in myriapods and pancrustaceans (crustaceans + hexapods, also called Tetracnata). Similar mandibular organization in these groups (Edgecombe et al. 2003; Edgecombe 2004) may be the result of functional adaptation (Hwang et al. 2001). The alternative view, that the mandible is a plesiomorphy of arthropods (Cook et al. 2001), implies a removal of the mandible from the chewing chamber of the mouth and its reversal to a biramous walking limb in chelicerates. Placement of the myriapods with the chelicerates also suggests that the first antenna has been transformed into the chelicerae of chelicerates (Scholtz & Edgecombe 2006) rather than sensory antennae, we favour the hypothesis of an independent origin of antennae in myriapods and pancrustaceans.

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