Arthropod phylogeny: onychophoran brain organization suggests an archaic relationship with a chelicerate stem lineage

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Neuroanatomical studies have demonstrated that the architecture and organization among neuropils are highly conserved within any order of arthropods. The shapes of nerve cells and their neuropilar arrangements provide robust characters for phylogenetic analyses. Such analyses so far have agreed with molecular phylogenies in demonstrating that entomostracans + malacostracans belong to a clade (Tetraconata) that includes the hexapods. However, relationships among what are considered to be paraphyletic groups or among the stem arthropods have not yet been satisfactorily resolved. The present parsimony analyses of independent neuroarchitectural characters from 27 arthropods and lobopods demonstrate relationships that are congruent with phylogenies derived from molecular studies, except for the status of the Onychophora. The present account describes the brain of the onychophoran *Euperipatoides rowelli*, demonstrating that the structure and arrangements of its neurons, cerebral neuropils and sensory centres are distinct from arrangements in the brains of mandibulates. Neuroanatomical evidence suggests that the organization of the onychophoran brain is similar to that of the brains of chelicerates.

Keywords: brain organization; arthropod evolution; Onychophora; Chelicerata

1. INTRODUCTION

The phylogenetic position of the onychophorans is considered by many to be central for understanding the origin and evolution of the arthropods. Modern taxa bear similarity to certain lower Cambrian forms (Ramsköld & Chen 1998), particularly *Onychodictyon* (Hou & Bergström 1995), and fossil evidence has been used to propose that extinct onychophoran taxa represent a basal lobopod zootype ancestral to arthropod lineages (Budd 1996). This placement of the Onychophora finds support from molecular characteristics (Aguinaldo *et al.* 1997; Colgan *et al.* 1998), respiratory proteins (Kusche *et al.* 2002) and *Hox* gene expression (Grenier *et al.* 1997). Combined molecular phylogenetics and morphological criteria generally place the Onychophora basal to the Arthropoda (Giribet *et al.* 2001) or as a sister group to the tardigrades and euarthropods (Zrzavy *et al.* 1998; Regier *et al.* 2005; Waloszek *et al.* 2005). In contrast, a study based on 12S ribosomal RNA supports the onychophorans not as basal arthropods but as modified euarthropods comprising a sister group to the chelicerates (Ballard *et al.* 1992).

Distinct from such analyses are recent comparisons of sensory and neural components of extant groups.

Neuromorphological analyses have led to the recognition that ommatidial development (Melzer *et al.* 2000) and brain anatomy (Strausfeld 1998, 2005) unite the insects and crustaceans and that arrangements of cerebral neuropils show the Remipedia to be phylogenetically closer to the malacostracans and hexapods than to basal crustaceans (Fanenbruck & Harzsch 2005). In the early 1900s, it was already recognized that the organization of the arthropod visual system and central brain was so highly conserved within different arthropod groups that these features could be used to suggest phylogenetic associations. Such neurophylogenetic studies pioneered by Holmgren (1916) and Hanström (1926) concluded that insects and crustaceans were monophyletic, derived from a common ancestor, and were not sister groups of the Myriapoda. This affinity was supported 80 years later by morphological and developmental studies allowing the recognition of a clade 'Pancrustacea' (Hexapoda + Crustacea; Stys & Zrzavy 1994) later termed the Tetraconata on the basis of common ommatidial organization across these groups (Dohle 2001; Richter 2002). Improved technologies for studying brain fibroarchitectures and neuron morphologies have demonstrated their robustness for phylogenetic reconstruction (Loesel *et al.* 2002) and have provided relational trees that are congruent with those achieved from molecular studies, while questioning the monophyly of certain groups such as the 'Myriapoda'.

Here, we employ neuroanatomical methods to describe salient features of the brain of an onychophoran and

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several chelicerates, to demonstrate similarities between their neuropils, and to distinguish them from brain organization in the Mandibulata. Support for a sister-group status for the Chelicerata and Onychophora is revealed by a cladistic analysis of 27 arthropod taxa employing 118 independent neuroarchitectural characters.

2. MATERIAL AND METHODS

(a) *Taxa and histological data production*

Taxa used for this study were purchased or collected in Arizona, at designated sites in New South Wales, Australia, and sites belonging to the Friday Harbor Marine Biology Laboratory, Washington, USA. Giant Tasmanian neanurid collembolans were provided by Dr Penny Greenslade at the CSIRO, Canberra. Dr Roy L. Caldwell, University of California, Berkeley, provided live stomatopods (*Pseudosquilla ciliata*). Liphistiomorph spiders (*Heptathela kimurai*) were obtained from designated sites on Mount Aso, Kyushu, Japan. The true spider *Cupiennius salei* was obtained from breeding colonies at the University of Frankfurt. Pycnogonids (*Lycythorhyncus* sp.) were collected from coastal habitats near Fukuoka, Japan; *Machilis germanicus* from vicinities in Würzburg, Germany; and *Scutigera coleoptrata* near Greve, Italy. Taxa used for this account are given in figure 5 and table 1.

For histology, animals were cooled to immobility, fixed and stained by reduced silver, ethyl gallate or Golgi impregnation (Strausfeld & Li 1999). Vibratome sections were treated with primary antibodies against serotonin and an antiserum raised in mice to *Diploptera punctata* allatostatin I (courtesy Dr Barbara Stay, University of Iowa), secondarily labelled and reconstructed using a Zeiss Pascal confocal microscope. Silver-stained images were captured using a Sony DKC 5000 CCD digital camera linked to an Apple G4 computer. For electron microscopy, tissue was fixed by double aldehyde and osmium methods and embedded in Epon resin. Sections were viewed in JEOL 2000EX transmission electron microscopes and images recorded on an SIS Megaview digital camera.

(b) *Character specification and analysis*

Characters used for phylogenetic analysis are (i) architectures of circumscribed neuropils; (ii) neuron morphologies; (iii) distribution, size and clustering of neuronal somata; (iv) certain configurations of neuropils, such as optic lobe regions scored as being twice, thrice or four times nested beneath a compound retina or glomerular neuropils with compound, spherical or wedged subunits; and (v) synaptic structures.

Of particular relevance here is the organization of unpaired midline neuropils in the first brain segment (protocerebrum), their modular origin from remotely positioned or immediate somata, and the presence or absence of satellite neuropils (see Loesel *et al.* 2002). Descriptions of the 118 characters used for this analysis are provided in the appendix (see electronic supplementary material).

(c) *Phylogenetic analyses*

Characters are treated as unlinked, unordered, equally weighted and either present or absent. Characters are not assigned character states. All characters are structural entities (see electronic supplementary material) without functional affiliations. Phylogenetic relationships were analysed using PAUP (phylogenetic analysis using parsimony; Swofford

2002) using a data matrix comprising 118 neural characters recorded as present or absent in 28 invertebrate taxa, including one annelid, the polychaete scale worm *Arctonoe fragilis*. Neuroanatomical analysis established that all species used were representative of their wider taxonomic classes. For example, three onychophoran species (two peripatopsids, *E. rowelli* and *Phallocephale tallagandensis* and an un-named peripatid species from the Mazatlan peninsula), three chilopod species (*Scutigera coleoptrata* and *Scolopendra alternans*, *Scolopendra polymorpha*) and two diplopods (*Archspirospireptus gigas* and *Orthoporus ornatus*) were screened, as were representatives of three suborders of spiders, several orders of palaeopteran and neopteran insects, eumalacostracans and two entomostracans (*Triops* and *Artemia*). As there was no difference within each group with respect to the data matrix, only one representative of each was included. Parsimony analysis was carried out via a heuristic search using 1000 random stepwise addition replicates. Trees were computed as unrooted.

3. CEREBRAL ORGANIZATION

(a) *Neuroanatomy: mushroom body organization and relationships with olfactory neuropils*

Neural architectures were used to compare the brains of the Australian onychophoran *E. rowelli* with arthropod taxa and the scale worm *A. fragilis*. In onychophorans and chelicerates, as in insects, the most anterior brain segment is equipped with elaborate higher centres, including the paired mushroom bodies (figure 1a,c). These centres, defined by Flögel (1876), were first recognized in neopteran insects where they possess cap-like neuropils called calyces that surmount and are contiguous with a stalk (pedunculus) and a system of lobes. Mushroom bodies are here scored as present if their pedunculi and lobes are composed of dense bundles of parallel intrinsic fibres originating from dense clusters of many thousands of uniformly small basophilic perikarya called globuli cells (Kenyon 1896).

Mushroom bodies are common to euarthropods and polychaetes and are thus likely to be symplesiomorphic, possessed by the last common ancestor of annelids and arthropods. Additional and specific neuroanatomical features of mushroom bodies characterize specific arthropod taxa as do the organization and location of primary olfactory neuropils associated with them. Mushroom bodies in chelicerates can have several lobe systems: lateral lobes that extend postero-ventrally through the protocerebrum; short vertical lobes that extend downwards from the globuli cells; and heterolateral lobes that extend across the midline, linking these neuropils in each side of the brain. In amblypygids, the lateral lobes are enormously extended and folded at the expense of the heterolateral and vertical lobes (Strausfeld *et al.* 1998). In *Limulus*, the short vertical lobes are reiterated many times at the expense of the lateral and heterolateral lobes. As a consequence, in xiphosurans much of the brain is filled with short pedunculus-like assemblies of parallel fibres that reach ventrally from a dorsal rind comprising millions of globuli cells (Fahrenbach & Chamberlain 1987). Heterolateral lobes are pronounced in araneans (Strausfeld & Barth 1993), and in the present study have been confirmed in *Delana cancerides*, *H. kimurai* and in salticids.

Figure 1. Brain regions shared by onychophorans ((a, b) *Euperipatoides rowelli*) and chelicerates ((c, d) *Eremobates pallipes*). (a, c) The paired mushroom body (MB) calyces (Ca), lobes (lo) and heterolateral commissures (com) shown green. VNC, ventral nerve cord. Olfactory glomeruli (bracketed, glom) are coloured purple. In *E. rowelli*, glomeruli are supplied by the protocerebral antennae (ant). In *E. pallipes* glomeruli are supplied by axons from the pedipalps (1) and from the alveoli (2). (b, d) Visual pathways from the eyes are shown in carmine, with the first and second optic neuropils indicated (vis 1, 2). In onychophorans and chelicerates, vis 2 is connected to the midline arcuate body (arc bo). (e–g) Shared morphology of (e, f) onychophoran and (g and inset to g) opilionid synapses at the mushroom body pedunculus compared with synaptic structure at an equivalent locus in (inset to e) the honey bee. Apposition of extended asymmetric membrane densities (arrows) characterize synapses in *E. rowelli* and the opilionid *Holonuncia* sp. Open arrowheads indicate smaller asymmetric synaptic clefts present at the same level, sometimes within the same profile. (inset e) The equivalent synaptic level of the insect mushroom body comprises patches of small ribbon- or button-like presynaptic densities that converge onto post-synaptic sites. Scales a, b = 200 μ m; c, d = 500 μ m. Scales e–g and inset c = 1 μ m; inset a = 0.5 μ m. (See electronic supplementary material for larger version.)

Histological preparations of brains of *E. rowelli* reveal paired lobed neuropils (figure 1a) that conform to the Flögel–Kenyon criteria for mushroom bodies and show structural similarity with mushroom bodies of

chelicerates. In *E. rowelli*, there are three lateral lobes, a more medial vertical lobe and a system of heterolateral lobes. Each lobe comprises many hundreds of parallel fibres derived from globuli cells lying dorsally over the brain. Contiguity of one lobe system across the midline, connecting the mushroom bodies on both sides, was also described by Holmgren (1916) and Schürmann (1987) and is typical of some chelicerates, here exemplified by the solfugid *Eremobates pallipes* (figure 1c).

Olfactory appendages provide chemosensory axons to glomerular olfactory lobes. With few exceptions, such as in isopods where mushroom bodies are lacking or araneans where mushroom bodies are supplied by second-order visual neuropils (Strausfeld & Barth 1993), olfactory glomeruli are connected by relay neurons to the mushroom bodies or, in malacostracan crustaceans, to the hemiellipsoid bodies thought to be equivalent to the mushroom bodies (Strausfeld *et al.* 1995; McKinzie *et al.* 2003). However, the arrangements of first-order olfactory neuropils in mandibulates and chelicerates show clear differences. In mandibulates, olfactory glomeruli are located in the deutocerebrum, the second preoral neuromere of the brain. In malacostracans, olfactory lobe glomeruli are supplied by receptor axons from the antennules (first antennae) and are connected by relays bilaterally to the hemiellipsoid bodies. In insects, antennal lobe relay neurons extend ipsilaterally to the mushroom body calyces. In chelicerates, although the mushroom bodies are in the protocerebrum, the olfactory glomeruli occur in the neuromere of whichever segment provides an appendage equipped with olfactory receptors. Relay neurons from glomeruli ascend ipsilaterally to the mushroom bodies. Olfactory appendages can be the pedipalps (in solfugids), the first leg pair (amblypygid, uropygids) or every leg pair as in pycnogonids. In the Onychophora, glomeruli are located in the protocerebrum as it is this brain segment that is supplied with sensory axons from the pair of frontal appendages ('antennae'; Eriksson *et al.* 2003). The presence of these appendages, which have been suggested to be modified legs (Mayer & Koch 2005), attests to the ancestral status of this taxon notwithstanding its proposed relationship with the chelicerates discussed below. In the Onychophora, relay neurons extend from the antennal glomeruli to the distal region of the ipsilateral mushroom body (figure 1a).

A microanatomical feature shared by the mushroom bodies of chelicerates and *E. rowelli* is seen at the boundary of the pedunculus and the lateral lobes arising from it. In *E. rowelli*, this location reveals elaborate synaptic structures comprising extensive membrane densities (figure 1e,f) that closely resemble synapses at the equivalent location in the opilionid *Holonuncia* sp. (figure 1g). Synapses at an equivalent location in insects have small and discrete presynaptic densities such as in the mushroom body of the honey bee *Apis mellifera* (inset to figure 1e).

(b) Neuroanatomy: organization of midline neuropils

In all euarthropods other than diplopods, the protocerebrum possesses a distinctive midline neuropil. In mandibulates, this neuropil differs from that of chelicerates and onychophorans. In mandibulate brains, the midline neuropil is embedded between the two protocerebral hemispheres (figure 2c–j), from which it recruits

Figure 2. Comparison of *Arctonoe fragilis* and examples of Mandibulata, with reference to midline neuropils. (a) Silver-stained brain of *A. fragilis*, showing the paired mushroom bodies (MB) and olfactory lobes (Olf Lo). Neuropils are bilaterally elaborated but there is no specialized midline region. Cell bodies of various sizes are clustered. (b) Allatostatin-stained midbrain of the millipede *Orthoporus ornatus*. Midline neuropil is absent (arrow). (c) Collaterals (bracketed) from heterolateral neurons extending across the brain define midline neuropil in the chilopod *Scutigera coleoptrata*. (d) Midline neuropil of the chilopod *Scolopendra polymorpha*. Neuropil is supplied by lateral fibres (arrowed), with perpendicular neurons (one indicated by arrowhead) extending through the neuropil. (e) Midline neuropil (bracketed) of *Triops longicaudatus*, showing the remote location of somata (arrows) supplying this neuropil. (f) Midline neuropil of the crab *Hemigrapsus oregonensis*. As in basal crustaceans, modular midline neuropil derives from heterolateral neurons (arrows) that originate from remote cell bodies. (g) The apterygote insect *Machilis germanicus*, showing similar arrangements with remote somata (arrows) providing midline neuropil. (h) An unidentified symphylan showing systems of heterolateral neurons, revealing (at arrow) midline modifications and some degree of lateralization (bracketed areas). (i) Fan-shaped body of a Tasmanian collembolan, *Neanura* sp, showing its associated heterolateral elements (arrows) supplying this midline neuropil. (j) Elaborate modular arrangements in the central complex of the cockroach *Periplaneta americana*. Heterolateral neurons supplying modular collaterals are arrowed. (k) Summary diagram showing the mandibulate (upper left) and chelicerate + onychophoran (upper right) midline neuropil ground patterns. Both are likely to have diverged from a simple rectilinear arrangement leading to the evolution of midline modules from remote heterolateral neurons (left) and from local midline columnar neurons (right). Scale bars = 50 μ m. (See electronic supplementary material.)

Figure 3. Arcuate midline neuropils shared by (a) onychophorans and (b–h) chelicerate taxa ((b) *Limulus polyphemus* (Xiphosura); (c) *Centruroides* sp. (Scorpionida); (d) *Eremobates pallipes* (Solfugida); (e) *Heptathela kimurai* (Aranaea); (f) *Pardosa* sp. (wolf spider, Aranaea); (g) *Mastigoproctus giganteus* (Uropygi); (h) *Holonuncia* sp. (Opilionidae)). Scales a–d, f, g = 100 µm; e = 50 µm; h = 25 µm. (See electronic supplementary material for larger version.)

afferents and to which it provides systems of efferent neurons that extend to descending pathways. Its modular organization derives from heterolateral neurons, the cell bodies of which are remote from the neuropil (figure 2c–j). In malacostracan crustaceans, including stomatopods, and pterygote insects, midline neuropils are elaborated into a complex of three discrete but interconnected parts: the superior arch, fan-shaped body and ellipsoid body. These unpaired neuropils are connected to paired satellite regions that are most numerous and elaborate in hexapod crown taxa (Loesel *et al.* 2002). The crescent- or arch-shaped midline neuropil of chelicerates (termed the arcuate body) extends across the protocerebrum superficially (figures 1d and 3b–h) rather than being embedded within it. Arcuate bodies do not form complexes with discrete satellite neuropils but comprise numerous and elaborate strata. These derive from columnar modules that are provided by hundreds (as in small opilionids) or thousands (as in ctenid spiders and solfugids) of perpendicular neurons supplied by perikarya lying immediately above or posterior to the neuropil. Species-specific differences of arcuate bodies relate to the breadth and depth of the neuropil, and the spacing of its strata (figure 3b–h). Such a central organization of columnar neurons is found in all chelicerates. Arcuate bodies also characterize the onychophoran brain (figures 1b, 3a, 4a,d,e). As in chelicerates, the onychophoran arcuate body is situated dorsally athwart the protocerebrum. Its relationship with the visual system and its internal architecture is like that of the chelicerate arcuate body. In *E. rowelli* (figure 1b), as in solfugids (figure 1d), second-order optic neuropil abuts the lateral edge of the arcuate body. In solfugids (figure 1d) a bundle of axons leaves the lenticular second optic neuropil in the brain and reaches the arcuate body's flank. In araneans, the second optic neuropil of each principal eye supplies a major

projection to this midline neuropil (Hanström 1926; Strausfeld *et al.* 1993). Studies of the spider *C. salei* (Strausfeld *et al.* 1993) show that visual relay neurons project tangentially across perpendicular neurons of the arcuate body without any evidence of retinotopic mapping. This suggests that columnar organization of the arcuate body is independent of the development of the eye and peripheral optic neuropils and has no affinity with the columnar organization of mandibulate optic lobe neuropils. The relationship between visual neuropils and arcuate midline neuropil thus distinguishes the chelicerate and onychophoran visual systems from those of tetraconates and scutigerid chilopods, in which compound eyes supply nested retinotopic neuropils in discrete optic lobes (Strausfeld 2005).

Silver staining and immunocytochemistry (Loesel *et al.* 2002) reveal that the neuroarchitecture of the arcuate body in *E. rowelli* (figures 3a and 4a,d,e) is similar in detail to that of the chelicerate arcuate body (figures 3b–h and 4b,c,f). The midline neuropil of *E. rowelli* (figure 4e) and the arcuate body of the New World hunting spider *Pardosa* sp. (figure 4f) both demonstrate successive strata of looped axons and tangential fibres that are intersected by ensembles of perpendicular axons. Immunostaining against allatostatin and serotonin reveals the same sequence of stratification in the midline neuropils of both Onychophora (figure 4a,d) and the aranean *D. cancerides* (figure 4b,c). These arrangements are characteristic of the Onychophora and chelicerates but not of other arthropod groups. That so many identical arrangements might be convergent homoplasies is highly unlikely, leading us to favour the view that arcuate body architectures are synapomorphies uniting the Onychophora and chelicerates. As is discussed below, the possibility that arcuate bodies might be plesiomorphic modifications of a midline neuropil ground pattern pertaining to all arthropods is argued as unlikely due to fundamental differences in the cellular identities of modules that make up midline neuropils in mandibulates and chelicerates.

(c) Phylogenetic relationships implied by brain organization

Phylogenetic relationships were examined using PAUP (see §2). Trees were computed as unrooted (figure 5a,b). Bootstrap values from 1000 replicates are shown in figure 5b. Neighbour joining and unweighted pair group method with arithmetic mean (UPGMA) showed congruence. No islands of trees were detected, indicating little conflict of signal in the data. Of the eight best trees (255 steps, CI=0.455) all resolve a monophyletic Chelicerate clade, which includes the Pycnogonidae and a sister-group relationship for the chelicerates and onychophorans. The latter has strong bootstrap support (90%). Enforcing polyphyly for this clade results in 17 shortest trees, each nine steps longer than the three best trees (264 steps, CI=0.439). Four independent characters (generally uniform cerebral somata, midline neuropils provided by medially positioned somata, midline neuropil superficial to protocerebrum, visual supply to the lateral margin of medial neuropil) and one suite of characters (stratification within midline neuropil) are synapomorphic for this group.

Figure 4. Homologous stratification in midline neuropils of (a, d, e) *E. rowelli*, the spiders (b, c) *Delana cancerides* and (f) *Pardosa* sp. (a) Anti-5HT staining of *E. rowelli* reveals a diffusely packed immunoreactive stratum (1) that is distinct from a palisade of immunoreactive columnar elements (2). (b, c) Anti-5HT staining of *D. cancerides* reveals homologous arrangements ((c) layers 1 and 2, and (b) purple in low resolution micrograph). (b) Anti-allatostatin staining (green) of *D. cancerides* reveals three levels: a graded concentration of profiles at level A, columnar processes at B (fibres detailed in inset, enlargement of bracketed area) and tangential processes at C. (d) Anti-allatostatin reveals homologous layers in *E. rowelli* (layer A–C). (e) Anti-5HT and (f) Bodian staining demonstrate identical layering (indicated by double arrows) through the midline neuropil of *E. rowelli* and arcuate body of *Pardosa* sp. Scales a–c, f=50 μ m. e adjusted to depth of f. (See electronic supplementary material for larger version.)

(d) Reliability of neuronal structures for phylogenetic analysis

Traditional use of morphological criteria to reconstruct phylogenies has focused on external features, such as limbs, mouthparts and segmental specializations. Such potentially adaptive characters can be difficult to interpret as a result of convergence, parallelism and other such processes. Further, homologous structures can vary greatly even among species recognized as related, as attested by gene expression showing the gnathobasic nature of mandibles in insects and crustaceans (Popadic *et al.* 1998). Nevertheless, that structural studies indeed provide reliable indicators for phylogenetic inference is evidenced by numerous analyses of fossil material that contribute to the resolution of crustacean stem lineages (see Walossek & Müller 1998), and many morphological features have been shown to unite taxa or provide crucial characters for phylogeny (Edgecombe *et al.* 2002; Giribet *et al.* 2002). But how reliable are neuroanatomical structures for inferring phylogenies? Many neurological features are indisputably consistent across taxa and reveal plesiomorphies. For example, uncrossed retinotopic axons linking the deepest optic neuropil with the next most

peripheral is a ground plan shared across the Tetraconata (Strausfeld 2005), supporting an origin of the hexapods from basal entomostracans. The presence of neuroblasts and a similar axonogenesis of pioneer neurons in insects and crustaceans, which show fundamental differences from chilopods (Whittington *et al.* 1991; Whittington 2004; Harzsch 2003), or the expression of the transcription factor gene *Even-skipped* in identical neurons across insects and crustaceans (Duman-Scheel & Patel 1999) also unite the assemblage Entomostraca+Hexapoda+Malacostraca.

The structure of neuropils is generally highly conserved within any crustacean or insect order (Sandeman & Scholtz 1995; Farris 2005). The following are just a few examples. Paired noduli of the central complex unite pterygote insects (Loesel *et al.* 2002). Male-specific antennal lobe glomeruli and grooved lobules in the optic lobes are uniquely lepidopteran synapomorphies. The spacing of columns in the third optic neuropil of eumalacostracans represents each facet of the compound eye, whereas in insects assemblies of retinotopic neurons in the homologous neuropil coarsen the retinotopic mosaic (Sinakevitch *et al.* 2003). In mushroom bodies,

Figure 5. (a) The results of a strict consensus of the three most parsimonious trees. All characters (see character matrix and character list, electronic supplementary material) are treated as unordered and unweighted. All trees support a sister relationship between Onychophora and Chelicerata. Hexapods and Crustacea show reciprocal monophyly and a sister relationship supporting the clade Tetraconata. (b) Bootstrap parsimony consensus tree showing those groups supported by a bootstrap value of 50% or more. Support for Onychophora + Chelicerata is strong at 90%.

centres thought to be concerned with learning and memory and therefore subject to strong selection pressure, the same morphological classes of intrinsic neurons differentiate in a characteristic sequence, but the size, layering and disposition of the calyces and lobes assume features that are characteristic of each neopteran order (Farris & Sinakevitch 2003).

Analysis of brain structure reveals a suite of neuroarchitectural arrangements shared between onychophorans and chelicerates that strongly support an affinity of these two groups. In scorpions, solfugids, araneans and onychophorans, heterolateral lobes connect the two mushroom bodies, a situation unknown in other arthropods other than *broad complex* mutants of *Drosophila* where one division of the mushroom bodies' medial lobes crosses the brain midline (Michel *et al.* 2004). The columnar and multi-stratified architectures and their immunoaffinities in the arcuate bodies of onychophorans correspond to those of araneans (figure 4). In addition to the many striking similarities between the supraoesophageal neuropils of onychophorans and chelicerates, the post-stomodaeal ganglia of onychophorans are unfused at the midline, with the two halves linked by numerous connectives, as is typical of juvenile xiphosurans (Mittman & Scholtz 2003). The feature of uniformly small neuronal perikarya is another characteristic of chelicerate and onychophoran brains that is not shared by the brains of Chilopoda and Tetraconata. Finally, a unique type of synaptic complex is common to a specific region of the onychophoran and opilionid mushroom bodies (figure 1e–g). Synaptic configurations as phylogenetically diagnostic elements have a precedent, as in the case of the T-shaped

presynaptic active zone that is diagnostic of the Diptera (Shaw & Meinertzhagen 1986).

4. EVOLUTIONARY IMPLICATIONS

The present neuromorphology-based cladogram (figure 5a,b; see also electronic supplementary materials) generally agrees with published analyses based on RNA and DNA data with regard to relationships within the Tetraconata and within the chelicerates *sensu stricto* (Ballard *et al.* 1992; Friedrich & Tautz 1995; Zrzavy *et al.* 1998). The exception is the position of the Onychophora, reminiscent of other ambiguous placements ranging from the Onychophora being nested within the 'pancrustaceans' (Peterson & Eernisse 2001), through their comprising (with the tardigrades) a sister group of the arthropods (Giribet *et al.* 2000; Regier *et al.* 2005), to their representing a lobopod stem taxon (Budd 1996). Both neuroanatomical observations and parsimony analysis suggest that this group should be integrated within the Euarthropoda as an ancient sister group of the Chelicerata.

This proposal conflicts with the received opinion that the Onychophora are not incorporated within the euarthropods but are a sister group. Supporting evidence includes the observation that onychophoran muscle possesses filament-like components similar to intermediate filaments of deuterostomes and lophotrochozoans (Bartnik & Weber 1989). The evolution of the haemocyanin superfamily (Immesberger & Burmester 2004) demonstrates that onychophoran respiratory haemocyanins were the first to diverge from ancestral phenoloxidasases thus suggesting that this group may be distinct from other arthropods. Onychophorans also retain other plesiomorphic features

Table 1. Ten characters relevant to midline neuropils in mandibulates, chelicerates and onychophorans with *Arctonoe fragilis*. Plus signs indicate the presence, minus signs the absence of a trait. Plus signs in parenthesis indicate that, in scolopendromorphs, modules of midline neuropil are defined by heterolateral remote neurons and a cluster of columnar neurons provided by uniform somata. (Annelida: 1, *Arctonoe fragilis*. Mandibulata: 2, *Orthoporus ornatus*; 3, *Scutigera coleoptrata*; 4, *Scolopendra polymorpha*; 5, *Triops longicaudatus*; 6, *Hemigrapsus nudus*; 7, *Machilis germanicus*; 8, *Collembola* (*Neanura* sp.); 9, *Periplaneta americana*. Onychophora: 10, *Euperipatoides rowelli*. Chelicerata: 11, *Limulus polyphemus*; 12, *Eremobates pallipes*; 13, *Tarantula* sp.; 14, *Cupiennius salei*; 15, *Centruroides* sp.).

	taxa														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 heterolateral midline neuropil	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+
2 modules defined by perpendicular local neurons	–	–	–	(+)	–	–	–	–	–	+	+	+	+	+	+
3 modules defined by heterolateral remote neurons	–	–	+	(+)	+	+	+	+	+	–	–	–	–	–	–
4 at least five discrete strata	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
5 looped axons provide local tangential connections	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
6 neuropil enclosed within protocerebrum	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–
7 visual supply to lateral margin of neuropil	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
8 neuropil supplied by uniform somata	–	+	–	(+)	–	–	–	–	–	+	+	+	+	+	+
9 somata supplying cerebral neuropils vary in size	(+)	–	+	+	+	+	+	+	+	–	–	–	–	–	–
10 satellite regions associated with midline neuropil	–	–	–	–	–	+	–	+	+	–	–	–	–	–	–

such as the lack of sclerotization, a sub-epidermal muscle layer and ciliated coelomoducts. A combined phylogenetic analysis, using ribosomal and nuclear protein coding genes, as well as mitochondrial genes, also places the onychophorans as a clade distinct from the Euarthropoda (Giribet *et al.* 2001), a conclusion similarly reached by Regier *et al.* (2005), by Mallatt *et al.* (2004) using 28S and 18S rRNA gene sequences and by Edgecombe *et al.* (2000) from histone H3 and U2 snRNA sequences.

On the other hand, a study based on 12S ribosomal RNA sequences (Ballard *et al.* 1992) includes Onychophora within the Euarthropoda, placing them as a sister group to the chelicerates, as does the present analysis using neural characters. The question remains whether neuroanatomical features here are sufficient to propose that the Onychophora are related to the chelicerates despite the many primitive features of the onychophoran body plan, including the paired protocerebral antennae, recently shown to be modified legs (Mayer & Koch 2005).

Brain anatomy suggests that if Onychophora are a sister group of the chelicerates, they probably represent an early branch of the chelicerate evolutionary trajectory. This would imply that cerebral organization characteristic of Onychophora has been essentially maintained throughout subsequent chelicerate evolution. Indeed, if there was no such relationship between the Chelicerata and Onychophora, then the similarities described in this account would demand an exceptional degree of homoplasy and, therefore, 'parallel evolution'.

But is the striking similarity between onychophoran and chelicerate arcuate bodies merely a plesiomorphic modification of a midline ground plan common to all arthropods? That this is unlikely is suggested by comparisons across mandibulates (figure 2) and chelicerates (figure 3) demonstrating fundamental differences of midline architectures and their contributing neurons (table 1). In mandibulates, midline neuropils are embedded within the protocerebral matrix and derived from heterolateral processes originating from a small population of remotely located somata (figure 2*c–j*). Elaboration at the midline is provided by heterolateral

neurons, the collaterals of which supply repeat units or 'modules' with a subsequent recruitment of perpendicular (often peptidergic) neurons. This arrangement is simplest in basal scutigerid chilopods (figure 2*c*), more elaborate in basal crustaceans (figure 2*e*) and most elaborate in pterygote insects (figure 2*j*). An organization that might represent an early stage of the evolution of mandibulate midline neuropils may have been retained in the Symphyla where some heterolateral neurons reveal subtle differences of branching at the midline (figure 2*h*).

In chelicerates and onychophorans, the midline neuropils are always located superficial to the rest of the protocerebrum and they derive from thousands of local somata at the midline (figure 3*b–h*). These provide the arcuate body's modular organization and also contribute to its tangential organization.

An ancestral character state that would reconcile differences between mandibulate and chelicerate/onychophoran midline architectures is a simple rectilinear network comprising heterolateral neurons intersected by perpendicular elements (figure 2*k*). Such a rectilinear network, exemplified by the post-stomodaeal ladder-like 'Markstrang' of certain annelids (Orrhage & Müller 2005), is likely to have existed as a ring-like structure around a mouth long before the origin of segmented invertebrates. An implication of this is that divergence of two distinct midline ground patterns is likely to have occurred very early in arthropod evolution.

In conclusion, we propose that the brains of onychophorans represent an archaic pattern of organization that has been maintained in chelicerates and which differs from the organization of mandibulate brains. That diagnostic features such as the arcuate bodies might be plesiomorphic characters of a basal arthropod ground pattern is unlikely considering the substantial differences between the arcuate bodies of chelicerates and onychophorans and the midline neuropils of mandibulates.

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