

Review article

Segmentation in Tardigrada and diversification of segmental patterns in Panarthropoda

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ABSTRACT

The origin and diversification of segmented metazoan body plans has fascinated biologists for over a century. The superphylum Panarthropoda includes three phyla of segmented animals—Euarthropoda, Onychophora, and Tardigrada. This superphylum includes representatives with relatively simple and representatives with relatively complex segmented body plans. At one extreme of this continuum, euarthropods exhibit an incredible diversity of serially homologous segments. Furthermore, distinct tagmosis patterns are exhibited by different classes of euarthropods. At the other extreme, all tardigrades share a simple segmented body plan that consists of a head and four leg-bearing segments. The modular body plans of panarthropods make them a tractable model for understanding diversification of animal body plans more generally. Here we review results of recent morphological and developmental studies of tardigrade segmentation. These results complement investigations of segmentation processes in other panarthropods and paleontological studies to illuminate the earliest steps in the evolution of panarthropod body plans.

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1. Introduction

1.1. The relationship of Tardigrada to Euarthropoda and Onychophora

The phylum Tardigrada consists of microscopic segmented animals, which are closely allied with the phyla Euarthropoda and Onychophora; together these three phyla make up the superphylum Panarthropoda. Unraveling the relationships of these phyla is critical for elucidating the evolution of segmental patterns and segmentation processes across Panarthropoda. However, these relationships remain debated. Morphological analyses based on extant Panarthropods and fossil remains of both crown and stem-group representatives of the panarthropod phyla have been performed. Some morphological analyses recover Tardigrada as the sister group of Euarthropoda (Fig. 1A; Budd, 1996; Smith and Ortega-Hernández, 2014; Smith and Caron, 2015; Murdock et al., 2016; Yang et al., 2016); the hypothesized tardigrade-euarthropod clade has been referred to as Tactopoda (Budd, 2001a,b). Trunk ganglia and a stomatogastric ganglion have been suggested to

represent synapomorphies of a tardigrade-euarthropod clade (Mayer et al., 2013a,b; Yang et al., 2016; see Sections 2.5 and 3.4). Other analyses recover Tardigrada as the sister group of Onychophora (Fig. 1B; Liu et al., 2011; Ma et al., 2014); this hypothesized clade has been referred to as Lobopodia (Dzik and Krumbiegel, 1989), although Lobopodia is often used in other contexts (Ortega-Hernández, 2014). The last possible topology, with Onychophora as the sister group of Euarthropoda, has also found support (Fig. 1C; Ma et al., 2009; Legg et al., 2012, 2013; Legg and Vannier, 2013). The hypothesized onychophoran-euarthropod clade has been referred to as Antennopoda (de Haro, 1998), although this designation has been used minimally, possibly because the euarthropod antenna and the antenna-like onychophoran frontal appendage are not direct segmental homologs (Eriksson and Budd, 2000; Eriksson et al., 2010). Under the Antennopoda hypothesis, Tardigrada was the first phylum to diverge within Panarthropoda.

Results of molecular analyses have been even more variable than morphological analyses. Some molecular analyses have even challenged the monophyly of Panarthropoda by supporting a clade consisting of tardigrades and nematodes (Philippe et al., 2005; Roeding et al., 2007, 2009; Lartillot and Philippe, 2008; Meusemann et al., 2010; Andrew, 2011). Equally surprising, an early molecular analysis placed Tardigrada within Euarthropoda

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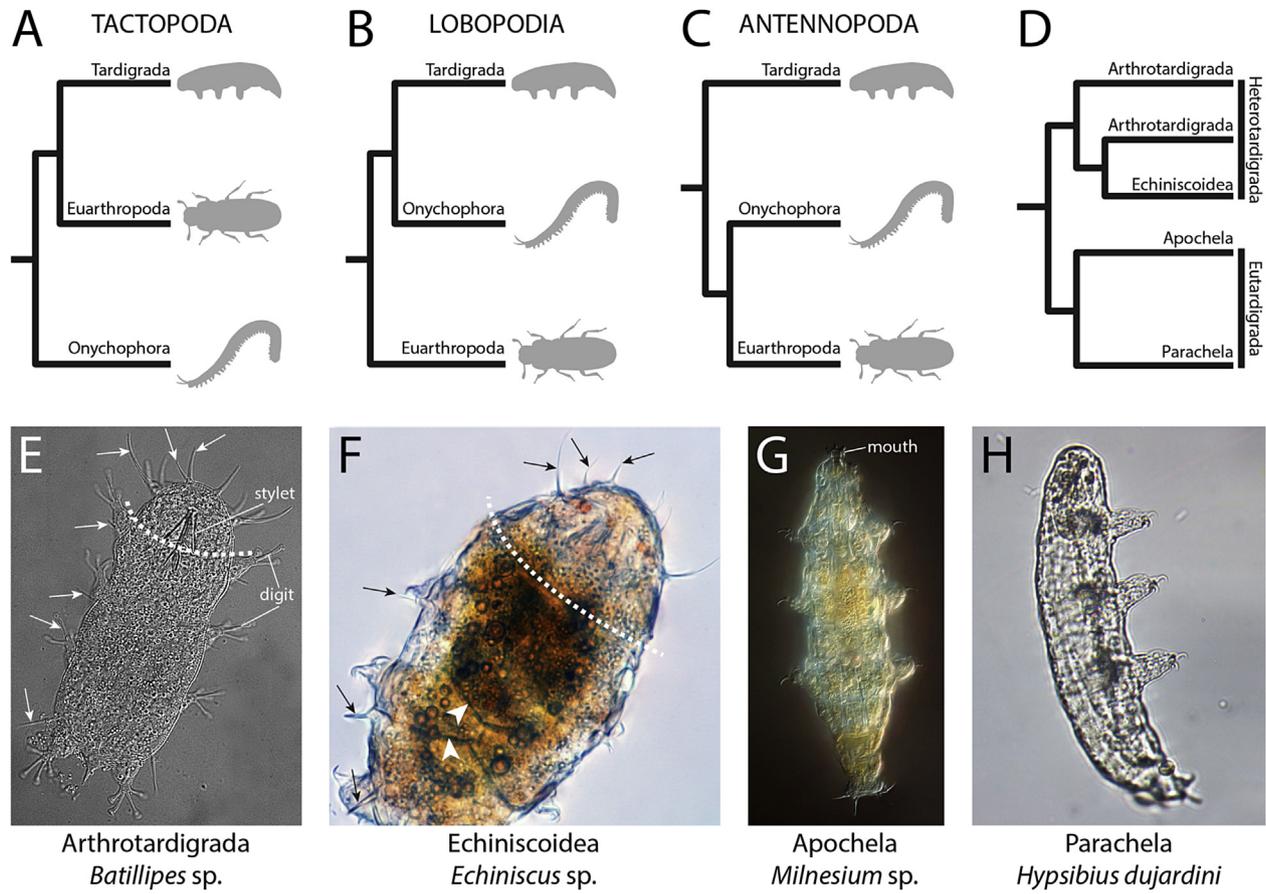


Fig. 1. Tardigrade phylogeny and diversity. (A–C) Different phylogenetic hypotheses for the relationships among panarthropod phyla. The names of hypotheses are provided above the phylogenies (Section 1.1). (D) Phylogeny of Tardigrada based on recent molecular analyses (Jørgensen et al., 2010; Guil and Giribet, 2012; Bertolani et al., 2014). Note that the order Arthrotardigrada comes out as paraphyletic in molecular analyses. (E–H) Representatives of tardigrade orders. (E, F) Dorsal view. Arrows point to cirri. Dashed line denotes estimate of head-trunk boundary. (F) Arrowheads denote in focus junctions between cuticular plates. (G) Ventral view. The everted mouth is labeled. (H) Lateral view. (E–G) By Thomas C. Boothby, used with permission.

(Aguinaldo et al., 1997), albeit with low support. The surprising relationships recovered by these molecular analyses are likely due to long branch attraction artifacts (Philippe et al., 2005; Rota-Stabelli et al., 2010). In recent phylogenomic analyses that are designed to deal with these potential artifacts, Panarthropoda is recovered as monophyletic, with good support. These analyses support either the Lobopodia hypothesis (Fig. 1B; Rota-Stabelli et al., 2010, 2011), or the Antennopoda hypothesis (Fig. 1C; Campbell et al., 2011; Borner et al., 2014). Given the prevalent skepticism regarding panarthropod phylogeny, in this review, where pertinent, we will present opposing character state reconstructions based on distinct phylogenetic hypotheses.

1.2. The invariant body plan of Tardigrada

Extant tardigrades include more than 1300 described species (Guidetti and Bertolani, 2005; Degma and Guidetti, 2007; Guil and Giribet, 2012; Degma et al., 2015), which are split into two classes—Eutardigrada and Heterotardigrada (Fig. 1D; Jørgensen et al., 2010; Guil and Giribet, 2012; Bertolani et al., 2014). Tardigrades of both classes share a conserved body plan that consists of a head and four leg-bearing trunk segments (Fig. 1E–H). This is a surprising degree of body plan conservation, given that these classes have ancient, possibly Precambrian, origins (Regier et al., 2004; Rota-Stabelli et al., 2013). The last common ancestor of Panarthropoda

is thought to have been composed of a head followed by a suite of homonomous leg-bearing trunk segments (Ma et al., 2009; Strausfeld, 2012). By contrast, representatives of Euarthropoda and Onychophora exhibit heteronomous segmental patterns, with anterior appendages serving sensory, prey capture, or food processing functions. Furthermore, different euarthropod classes exhibit distinct tagmosis patterns. There is general agreement that the heteronomous segmental patterns exhibited by onychophorans and euarthropods are derived relative to the panarthropod common ancestor (Ma et al., 2009; Eriksson et al., 2010). However, the degree to which the relatively homonomous segmented body plan of Tardigrada is conserved or derived relative to the panarthropod common ancestor remains highly debated (see Section 3).

1.3. The problem: how do tardigrade segments relate to segments of other panarthropods?

In principle, the differences in the degree to which the panarthropod phyla have diverged from their last common ancestor should facilitate research that focuses on how segmental diversity arose in this lineage. In order to formulate a coherent model of panarthropod segment diversification, first we need a clear understanding of the evolutionary relationships of segments across this lineage. A consensus is beginning to emerge regarding the relationship of segments among representatives of the disparate

euarthropod classes and between representatives of the euarthropod and onychophoran phyla (Damen et al., 1998; Telford and Thomas, 1998; Jager et al., 2006; Eriksson et al., 2010). However, several conflicting models exist regarding the relationship of tardigrade segments to those of other panarthropods. Here, we review the general morphological aspects of segmental organ systems in Tardigrada (Section 2), different models of the relationship of tardigrade segments to segments in other panarthropods (Section 3), and recent studies of tardigrade development that weigh-in on how tardigrade segments relate to those of other panarthropods (Section 4). We end with a model regarding the evolution of segmented body plans in Panarthropoda, based in part on our investigations of tardigrade development (Section 5).

2. Segmental organ systems of Tardigrada

2.1. What is a segment?

Defining a segment has been notoriously difficult (Budd, 2001b; Scholtz, 2002; Hannibal and Patel, 2013); disagreements arise concerning the suite of organ systems that must share repeated morphological structure in order for an animal to be considered truly segmented. For understanding the origin and diversification of body plans, Budd (2001b) argues that segmentation is best viewed as a property of individual organ systems, which circumvents debates about what constitutes a true segment and which animals are truly segmented. We agree with this view of segmentation. With this in mind, when we refer to a segment, we are referring to the sum of segmented organ systems that comprise a metameric unit. In the archetypal segmented animals, the euarthropods, the epidermis (in terms of visible segment boundaries), the nervous system (in terms of ganglia), the excretory system (in terms of nephridia), the muscle system, and appendages are segmental (Scholtz, 2002). In this section, we compare the segmented organ systems of tardigrades to those of other panarthropods, and discuss their implications for panarthropod body plan evolution.

2.2. The missing segmental components of tardigrades

As presented below, some organ systems that exhibit segmentation in the panarthropod relatives of tardigrades are also segmented in tardigrades. It is likely that these organ systems were also segmented in the panarthropod ancestor. However, there are cases where tardigrades differ from other panarthropods, which, in principle, could make it difficult to reconstruct panarthropod ancestral states. In the first case, in euarthropods and onychophorans, a pair of nephridia—organs involved in waste removal—is typically associated with each segment (Scholtz, 2002; Mayer and Koch, 2005). By contrast, tardigrades completely lack nephridia. In the second case, segments of onychophorans and euarthropods typically contain a coelomic sack (Schmidt-Rhaesa, 2001; Scholtz, 2002), at least during some ontogenetic stage (Budd, 2001b). By contrast, the only possible remnant of a true coelom in tardigrades is the mesodermally lined gonadal cavity (Dewel and Dewel, 1998); the main tardigrade body cavity is considered to be an unsegmented pseudocoelom.

Two explanations exist for the lack of these segmental organ systems in tardigrades (Schmidt-Rhaesa, 2001). First, small body size could be a synapomorphy of Tardigrada, with the loss of both nephridia and coelomic sacks associated with size reduction; for example, nephridia may not be required for waste removal in very small animals. Second, nephridia and segmental coelomic sacks could be a synapomorphy of the lineage leading to Euarthropoda and Onychophora. Distinguishing between these possibilities has

been difficult. On one hand, non-panarthropod phyla within Ecdysozoa also lack these structures (Schmidt-Rhaesa, 2001), suggesting that they could indeed be synapomorphies of a euarthropod-onychophoran clade. On the other hand, nephridia and coeloms are common features of more distantly related invertebrate phyla. If nephridia and coeloms are homologous between euarthropods/onychophorans and more distantly related phyla, as has been suggested (Scholtz, 2002), then these features must have been lost in the tardigrade lineage. Recent phylogenetic analyses suggest that a relatively large Cambrian lobopod, *Onychodictyon ferox*, is a stem-group tardigrade (Smith and Ortega-Hernández, 2014; Smith and Caron, 2015); this relationship supports small body size as a synapomorphy of Tardigrada, and allows for the possibility that size reduction preceded the loss of nephridia and segmental coelomic sacks in the tardigrade lineage.

2.3. The segmented cuticle of tardigrades

The tardigrade cuticle exhibits signs of segmentation in heterotardigrades, but not typically in eutardigrades, although the eutardigrade *Ramazzottius tribulosus* exhibits transversal brown-red lines in the epidermis (Bertolani and Rebecchi, 1988), which could be interpreted as evidence of epidermal segmentation. Within heterotardigrades, the dorsal cuticle is typically arranged into segmental cuticular plates, while the ventral cuticle more rarely exhibits this arrangement (Fig. 1F; Kristensen and Higgins, 1984). Junctions between plates may be homologous to the epidermal segment boundaries of euarthropods (Budd, 2001b). Furthermore, many heterotardigrade species exhibit segmentally reiterated cuticular spines (cirri), emanating from the body and/or legs (Fig. 1E, F; Kristensen and Higgins, 1984). Although eutardigrades lack them (Fig. 1G, H), segmental cuticular plates may be a plesiomorphy of Tardigrada (Kristensen and Higgins, 1984), sharing homology with sclerotized plates of some Cambrian lobopods (Dewel and Dewel, 1998). If so, then cuticular plates must have been lost in the stem-lineage leading to Eutardigrada. The loss of cuticular plates is not without precedent in Tardigrada; within Heterotardigrada, cuticular plates are absent in members of the family Halechiniscidae (Kristensen and Higgins, 1984). Given that this family is polyphyletic (Jørgensen et al., 2010), cuticular plates were most likely lost independently in the multiple evolutionarily distinct lineages of Halechiniscidae.

2.4. The muscle system of tardigrades

The muscle system of tardigrades is primarily composed of a series of muscle strands that extend between attachment points (Fig. 2A). Muscle strands are typically composed of one or a few muscle cells (Walz, 1974). Most muscle strands extend between epidermal attachment points (Schmidt-Rhaesa and Kulesa, 2007; Halberg et al., 2009; Schulze and Schmidt-Rhaesa, 2011; Halberg et al., 2013; Marchioro et al., 2013; Smith and Jockusch, 2014); these muscles are referred to as somatic muscles. Somatic muscles function in locomotion or bending of the body by working against the fluid-filled pseudocoelom, which functions as a hydrostatic skeleton. Visceral muscle strands function in feeding or digestion. Visceral muscles involved in feeding include the pharynx and stylet muscles (sm, Fig. 2A) associated with the tooth-like stylets (Schmidt-Rhaesa and Kulesa, 2007; Halberg et al., 2009; Guidetti et al., 2013; Smith and Jockusch, 2014). Several additional visceral muscles are found in the gut cavity, and probably play roles in digestion (Schmidt-Rhaesa and Kulesa, 2007; Smith and Jockusch, 2014).

The muscle system of Tardigrada has diverged in an interesting mosaic pattern, with the dorsal muscle group exhibiting the most

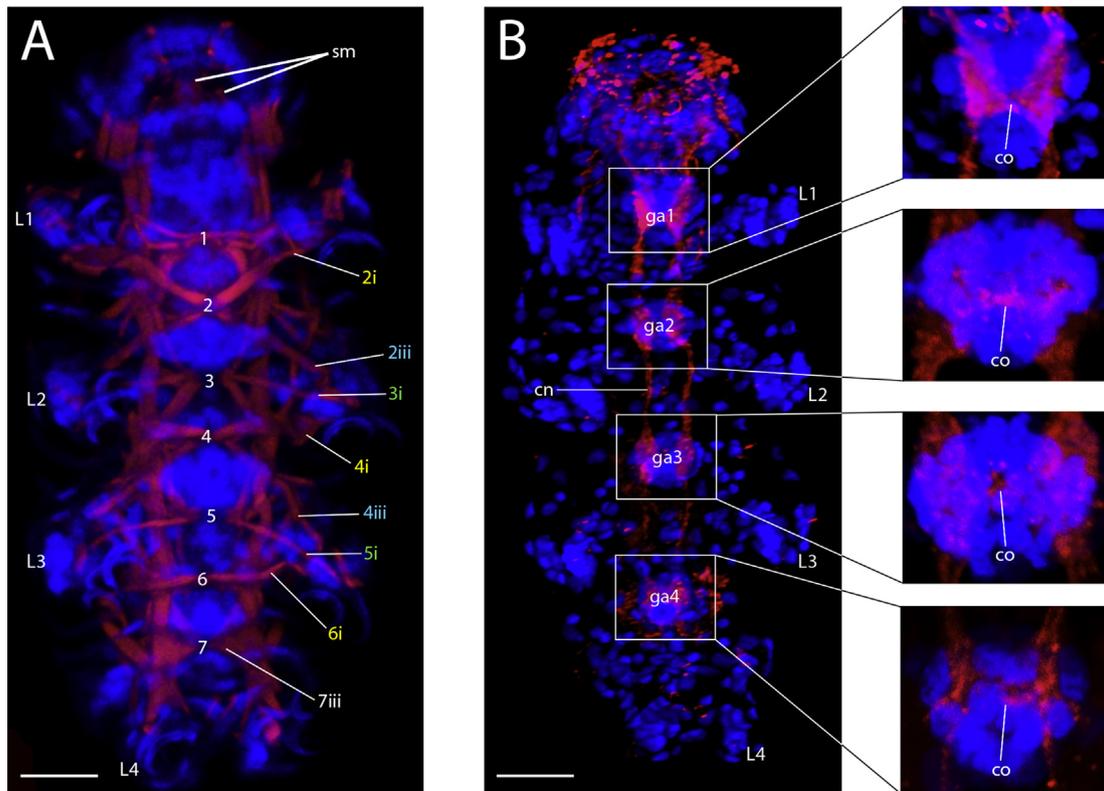


Fig. 2. Segmental organ systems of Tardigrada. All panels show ImageJ Volume Viewer renderings of laser-scanning confocal Z-series (data collected as in Smith et al., 2016). DAPI (blue) stains nuclei. Scale bar = 10 μ m. (A) The ventral muscle system of *Hypsibius dujardini*. Alexa flour 488 conjugated phalloidin (red) was used to visualize muscles. Ventral attachment sites are numbered (1–7). Leg muscles are labeled according to Smith and Jockusch (2014). Serially homologous leg muscles are labeled in the same color; note the similarity between leg-pairs 2 and 3. (B) The nervous system of *H. dujardini* (ventral view). An anti- β -tubulin antibody (red) was used to visualize the nervous system. Panels to the right show individual ganglia. Abbreviations: cn, connective; co, commissure; ga1–ga4, ganglion 1–ganglion 4; L1–L4, leg 1–4; sm, stylet muscles.

variation, the lateral muscle group exhibiting less variation, and the ventral muscle group, which includes leg muscles, exhibiting very little variation (Marchioro et al., 2013). The extensive variation in the architecture of the dorsal muscle group is likely related to variation in the presence of dorsal cuticular plates (Marchioro et al., 2013). Some heterotardigrades possess thick dorsal cuticular plates, which provide reinforced attachment points for a relatively few thick muscles; by contrast, eutardigrades lack cuticular plates, and exhibit many more dorsal attachment points for correspondingly thinner muscles (Marchioro et al., 2013).

In tardigrades, the leg muscles are the most obvious segmental component of the muscle system (Fig. 2A). It has been suggested that the stylets of the bucco-pharyngeal apparatus and their associated muscles are homologous to leg claws and leg muscles (Nielsen, 2001), which is supported by the fact that only leg and stylet muscles are cross-striated (Halberg et al., 2009). If so, then stylet muscles (sm, Fig. 2A) might represent the segmental component of tardigrade head musculature. Leg musculature is fairly conserved across Tardigrada, and is organized in an intriguing heteronomous pattern (Schmidt-Rhaesa and Kulesa, 2007; Halberg et al., 2009; Schulze and Schmidt-Rhaesa, 2011; Marchioro et al., 2013; Smith and Jockusch, 2014). Within any given tardigrade, the leg musculature of the second and third leg pairs is nearly identical. The leg musculature of the first leg pair is moderately divergent compared to that of the more posterior two leg pairs. The posterior-most leg pair is the most divergent, in terms of muscle anatomy, with the most obvious differences being that the posterior legs house far fewer muscles than the more anterior legs, and they are rotated posteriorly relative to the more anterior

legs. This heteronomous pattern likely reflects the degree of similarity in locomotive function among leg pairs. It is likely, based on comparisons of leg musculature between species, that the last common ancestor of crown group Tardigrada already exhibited the differentiated segmental leg musculature characteristic of extant tardigrades (Marchioro et al., 2013; Smith and Jockusch, 2014). However, this pattern is most likely specific to Tardigrada; if so, then it cannot be used to homologize tardigrade segments to segments of other panarthropods.

2.5. The nervous system of tardigrades

The central nervous system of Tardigrada is composed of a brain and four ventral trunk ganglia—one ganglion per trunk segment (Fig. 2B; ga1–ga4). The four trunk ganglia are positioned just anterior to each corresponding leg pair, in a pattern that has been described as parasegmental (Mayer et al., 2013b). Each trunk ganglion is morphologically unique (Fig. 2B); they are composed of different numbers of cells (Zantke et al., 2008), and exhibit unique patterns of neuropeptide expression (Mayer et al., 2013b). Ganglia are composed of paired hemiganglia, i.e., clusters of neuronal cell bodies. From each hemiganglion, tubulin rich connectives (cn), presumably composed of axons, extend to hemiganglia in neighboring segments (Fig. 2B). Interpedal commissures extend between the paired connectives (Mayer et al., 2013b). Inner (ic) and outer (oc) connectives extend from the hemiganglia of the first trunk segment to the brain (Fig. 3A). Nerves also extend from each hemiganglion into its corresponding leg, while each leg includes a cluster of neuronal cell bodies that is referred to as a leg ganglion

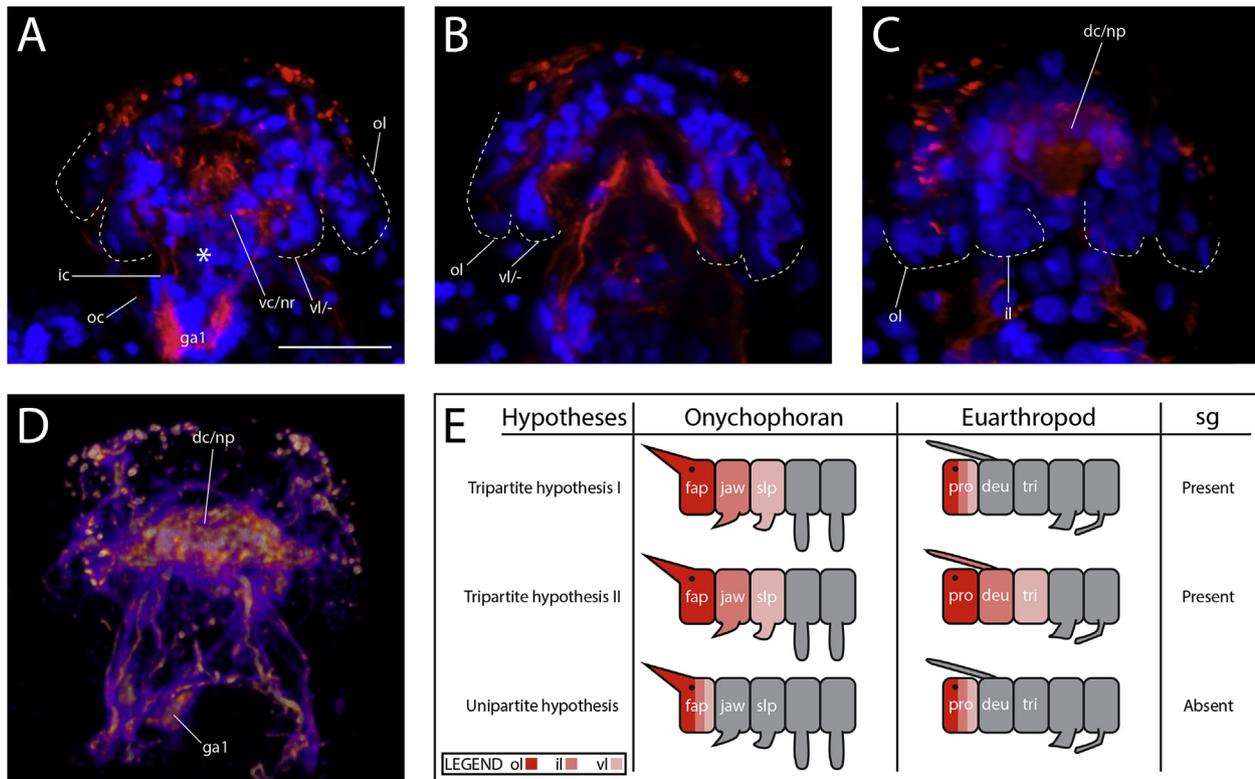


Fig. 3. The brain of *Hypsibius dujardini*. (A–D) ImageJ Volume Viewer renderings of laser-scanning confocal Z-series (data collected as in Smith et al., 2016). Opposing interpretations are given for some brain structures (see Section 3). (A–C) An anti- β -tubulin antibody (red) stains the nervous system. DAPI (blue) stains nuclei. (A) Ventral section. The asterisk marks the predicted position of a subpharyngeal ganglion (Persson et al., 2012; Persson et al., 2014), which is not apparent in *H. dujardini*, *Macrobiotus* (Zantke et al., 2008; Mayer et al., 2013b), or several heterotardigrade species (Schulze and Schmidt-Rhaesa, 2013; Schulze et al., 2014). Ventral lobes (vl) are apparent in *H. dujardini* and *H. crispae* (Persson et al., 2012), but not in *Macrobiotus* (-). Scale bar = 10 μ m. (B) Intermediate section. (C) Dorsal section. (D) Dorsal view of brain stained with an anti- β -tubulin antibody. Image was rendered using Fire LUT Transfer Function in ImageJ. (E) Hypotheses for the segmental composition of the tardigrade head. Segments of an onychophoran and a euarthropod (Diplopoda) are colored based on their homology to the segments hypothesized to have given rise to the brain lobes of tardigrades (see references in Section 3). For each hypothesis, we label whether a subpharyngeal/subesophogeal ganglion is present (sg column). Abbreviations: dc, dorsal commissure; deu, deutocerebral segment; fap, frontal appendage-bearing segment; ga1, first trunk ganglion; ic, inner connective; il, inner lobe; jaw, jaw-bearing segment; np, central brain neuropil; nr, nerve ring; oc, outer connective; ol, outer lobe; pro, protocerebral segment; sg, subpharyngeal/subesophogeal ganglion; slp, slime papilla-bearing segment; tri, tritocerebral segment; vc, ventral commissure; vl, ventral lobe.

(Persson et al., 2012; Mayer et al., 2013b). Within each trunk ganglion, tubulin rich commissures (co) connect each hemiganglion to its contralateral neighbor (Fig. 2B). The connectives between ganglia and the commissures within give the ventral nervous system of Tardigrada a rope ladder-like appearance (Mayer et al., 2013b; Smith and Jockusch, 2014).

Like tardigrades, euarthropods exhibit a rope ladder-like ventral nervous system. By contrast, Onychophora lack trunk ganglia (Mayer and Harzsch, 2007; Mayer and Whittington, 2009). It has been suggested that a rope ladder-like nervous system is a synapomorphy of a monophyletic euarthropod-tardigrade clade (Mayer et al., 2013b; Yang et al., 2016). In contrast to this view, a proposed onychophoran stem-group, *Paucipodia inermis* (Smith and Ortega-Hernández, 2014; Smith and Caron, 2015), has been proposed to have possessed trunk ganglia (Hou et al., 2004; see also Edgecombe et al., 2015). If so, then a rope ladder-like nervous system would not be a synapomorphy of a euarthropod-tardigrade clade; instead, this architecture could be a plesiomorphy of Panarthropoda, having been lost in crown group Onychophora. However, skepticism exists regarding the neuronal identity of the presumptive trunk ganglia in *P. inermis* (Smith and Ortega-Hernández, 2014; see also Edgecombe et al., 2015), leaving open the possibility that, like crown group representatives, stem-group onychophorans lacked trunk ganglia. In sum, recent morphological analyses have concluded that trunk ganglia are a

synapomorphy of a tardigrade-euarthropod clade (i.e., Tactopoda; Mayer et al., 2013b; Yang et al., 2016), a conclusion that is at odds with recent molecular analyses, which do not support the Tactopoda hypotheses (Rota-Stabelli et al., 2010, 2011; Campbell et al., 2011; Borner et al., 2014). Until a consensus has been reached regarding the relationship of the panarthropod phyla, reconstructions of the evolution of ganglionated nervous system architecture within Panarthropoda will likely remain contentious.

3. Evolutionary models for the segmental composition of the tardigrade head

3.1. The enigmatic tardigrade brain

Brain morphology has been investigated in several representatives of each class of Tardigrada, most recently by combining immunohistochemistry to visualize neuronal peptides with laser-scanning confocal microscopy (Zantke et al., 2008; Persson et al., 2012; Mayer et al., 2013a; Schulze and Schmidt-Rhaesa, 2013; Persson et al., 2014; Schulze et al., 2014; Smith and Jockusch, 2014). Tardigrade brains appear to be composed of a series of lobes—dense clusters of cells (Fig. 3A–C), and brain lobes have been homologized across the phylum (Persson et al., 2014; Smith and Jockusch, 2014). While there is little debate concerning the lobe-like structure of the tardigrade brain, the nature of brain lobes

is highly debated. One suite of hypotheses suggests that brain lobes represent modified trunk ganglia. If so, then the tardigrade brain has a multi-segmented composition. Alternatively, it has been suggested that the tardigrade brain has a single-segment origin. Distinguishing between these possibilities would have important implications for our understanding of the degree of cephalization exhibited by the last common ancestor of Panarthropoda. Below we summarize available hypotheses and present the arguments favoring or contradicting these hypotheses.

3.2. Tripartite hypothesis I

Hypotheses regarding the segmental composition of the tardigrade head can be broken down into two categories. In the first category are hypotheses that predict that the head is composed of multiple segments; in general, these hypotheses also predict the existence of a subpharyngeal ganglion between the brain and first trunk ganglion (Fig. 3E; Kristensen, 1983; Dewel and Dewel, 1996; Nielsen, 2001; Persson et al., 2012, 2014). In one hypothesis (tripartite hypothesis I), the tardigrade brain is homologous to the protocerebrum of euarthropods (Dewel and Dewel, 1996). Typically, the arthropod protocerebrum is thought to be constructed of a ganglion from a single segment (reviewed in Strausfeld, 2012). Intriguingly, tripartite hypothesis I predicts that both the tardigrade brain and euarthropod protocerebrum are composed of ganglia from three segments. In this interpretation, the tardigrade head and the euarthropod protocerebrum are homologous to the anterior three segments of Onychophora (Fig. 3E). This hypothesis is primarily based on comparisons of the neural anatomy associated with cephalic sense organs of a heterotardigrade (*Echiniscus viridissimus*) to brain anatomy in arthropods (Dewel and Dewel, 1996).

3.3. Tripartite hypothesis II

A second hypothesis (tripartite hypothesis II) agrees with tripartite hypothesis I that the tardigrade head is composed of multiple segments, but disagrees on the affinities of these segments to those of other panarthropods (Kristensen, 1983; Nielsen, 2001; Persson et al., 2012, 2014). In this hypothesis, the tardigrade brain is homologous to the proto-, deuto-, and tritocerebral ganglia of euarthropods (Fig. 3E), rather than being homologous to just the protocerebral ganglion as predicted by tripartite hypothesis I. Furthermore, in this hypothesis, the proto-, deuto-, and tritocerebral segments align one-to-one with the first three segments of Onychophora, rather than just the protocerebrum aligning with these segments as predicted by tripartite hypothesis I. This hypothesis is based on the idea that brain lobe pairs represent evolutionarily modified trunk ganglia. In favor of this interpretation, serotonergic immunolabeling marks trunk ganglia and brain lobes, and tubulin dense extensions traverse contralateral brain lobes (dc, vc) in a pattern reminiscent of ganglion commissures (Fig. 3A,C,D; Persson et al., 2012, 2014).

3.4. Unipartite hypothesis

In contrast to the tripartite hypotheses, it has been argued that the tardigrade head is composed of a single segment (unipartite hypothesis). In this interpretation (Fig. 3E), the tardigrade head is homologous to the protocerebrum of euarthropods and the anteriormost segment of onychophorans (Ou et al., 2012; Mayer et al., 2013a). Furthermore, adherents of this model generally question the existence of a subpharyngeal ganglion in the tardigrade nervous system (Fig. 3E; Zantke et al., 2008; Mayer et al., 2013a; Schulze and Schmidt-Rhaesa, 2013; Schulze et al., 2014; Smith and Jockusch, 2014). The unipartite hypothesis is based on several challenges to the

notion that each brain lobe-pair in tardigrades represents a modified trunk ganglion (Zantke et al., 2008; Mayer et al., 2013a; Schulze and Schmidt-Rhaesa, 2013; Schulze et al., 2014). For example, rather than representing ganglion-associated commissures, it has been suggested that the prospective dorsal (dc) and ventral (vc) brain commissures represent neuropil (np) and part of a circumesophageal nerve ring (nr), respectively (Fig. 3A,C,D; Mayer et al., 2013a). Additionally, Mayer et al. (2013a) identified a structure in *Macrobotus cf. harmsworthi*, with possible homology to a structure associated with the tritocerebrum of mandibulate euarthropods—the stomatogastric ganglion. The prospective stomatogastric ganglion of *Macrobotus cf. harmsworthi* is innervated by the second trunk ganglion, as expected if this ganglion is directly homologous to the tritocerebrum, which is a prediction of the unipartite brain hypothesis (Mayer et al., 2013a). However, a stomatogastric ganglion has not been identified in other tardigrade species (Schulze et al., 2014; Smith and Jockusch, 2014). Identification of a homolog of this structure in additional tardigrade species would strengthen the case for its utility in inferring segment homologies between Tardigrada and Euarthropoda (Smith and Jockusch, 2014).

4. An evolutionary developmental biology (evo-devo) solution to the tardigrade head problem

4.1. An old problem with a new solution

The three models regarding the segmental composition of the tardigrade head disagree on how many segments comprise the tardigrade head and/or how the head is related to segments in other panarthropods; these disagreements emerge from different evolutionary interpretations of brain morphology. The tardigrade head problem mirrors disagreements that underlie a classic, century old, zoological dispute referred to as the arthropod head problem (see Rempel, 1975); in this case, researchers disagreed about the number of segments that comprise euarthropod heads, how head segments of different euarthropod classes are related, and the nature of the euarthropod labrum (Rempel, 1975; reviewed in Scholtz and Edgecombe, 2006). Recently, an evo-devo approach has allowed researchers to test different hypotheses regarding the segmental composition of the euarthropod head in a manner that is nearly independent of morphological interpretations. In one example, Engrailed expression was found to mark the posterior border of segments during euarthropod development (Patel et al., 1989; Fleig, 1990, 1994; Scholtz, 1994; Damen, 2002; Hughes and Kaufman, 2002; Chipman et al., 2004; Janssen et al., 2004); three Engrailed stripes traverse the developing euarthropod brain, supporting a model in which the euarthropod brain is composed of ganglia from three segments (Scholtz and Edgecombe, 2006). In a second example, segment alignments based on embryonic expression patterns of Hox genes support a model in which the cheliceral segment in Chelicerata is homologous to the antennal segment in Mandibulata (Damen et al., 1998; Telford and Thomas, 1998), rather than supporting a more traditional view, which proposed that chelicerates lack a homolog of the antennal segment (Weygoldt, 1985). In a third example, results of investigations of Hox genes and *six3* in the onychophoran *Euperipatoides kanangrensis* support a compelling evolutionary model for the origin of the mysterious euarthropod labrum; these studies suggest that the labrum evolved from an appendage pair homologous to the onychophoran frontal appendages (Eriksson et al., 2010, 2013). By utilizing an evo-devo approach, researchers have made significant advances towards resolving the arthropod head problem, after more than a century of debate. In this section, we discuss the implications of recent results of developmental studies in tardigrades for the tardigrade head problem.

4.2. Segment embryogenesis

Embryogenesis has been recently studied in two tardigrade species—*Hypsibius dujardini* and *Thulinus stephaniae*. During tardigrade development, segments appear nearly simultaneously (Hejnal and Schnabel, 2005). During *H. dujardini* embryogenesis, the first clear evidence of segmentation appears in the form of four pouches (compare Fig. 4A and B)—one for each trunk segment (Gabriel and Goldstein, 2007; Gabriel et al., 2007). Pouches appear shortly after elongation of the anteroposterior body axis and primarily give rise to the gut (Gabriel and Goldstein, 2007). During *T. stephaniae* embryogenesis (Hejnal and Schnabel, 2005), mesodermal bands appear to the left and right of the developing gut (Fig. 4B); these bands later form somites near the developing limb buds (Fig. 4D). Although clear morphologically defined, segment boundaries are not visible in the epidermis of adult *H. dujardini*; during development, segment boundaries do become apparent as indentations in the ectoderm shortly after endomesodermal pouches appear (Gabriel et al., 2007). An ectodermal indentation can be detected between the head and the first trunk segment, and between the four trunk segments (Fig. 4C). After the appearance of ectodermal indentations, leg buds appear, and later the brain and ganglia become apparent (Fig. 4E; Gabriel et al., 2007). Interestingly, unlike other segmental structures in tardigrades, the brain

and trunk ganglia develop in anterior-to-posterior order (Gross and Mayer, 2015).

The embryological studies discussed above do not support a multi-segmented origin for the tardigrade head. For example, although ectodermal indentations appear between each trunk segment and between the head and the first trunk segment (Gabriel et al., 2007), they do not appear within the head during development as might be expected if the head were composed of multiple segments. Furthermore, only a single brain commissure is detected during development in the tardigrade *H. dujardini* (Gross and Mayer, 2015), rather than the several commissures expected if the brain were composed of ganglia from multiple segments.

4.3. Expression patterns of a paired box ortholog(s) and Engrailed

Although results of recent studies of tardigrade embryogenesis do not support the existence of a tripartite tardigrade brain, it may be difficult to detect distinct head segments by utilizing embryological methods. With this in mind, the expression patterns of segmentation genes in *H. dujardini* have been suggested to provide independent support for the unipartite hypothesis (Zantke et al., 2008; Mayer et al., 2013a). An antibody that detects a subset of Paired box proteins in euarthropods (Davis et al., 2005) marks the developing ganglia and brain in *H. dujardini* (Gabriel and Goldstein,

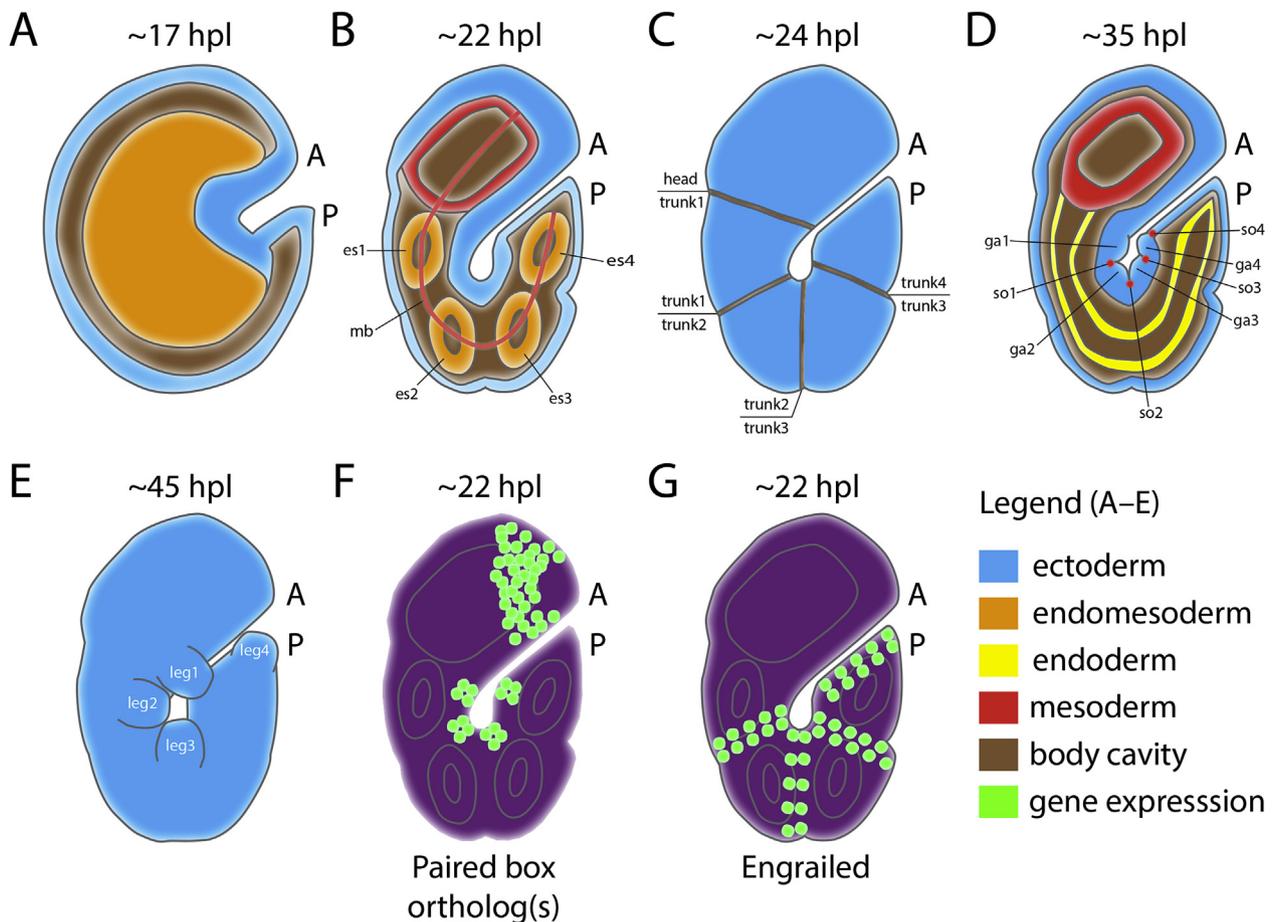


Fig. 4. Development of segments in tardigrades. (A–E) Based primarily on description of *Hypsibius dujardini* development (Gabriel et al., 2007). (B) The endomesodermal identity of the sacks (es1–es4) is based on the interpretation of Gabriel and Goldstein (2007). Mesodermal bands (mb) were detected to the left and right side of the developing gut in the eutardigrade *Thulinus stephaniae* (Hejnal and Schnabel, 2005). (C) Ectodermal indentations mark the boundaries between segments. (D) Mesodermal somites (so) develop above the position of the prospective limb anlagen in *T. stephaniae* (Hejnal and Schnabel, 2005). (F, G) Based on antibody staining (Gabriel and Goldstein, 2007). (F) The expression pattern shown was originally attributed to a Pax3/7 ortholog. However, it is unclear which Paired box ortholog(s) were actually detected in this study, because the cross-reactive antibody used detects several distinct Paired box orthologs in euarthropods (Davis et al., 2005).

2007). A single expression domain marks the developing brain of *H. dujardini* (Fig. 4F), a fact that argues against a multi-ganglionic origin of the tardigrade brain (Zantke et al., 2008; Mayer et al., 2013a). Furthermore, Engrailed is expressed in the posterior ectoderm of each trunk segment during segmentation in *H. dujardini* (Fig. 4G), while its expression in the head is restricted to a later period, after segmental expression has ceased (Gabriel and Goldstein, 2007). This pattern has been taken as evidence against the tripartite hypotheses (Zantke et al., 2008; Mayer et al., 2013a; Gross and Mayer, 2015), i.e., these hypotheses predict that Engrailed should be expressed in multiple stripes in the developing tardigrade head, which does not appear to be the case in *H. dujardini*.

There are important caveats to consider before drawing strong conclusions about the composition of the tardigrade head based on the expression patterns of segmentation genes. First, regarding these genes, a brain composed of multiple conjoined trunk ganglia in tardigrades might have evolved via reduction in the number of discrete anterior expression domains and/or the union between previously discrete expression domains. If so, then the number of discrete expression domains of these genes would not reflect the number of segments that comprise the brain. This possibility highlights the fact that morphological evidence and developmental evidence should not necessarily be weighed independently in favor or against an evolutionary hypothesis. Second, a stripe of Engrailed expression was not detected between the developing head and the first trunk segment of *H. dujardini* (Gabriel and Goldstein, 2007), although all hypotheses agree that a true segmental boundary exists at this position. The fact that an Engrailed stripe is not associated with this segment boundary suggests that it may be an imperfect molecular marker of segment boundaries in *H. dujardini*. Therefore, the absence of Engrailed stripes within the head is not necessarily indicative of a single segment origin of the tardigrade head.

4.4. Expression patterns of Hox genes and orthodenticle

Drawing conclusions about the segmental composition of the tardigrade brain based on brain morphology or the expression of segmentation genes in the brain has been problematic (Section 3, 4.3). With this in mind, we recently set out to infer the relationship of tardigrade segments with those of other panarthropods based on comparisons of the expression patterns of Hox genes. Hox genes are expressed downstream of segmentation genes and typically specify segment identities (reviewed in Hughes and Kaufman, 2002). Their expression patterns have been used to homologize segments of different panarthropods, even in cases where segment morphologies are highly divergent (Damen et al., 1998; Telford and Thomas, 1998; Jager et al., 2006; Eriksson et al., 2010). We found that the anterior segmental expression boundaries of the *H. dujardini* orthologs of the Hox genes *Hox3*, *Deformed*, and *fushi tarazu*, align near perfectly with their anterior segmental expression boundaries in euarthropods and onychophorans, when tardigrade segments are aligned one-to-one in anteroposterior order with arthropod and onychophoran segments (Smith et al., 2016). This alignment supports the unipartite hypothesis for the composition of the tardigrade head (Fig. 5A and B). By contrast, based on the tripartite hypotheses, predicted expression domains of Hox genes are out of register with observed expression patterns (Fig. 5C and D).

To further test the unipartite hypothesis, we investigated the embryonic expression pattern of the *H. dujardini* ortholog of the transcription factor coding gene *orthodenticle* (*otd*). Expression of this gene is restricted to the anteriormost segment during early stages of segment development in onychophorans and most euarthropods investigated (Telford and Thomas, 1998; Eriksson et al.,

2013; Janssen et al., 2011). Expression of the *H. dujardini* ortholog of *otd* is confined to the head, shortly after this structure becomes morphologically discernable during *H. dujardini* development (Smith et al., 2016), as expected if the tardigrade head is homologous to the anteriormost segment of euarthropods and onychophorans (Fig. 5A and B). In sum, Hox gene and *otd* expression patterns support direct alignment of anterior segments across Panarthropoda. This alignment supports the unipartite hypothesis regarding the composition of the tardigrade head (Fig. 5A and B).

5. The evolution of panarthropod segmented body plans: a tardigrade's point of view

5.1. Divergence from a homonomous ancestral panarthropod

The consensus model of panarthropod body plan diversification suggests that the panarthropod ancestor was composed of a head that housed a pair of frontal appendages that served sensory roles, and a series of nearly homonomous trunk segments that each housed a pair of lobopodal legs (Snodgrass, 1935; Manton, 1977; Waloszek et al., 2007; Strausfeld, 2012). This model finds its strongest support from stem-group representatives of the extant panarthropod phyla, which typically exhibit this arrangement (Ma et al., 2009; Ou et al., 2012; Murdock et al., 2016). While there is general agreement regarding the nature of appendages in the ancestral panarthropod, the state of the central nervous system in this ancestor remains vigorously debated. Specifically under debate is whether the ancestral panarthropod's ventral nervous system exhibited a ganglionic organization (see Section 2.5), and the degree to which this ancestor was cephalized (see Section 3.1; Mayer et al., 2010; Whittington and Mayer, 2011).

According to our model, based on expression patterns of Hox genes and other axis patterning genes (Section 4.4; Fig. 5; Smith et al., 2016), tardigrades have diverged the least from the proposed panarthropod ancestor in two important respects. First, like the panarthropod common ancestor, tardigrades retain legs on all trunk segments. Second, our model suggest that tardigrades possess a unipartite brain, which supports a reconstruction in which the last common ancestor of Panarthropoda also exhibited this degree of cephalization (Fig. 6). According to our model, the diversification of ancestral trunk segments that gave rise to the plethora of appendage types and multipartite brains of euarthropods, and to a lesser extent in onychophorans, likely occurred after the divergence of tardigrades from the other panarthropod phyla (Fig. 6). By contrast, two models based on morphology suggest that tardigrades possess a tripartite brain and that the head of tardigrades is composed of three fused segments (see Sections 3.2 and 3.3; Dewel and Dewel, 1996; Persson et al., 2012, 2014). If so, then the tardigrade head could be considered a true tagma. Under these models, the panarthropod common ancestor could have exhibited a higher degree of cephalization than our model predicts. Furthermore, under these models, it would be less clear if stem-group tardigrades exhibited homonomous appendages, since, according to these models, several head appendages have been lost in the tardigrade lineage. However, given the discordance between the tripartite hypotheses and available data on tardigrade development (Section 4), the existence of a highly cephalized panarthropod ancestor now seems unlikely.

Unlike the predicted euarthropod ancestor (Budd, 2002; Cong et al., 2014), or extant onychophorans (Eriksson and Budd, 2000; Janssen et al., 2010), tardigrades lack frontal appendages on the head. It has been suggested that the tardigrade stylet apparatus evolved from a pair of head appendages (Section 2.4; Nielsen, 2001); therefore, the stylet apparatus may represent the homolog of the ancestral frontal appendage in tardigrades (Fig. 6; Ou et al.,

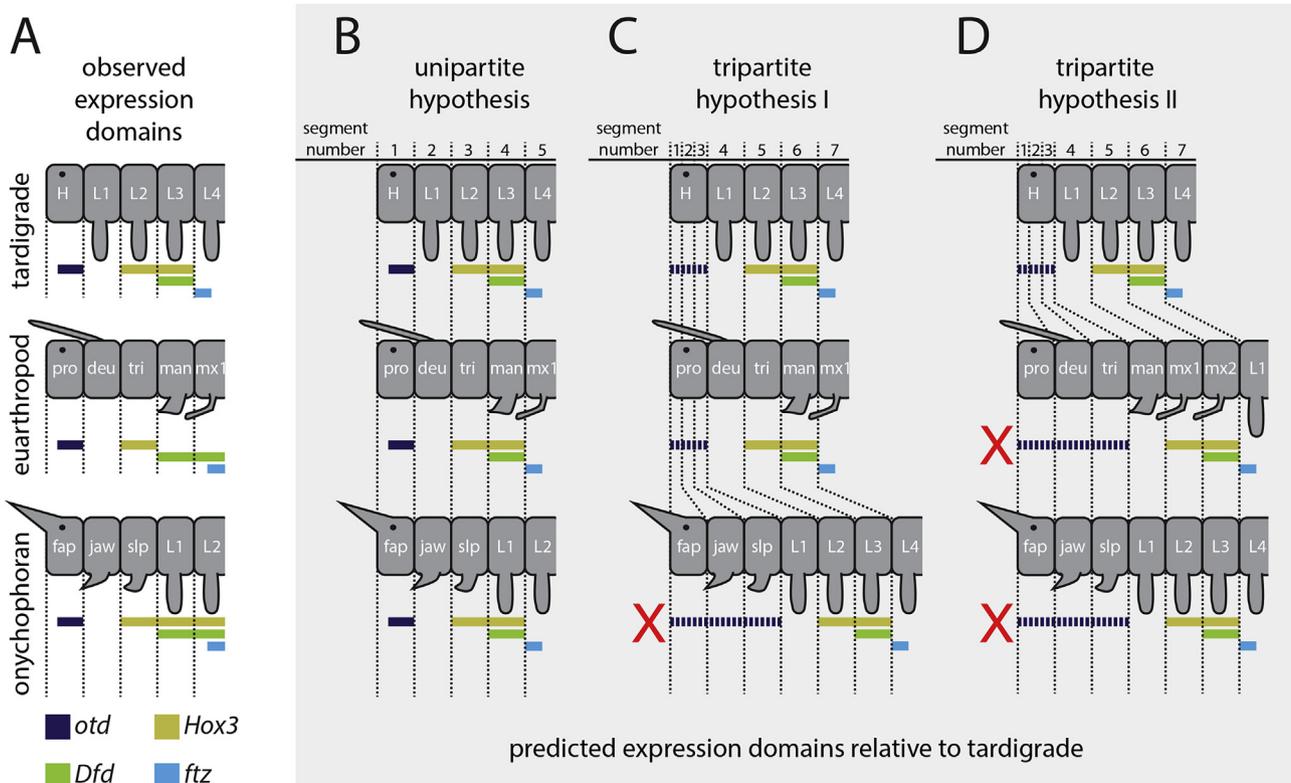


Fig. 5. Expression of Hox genes and *orthodenticle* in *Hypsibius dujardini* support the unipartite hypothesis for the origin of the tardigrade head. (A) Observed embryonic expression patterns of Hox genes and *otd* in the tardigrade *H. dujardini* (Smith et al., 2016), the euarthropod (millipede) *Glomeris marginata* (Janssen and Damen, 2006; Janssen et al., 2011), and the onychophoran *Euperipatoides kanangrensis* (Eriksson et al., 2010; Eriksson et al., 2013; Janssen et al., 2014). (B–D) Predicted expression patterns of Hox genes in euarthropods and onychophorans, relative to tardigrades, based on proposed models of panarthropod segment homology. The anterior expression borders of Hox genes and the posterior expression border of *otd* (A) closely match predictions of the unipartite head hypothesis (B). (C, D) Predicted expression patterns do not match observed expression patterns. Red X denotes falsified predictions. *otd* expression is shown as hatched to reflect uncertainty in how to interpret its expression in terms of multi-segmented head hypotheses. Abbreviations: deu, deutocerebral segment; fap, frontal appendage-bearing segment; H, head; jaw, jaw-bearing segment; L1–L4, leg-bearing segment 1–4; man, mandible-bearing segment; mx1, first maxilla-bearing segment; pro, protocerebral segment; slp, slime papilla-bearing segment; tri, tritocerebral segment.

2012). The evolutionary mystery concerning the fate of the frontal appendage in tardigrades mirrors an earlier mystery concerning its fate in euarthropods. In the euarthropod case, it has been suggested that the frontal appendages gave rise to a diminutive appendage referred to as the labrum (see Section 4.1; Budd, 2002; Cong et al., 2014), although alternative hypotheses have been proposed (Boyan et al., 2002; Frase and Richter, 2013). During development, the anterior specification gene *six3* is expressed in both the frontal appendages of onychophorans (Eriksson et al., 2013) and the labrum of euarthropods (Posnién et al., 2009), supporting homology of these appendage types. Elucidating the role of *six3* in patterning the tardigrade stylet apparatus may illuminate the relationship of this structure to the frontal appendages of onychophorans and the labrum of euarthropods.

5.2. An elongate ancestor gave rise to a compact tardigrade

One question that was previously unclear, given that Tardigrada could be the first crown group lineage to diverge within Panarthropoda (Campbell et al., 2011; Legg et al., 2013), is whether the ancestor of Panarthropoda was a relatively elongate animal, like most onychophorans and euarthropods, or a relatively compact animal, like a tardigrade. Our analysis of Hox gene expression in *H. dujardini* indicates that tardigrade segments align nearly one-to-one with the first five segments of other panarthropods (Smith et al., 2016, Figs. 5A,B and 6B), segments that are part of head/prosoma tagmata of euarthropods (see below for discussion of the

posterior most region). Intriguingly, Hox genes that pattern intermediate trunk segments in euarthropods and onychophorans are not found in the genome of *H. dujardini*, suggesting that intermediate trunk identity is missing in tardigrades relative to other panarthropods. As with euarthropods and onychophorans, these genes are present in the genome of the lophotrochozoan *Capitella* sp., a polychaete worm, and are expressed in an intermediate region of the anteroposterior body axis in this species (Fröbius et al., 2008). Based on this fact, it is clear that these Hox genes were present in the ancestral panarthropod and almost certainly patterned an intermediate region of its body axis. This reconstruction polarizes body axis evolution in Panarthropoda: the ancestor was relatively elongate; onychophorans and euarthropods inherited this condition from the ancestor, while intermediate segments were lost in the tardigrade stem-lineage (Fig. 6; Smith et al., 2016). This reconstruction is supported by recent phylogenetic analyses that suggest that the elongate Cambrian lobopod *O. ferox* is a stem-group tardigrade (Smith and Ortega-Hernández, 2014; Smith and Caron, 2015).

Terminal addition is likely an ancestral developmental mode for Bilateria (Jacobs et al., 2005; Gold et al., 2015), and is a common mode of development in onychophorans and euarthropods. By contrast, tardigrades lack terminal addition during development (Jacobs et al., 2005; Hejnal and Schnabel, 2005). We previously speculated that it was the loss of terminal addition in the tardigrade lineage that caused the loss of intermediate segments in tardigrades (Smith et al., 2016). Typically, terminal addition is

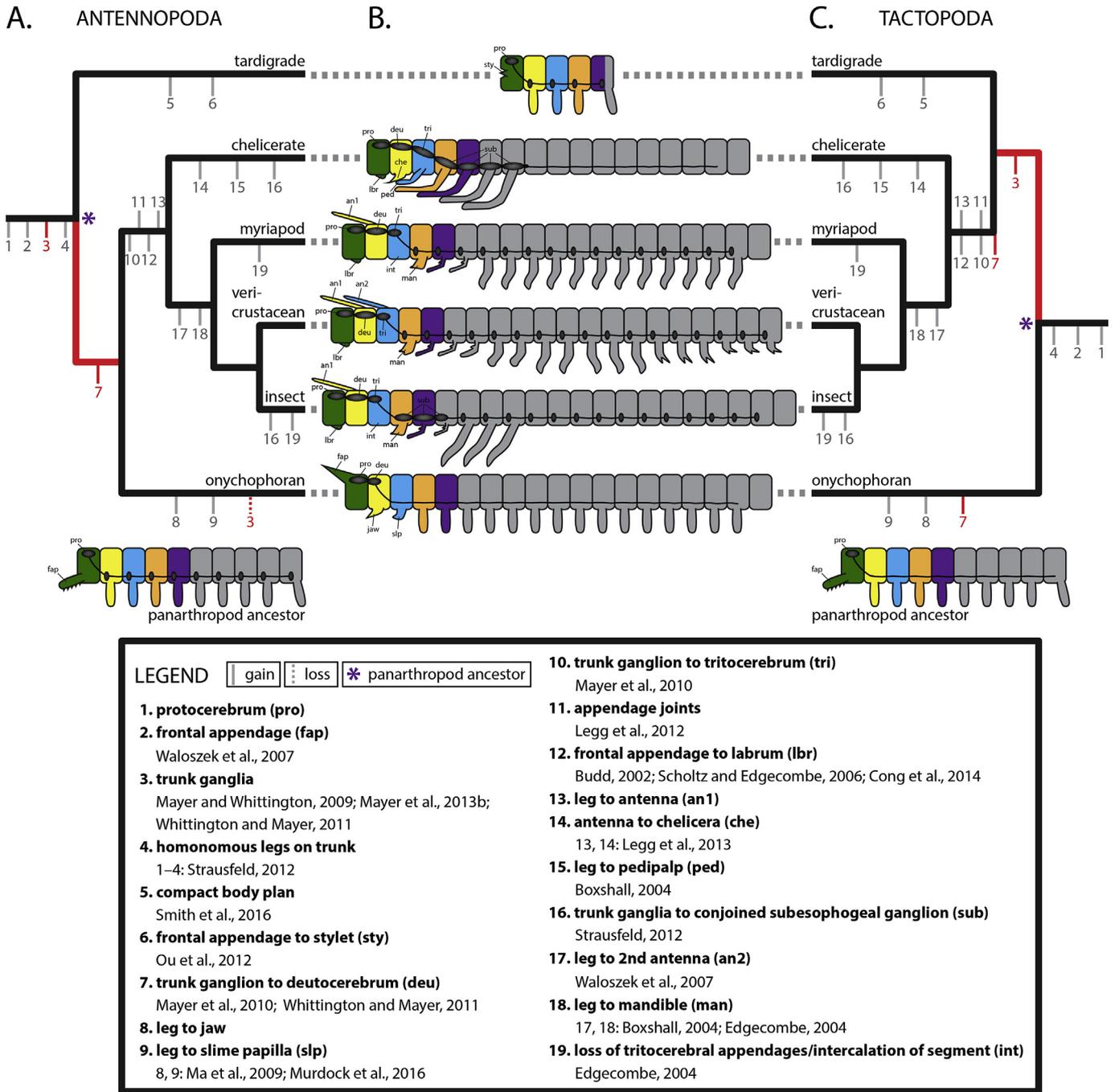


Fig. 6. Diversification of serial homologs across Panarthropoda. See legend for characters/character states mapped and a selection of pertinent references that present arguments in favor and/or against reconstructions shown. For cases where conflicting reconstructions have been presented, we present the reconstruction that we consider best supported. For the purposes of our character state reconstruction, anterior appendages that are morphologically similar to trunk appendages, in any given species, are considered to be legs, irrespective of whether they perform(ed) a locomotive function. (A, C) Relationships of euarthropods are based on Campbell et al. (2011). Red color highlights differences between the topologies of the two phylogenies and corresponding character state reconstructions. Purple asterisks mark the position of the last common panarthropod ancestor in the phylogenies. A hypothesized body plan model for the panarthropod ancestor is provided below both phylogenies. (A) Characters/character states mapped onto the Antennopoda hypothesis phylogeny. (B) Body plan models for extant panarthropods. Homologous segments are represented in the same color. Segment coloring scheme is based on results of Smith et al. (2016). (C) Characters/character states mapped onto the Tactopoda hypothesis phylogeny. Of the characters and character states mapped, the placement of Tardigrada within Panarthropoda only affects the mapping of trunk ganglia (3) and the deutocerebrum (7). Note: if Tardigrada is sister to Onychophora—the Lobopodia hypothesis (Section 1.1, Fig. 1B)—several equally parsimonious possibilities emerge for the evolution of trunk ganglia and the deutocerebrum.

regulated by a genetic pathway involving the ParaHox gene *caudal* (*cad*), Notch signaling, and Wnt signaling (McGregor et al., 2009). In many euarthropods, this genetic pathway regulates growth of the trunk region during development, while some or all head segments are specified earlier through mechanisms that are

independent of terminal addition (reviewed in McGregor et al., 2009). This mechanism has also been implicated in posterior elongation during onychophoran development (Janssen and Budd, 2016). A disruption of the terminal addition pathway could underlie the loss of segments that are homologous to

euarthropod trunk segments in the tardigrade lineage, while explaining the retention of segments that are homologous to euarthropod head segments. Intriguingly, the *H. dujardini* ortholog of *cad* is expressed in a small posterior fraction of the last segment of the body axis during development (Smith et al., 2016), suggesting that loss of posterior expression of this gene does not account for the loss of terminal addition in tardigrades; rather, a disruption of some other aspect of the terminal addition network may account for the loss of this process in the tardigrade lineage. This result also suggests that the tardigrade body axis retains a posterior region homologous to the posterior most regions of other bilaterians, a conclusion that finds additional support by the expression pattern of an *Abdominal-B* ortholog in *H. dujardini*. The expression of this gene is restricted to the posterior most region of the *H. dujardini* body axis, as it is in most other bilaterians (Smith et al., 2016). In sum, relative to euarthropods, tardigrades appear to retain some head segments and a posterior region, but have lost intermediate trunk segments.

6. Conclusions

Evo-devo studies of tardigrades have yielded important insights into the evolution of the tardigrade body plan, and, more generally, the evolution of panarthropod body plans. This approach promises to yield additional insights into several outstanding questions concerning tardigrade body plan evolution specifically and panarthropod body plan evolution more generally. Concerning tardigrade body plan evolution, this approach might provide valuable insight into the gene regulatory changes that account for the loss of terminal segment addition in the tardigrade lineage. This approach could also help determine the evolutionary fate of the ancestral panarthropod's frontal appendages in the tardigrade lineage; did the frontal appendages give rise to the stylet apparatus of tardigrades (Ou et al., 2012), or is the stylet apparatus derived from a pair of legs (Nielsen, 2001)? This raises a more general question that studies of the tardigrade stylet apparatus might clarify—were the frontal appendages derived from a pair of legs (Mayer and Koch, 2005) or does their origin predate the origin of legs (Strausfeld, 2012)? Solving this question might have important implications for our understanding of the origin of the euarthropod labrum, for which several competing hypotheses exist (see Sections 4.1 and 5.1; Budd, 2002; Scholtz and Edgecombe, 2006; Posnien et al., 2009; Frase and Richter, 2013; Smith et al., 2014). On the other hand, possibly the stylet apparatus has a non-appendicular origin, which may become apparent via studies of canonical appendage patterning genes in tardigrades. Additionally, comparisons between tardigrade development and development of other panarthropods might yield insight into the developmental innovations that underlie the evolution of the hyperdiverse head appendages of euarthropods (see Boxshall, 2004; Edgecombe, 2004), and to a lesser extent onychophorans, and the evolution of multipartite brains in these phyla. Furthermore, comparison of development between tardigrades and other panarthropods provides an additional avenue for testing hypotheses regarding the relationships of the panarthropod phyla; a solution to this problem is critical for polarizing evolutionary transitions in body plan architecture in Panarthropoda, such as whether a ganglionated ventral nervous system is a plesiomorphy of Panarthropoda or a synapomorphy of a tardigrade-euarthropod clade (see Section 2.5). Future studies of tardigrade development will continue to complement the efforts of paleontologists, morphologists, phylogeneticists, and developmental biologists working on panarthropod systems to illuminate the diversification of segmented body plans.

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