

Arachnid monophyly: Morphological, palaeontological and molecular support for a single terrestrialization within Chelicerata

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ABSTRACT

The majority of extant arachnids are terrestrial, but other chelicerates are generally aquatic, including horseshoe crabs, sea spiders, and the extinct eurypterids. It is necessary to determine whether arachnids are exclusively descended from a single common ancestor (monophyly), because only that relationship is compatible with one land colonization in chelicerate evolutionary history. Some studies have cast doubt on arachnid monophyly and recast the origins of their terrestrialization. These include some phylogenomic analyses placing horseshoe crabs within Arachnida, and from aquatic Palaeozoic stem-group scorpions. Here, we evaluate the possibility of arachnid monophyly by considering morphology, fossils and molecules holistically. We argue arachnid monophyly obviates the need to posit reacquisition/retention of aquatic characters such as gnathobasic feeding and book gills without trabeculae from terrestrial ancestors in horseshoe crabs, and that the scorpion total-group contains few aquatic taxa. We built a matrix composed of 200 slowly-evolving genes and re-analysed two published molecular datasets. We retrieved arachnid monophyly where other studies did not - highlighting the difficulty of resolving chelicerate relationships from current molecular data. As such, we consider arachnid monophyly the best-supported hypothesis. Finally, we inferred that arachnids terrestrialized during the Cambrian–Ordovician using the slow-evolving molecular matrix, in agreement with recent analyses.

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1. Introduction

Chelicerata is an ancient and highly diverse clade, representing one of the two subphyla of Arthropoda (its counterpart being Mandibulata, containing myriapods and pancrustaceans). Discerning whether there was a single terrestrial common ancestor of the extant terrestrial chelicerates (Arachnida – spiders, scorpions, mites, ticks, etc.) is an intriguing question in arthropod macroevolution and palaeobiology. This is because it allows an inference of how many terrestrialization events there are likely to

have been in chelicerate evolutionary history - one vs. multiple (Scholtz and Kamenz, 2006). The answer to that question is pertinent because arachnids are among the most numerous and diverse land animals, and therefore their adaptation to living on land is of great evolutionary interest.

Among extant Chelicerata, three subclades are traditionally recognised: the marine sea spiders (Pycnogonida) and horseshoe crabs (Xiphosura), and the terrestrial Arachnida. Arachnida and Xiphosura, along with the fossil groups Eurypterida, Chasmataspida and “synziphosurines” – the latter a polyphyletic assemblage of horseshoe crab-like forms (Lamsdell, 2013, 2016) – are grouped together as Euchelicerata. Synziphosurines are resolved in the stem groups of Xiphosura and Dekatriata (the proposed arachnid–eurypterid–chasmataspidid clade), as well as some of the most basal branches within Euchelicerata (Lamsdell, 2013, 2016). Most studies indicate that Pycnogonida and Euchelicerata are sister taxa (Giribet, 2018), and that Euchelicerata is monophyletic – even

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when more stemward Cambrian chelicerate fossils are analysed (Legg et al., 2013; Aria & Caron, 2017, 2019). Exceptionally preserved stem-group chelicerates and/or stem-group euchelicerates (depending on the relative position of Pycnogonida, which is problematic to resolve due to a highly autapomorphic bodyplan) from Cambrian *Konservat-Lagerstätten* include *Sanctacaris uncata* (Briggs and Collins, 1988; Legg, 2014), *Habelia optata* (Aria and Caron, 2017) and *Mollisonia plenovenatrix* (Aria and Caron, 2019) from the Burgess Shale.

The phylogenetic relationships between extant euchelicerates have proved highly problematic to resolve by morphology and molecules alike – particularly the relationships between the arachnid orders (Wheeler and Hayashi, 1998; Giribet et al., 2002; Shultz, 2007; Regier et al., 2010; Sharma et al., 2014; Giribet, 2018; Ballesteros and Sharma, 2019; Lozano-Fernandez et al., 2019; Ballesteros et al., 2019). This presents a great challenge when reconstructing the history of chelicerate terrestrialization, because the inferences of historic land colonisations in any group of animals are dependent on the branching order of the evolutionary tree. Conflicting phylogenetic hypotheses can confound the position of nodes representing a shift to a terrestrial lifestyle, which has led to ambiguity regarding how many terrestrialization events have occurred in Chelicerata. As all living arachnids are terrestrial – bar a few obviously secondarily aquatic groups such as the marine and freshwater mites (Pepato et al., 2018) – it is traditionally inferred that they could have originated from a terrestrial ancestor if they are monophyletic. As this common ancestor is unknown, support for this hypothesis relies on the identification of apomorphic characters for monophyletic Arachnida that are demonstrably adaptations to a terrestrial lifestyle (Scholtz and Kamenz, 2006). However, it has been argued that this is an overly simplistic line of reasoning (Shultz, 2007). This is due to the difficulties in discriminating such characters as exclusively associated with terrestrial taxa, the possibility of convergent evolution, as well as the assumption of a dichotomy between aquatic and terrestrial living – as opposed to the amphibiousness seen in horseshoe crabs, eurypterids (Lamsdell et al., 2020), and various pancrustaceans. Furthermore, arachnid monophyly has been questioned historically (see review in Giribet, 2018), but particularly more recently (Ballesteros and Sharma, 2019), which fully opens up the possibility that there have been cases of independent terrestrialization among different arachnid groups.

Morphology-based phylogenetic analyses generally support arachnid monophyly (Wheeler and Hayashi, 1998; Giribet et al., 2002; Shultz, 2007; Legg et al., 2013; Garwood and Dunlop, 2014a), and substantial lists of morphological autapomorphies have been proposed to support Arachnida, e.g., 11 characters listed by Shultz (2001). However, in explicit analyses of morphological datasets, arachnid monophyly is sometimes based on only a few apomorphic characters (Garwood and Dunlop, 2014a); or by effectively assuming arachnid monophyly by rooting the trees between Xiphosura and Arachnida (Shultz, 2007); or by sampling only a subset of crown group arachnid diversity, such that arachnid monophyly was not severely tested (Lamsdell, 2013, 2016). Some palaeontological studies contested arachnid monophyly by allying scorpions with eurypterids, but this relationship was either not based on an explicit optimality criterion (Selden and Jeram, 1989; Dunlop and Webster, 1999) or was in fact found to be unparsimonious (Dunlop and Braddy, 2001). More serious challenges to arachnid monophyly have come from molecular datasets (Sharma et al., 2014; Pepato and Klimov, 2015; Ballesteros and Sharma, 2019; Ballesteros et al., 2019; Noah et al., 2020). Molecular evidence has been presented recently to support a derived position of the marine Xiphosura within the terrestrial arachnid lineages (Ballesteros and Sharma, 2019; Ballesteros et al., 2019; Noah et al.,

2020) – with some topologies suggesting lung-bearing arachnids (Arachnopulmonata – e.g. scorpions and spiders) could have terrestrialized from xiphosuran-like ancestors independently of apulmonate arachnid groups (e.g. Acari, Opiliones, Solifugae, etc.). However, another recent molecular study has on the contrary supported arachnid monophyly and suggested that placement of xiphosurans within Arachnida could be artefactual (Lozano-Fernandez et al., 2019). Another source of controversy comes from the presumptive aquatic nature of some stem-group scorpions (Dunlop et al., 2008; Poschmann et al., 2008; Kühl et al., 2012; Dunlop and Selden, 2013; Waddington et al., 2015; Wendruff et al., 2020), which may imply scorpions invaded terrestrial environments independently of other arachnids – regardless of the phylogenetic position of Xiphosura. As such, there are clear issues impeding inferences of chelicerate terrestrial evolutionary history that require clarification.

Within the context of this special issue – arthropod terrestrialization – we critically discuss the possibility of a monophyletic Arachnida and a single land colonisation in Chelicerata. Approaching the question holistically, we consider both fossil/morphological data and molecular sequence data. We discuss four key morphological character systems that bear significance for chelicerate terrestrialization. These include 1) the occurrence of gnathobasic feeding in horseshoe crabs, aquatic fossil chelicerates, and relevant outgroups, 2) the reduction of the compound lateral eye in arachnids, 3) the homology and occurrence of lamellate respiratory structures in euchelicerates (book gills and book lungs), 4) the aquatic origin of the scorpion total-group. From a molecular standpoint, we built a molecular matrix based on 200 slowly evolving genes and re-analysed two molecular datasets, from Regier et al. (2010) and Sharma et al. (2014), that recovered Xiphosura within a paraphyletic Arachnida in recently published studies – in Noah et al. (2020) and Ballesteros and Sharma (2019) respectively. In contrast to the original studies, we recover arachnid monophyly from each matrix and discuss the reasons for this discordance. We conclude that the difficulty in resolving arachnid phylogeny from current molecular datasets justifies precedence to morphological arguments for the monophyly of Arachnida.

2. Morphology & palaeontology

2.1. The significance of gnathobasic feeding

Some of the most significant differences between the appendages of arachnids and xiphosurans involve the structure and function of the coxa in feeding, specifically whether or not a series of prosomal coxae bears a row of spinose projections known as gnathobases, and whether or not the coxa-body joint is mobile. Gnathobases are masticatory endites of the protopodite/coxa (and, in fossils, sometimes additional podomeres of the endopodite/telopodite distal to the protopodite) of a range of arthropods. A homonomous series of these structures is exclusively associated with aquatic taxa including Xiphosura, Eurypterida, and a range of other fossil euchelicerates, wherein they flank a groove along the ventral anterior–posterior axis of the prosoma.

The morphology of the spinose gnathobases of xiphosurans, the adduction of which crushes or shreds food (Manton, 1964), is very similar to those of the corresponding coxae in eurypterids (Selden, 1981; Bicknell et al., 2018a). If eurypterids are stem-group arachnids, then the close correspondence in their prosomal gnathobases to those of Xiphosura serves to attract these taxa. This attraction is not necessarily because these lineages form a clade (the traditional Merostomata), since a homonomous series of coxal gnathobases is inferred to be a plesiomorphic character for Euchelicerata.

Gnathobasic coxae are widely distributed in trilobite-allied Palaeozoic arthropods (Artiopoda), an assemblage that is often resolved deeply in the chelicerate stem group. These gnathobases have indeed been cited as a possible apomorphic character of an “arachnomorph” clade that unites artiopodans and chelicerates (Aria and Caron, 2017). Among artiopodans, *Sidneyia* exemplifies “merostome”-like gnathobasic coxae, and has been interpreted as being a xiphosuran-like durophagous predator/scavenger on the basis of functional morphology of the appendages (Bicknell et al., 2018b), the structure of its gnathobasic spines in comparison to those of *Limulus* (Bicknell et al., 2018a), and from observations on shelly gut contents (Zacai et al., 2016). As such, gnathobasic feeding would be a character acquired in the chelicerate stem lineage and retained in crown group Euchelicerata (plesiomorphy of Xiphosura) and stem-group arachnids (Eurypterida).

A coxal gnathobase was attributed to appendage 3 (arachnid leg 1) in the Silurian scorpion *Proscorpius* (see discussion of character 52 in Shultz, 2007) and underpinned a theory of xiphosuran- or eurypterid-like feeding in stem-group scorpions (Kjellsvig-Waering, 1986). However, this claim was dismissed as erroneous when restudy of the specimen purported to exhibit a gnathobase provided no evidence for its presence (Dunlop et al., 2008, text-Figure 2), nor did other specimens of *Proscorpius*. Some arachnids do nevertheless employ modified coxal endites in feeding, for example, endites that function in a preoral tube known as a stomotheca in scorpions and opilions. In this case, movements of the pedipalp and leg 1 coxae modify the shape of the preoral cavity into which they project. These coxal projections do not, however, closely resemble the coxal gnathobases in Xiphosura, which are serially repeated (with segmentally specific details) on prosomal limbs 2–6. Stomothecae were previously interpreted as a synapomorphic character to unite scorpions and opilions (Stomothecata, e.g. Shultz, 2007). However, phylogenomic studies agree that scorpions are the sister group to tetrapulmonates (Sharma et al., 2014; Lozano-Fernandez et al., 2019) indicating the stomotheca is likely a convergently evolved character, which is also endorsed by the absence of coxal endites in early scorpions (Dunlop et al., 2008; Kühl et al., 2012).

A few other arachnid groups exhibit endites on particular legs in feeding, including some spiders and trigonotarbids. In the case of spiders, a masticatory endite is present on a movable pedipalp coxa. Known as the serula/gnathocoxa (Kästner, 1925), it is present only on a single pair of appendages and in certain derived spider groups. Therefore, the serula/gnathocoxa is autapomorphic and does not reflect a stepwise reduction of the gnathobase series within Arachnida. Two families of the extinct Palaeozoic arachnid order Trigonotarbida also exhibit coxal endites (Palaeocharinidae and Anthracomartidae). Setose coxal endites are reported in the Devonian palaeocharinid *Palaeocharinus* (Garwood and Dunlop, 2014b, text-Figure 1(5); Haug, 2020, text-Figures 7D, 8A), but they are restricted to walking leg 1, and their ornamentation does not resemble the battery of gnathal spines of xiphosuran or eurypterid gnathobases. Among Carboniferous anthracomartids, a series of flattened to tubular endites without ornament is developed on the walking legs of *Anthracomartus hindi* (Garwood et al., 2009, text-Figure 1b and 1g; Garwood and Dunlop, 2011, text-Figures 1B, 2B) and a series of weakly developed endites is reported in *Maiocercus celticus* (Garwood and Dunlop, 2011, Figure 3B). As the endites in anthracomartids form a series, they have warranted comparison to the gnathobasic endites of Xiphosura (Garwood et al., 2009; Garwood and Dunlop, 2011), and these authors considered the possibility that the trigonotarbid endites are plesiomorphically retained gnathobases. However, we consider their homology to be unlikely. Not only do the endites of anthracomartids lack a battery of gnathal spines, the deeply nested

position of Trigonotarbida (i.e. Pantetrapulmonata within Arachnopolmonata) makes it highly unparsimonious that Anthracomartidae has retained coxal endites, where all other arachnid lineages have independently lost them. Indeed, Garwood and Dunlop (2011) recognised this as unlikely, and suggested the presence of coxal endites in anthracomartids and palaeocharinids is a synapomorphy linking the two families. This character is restricted to a clade that unites these families in phylogenetic analysis of Trigonotarbida (character 36 of Jones et al., 2014).

If it is accepted that the prosomal gnathobase series is a plesiomorphic character for Euchelicerata, then their presence in horseshoe crabs is surprising if Xiphosura is nested within Arachnida. The most recent and comprehensive morphological phylogeny of fossil and extant horseshoe crabs shows that despite several Palaeozoic and Mesozoic marine to freshwater invasions within Xiphosurida, a marine lifestyle is clearly plesiomorphic for the crown group - with that lineage and marine occurrence tracing back to the Early Ordovician (Van Roy, 2010; Lamsdell, 2016). As such, an argument that horseshoe crabs are secondarily marine arachnids would rely on the inference of an extremely geologically early re-invasion of the marine realm from established terrestrial diversity, with horseshoe crab gnathobases representing a convergent reacquisition. As such, multiple land colonisations among arachnids - with horseshoe crabs retaining typically aquatic characters such as gnathobases - is the more stratigraphically realistic scenario if Xiphosura is nested within Arachnida. Under this scenario, multiple aquatic ancestors of extant arachnid orders are expected in the fossil record. The alternative is that the common ancestor of extant arachnids (excluding horseshoe crabs) already lived on land (or was semi-aquatic, see Lamsdell et al., 2020), having lost or reduced its gnathobase series to feed in terrestrial environments. This would be an apomorphy representing adaptation to a terrestrial lifestyle for crown group Arachnida (which supports a single terrestrialization according to Scholtz and Kamenz, 2006).

Associated with a shift from gnathobasic feeding, arachnids exhibit reduced mobility of their coxae compared to Xiphosura - including the endite-bearing trigonotarbids (Garwood and Dunlop, 2014b). Mobility of the coxae is necessary to facilitate the synchronous crushing/shredding action along the food groove required for aquatic mastication by coxal endites. Haug et al. (2019) provide an account of coxal (“basipodal” in their general euarthropod terminology) mobility in euchelicerates that involves a stepwise reduction from Xiphosura to a clade that unites eurypterids and arachnids to a monophyletic Arachnida. Gnathobasic feeding is facilitated in Xiphosura by movement of the coxae against the body in prosomal (postocular segments 2–7) and opisthosomal segments (postocular segments 8–13); and the promoter-remoter swing in the prosomal appendages is between the coxa and the body. In Eurypterida (where gnathobasic endites are still present) most movement in the appendages of segments 2–7, including promotion and remotion, is between the coxa and trochanter rather than the coxa and body (Selden, 1981), and mobility is hardly present at the coxa-body joint in segments 8–13. Finally, in Arachnida the coxa has a broad attachment to the body wall in segments 2–6, with segments 5–7 not employed in feeding. As in eurypterids, promoter-remoter swing in arachnids is post-coxal, and the opisthosomal segments in arachnids have immobilised coxae essentially forming sternitic plates.

2.2. The homology of book gills and book lungs, and its bearing on euchelicerate phylogeny

The respiratory appendages of the opisthosoma have drawn much phylogenetic discussion regarding horseshoe crabs and

arachnids – or rather pulmonate arachnids (Scholtz and Kamenz, 2006; Farley, 2010, 2011, 2012, 2015, 2016). Horseshoe crabs respire through book gills, which also aid their propulsion in swimming, and are located in pairs associated with segments 8–13. These lamellate appendicular structures have been regarded as homologues to arachnid book lungs since the 19th century (Kingsley, 1885) – which are similarly lamellate, but inverted, internal, and function in air rather than water. Lamellate book gill pairs on the opisthosoma have also been reported for Eurypterida (Braddy et al., 1999) and are believed to be homologous with those of Xiphosura. As such, authors have regarded book gills as the precursor to book lungs, and the transition between them is hypothesised to be associated with terrestrialization (Selden and Jeram, 1989). This hypothesis is supported by the recent description of trabeculae between the book gill lamellae of the Carboniferous eurypterid *Adelophthalmus pyrrrhae* (Lamsdell et al., 2020). Trabeculae are columnar structures that prevent the collapse of the lamellae in air, which suggests that *Adelophthalmus* was an amphibious eurypterid capable of air-breathing. Trabeculae are similarly present between the lamellae of the book lungs of pulmonate arachnids, their conical bases and narrow pillars supporting homology with those of *Adelophthalmus*. Trabeculae are, crucially, lacking in the book gills of horseshoe crabs – which are capable of only limited amphibious excursions. Several other lines of evidence suggest eurypterids were a part-amphibious group, including subaerial locomotory trace fossils (Whyte, 2005; Draganits et al., 2001) and spermatophore-mediated reproduction (Kamenz et al., 2011). These data suggest that a set of terrestrial adaptations were accrued within the stem lineage of Arachnida and were present in the last common ancestor of arachnids and eurypterids (Lamsdell et al., 2020).

A number of areas of research further indicate that the long-standing book-gill to book–lung transition is likely. Though studies were inconclusive as to whether the embryological development of book gills in the horseshoe crab *Limulus polyphemus* and book lungs in the scorpion *Centruroides gracilis* are consistent with them being homologous (Farley, 2010, 2011), similar studies on the spider *Parasteatoda tepidariorum* (Farley, 2015, 2016) concluded that book lung/book gill homology is well supported. Furthermore, the transformation from typical biramous trunk appendages in stem-group chelicerates to lamellate respiratory structures with an accompanying operculum in crown group Euchelicerata can be reconstructed through a combination of palaeontological and evolutionary developmental methods. Euchelicerate trunk appendages are plesiomorphically biramous, comprising both an inner endopod (the telopodite in uniramous appendages) and outer flap-like exopod, which is exemplified in taxa at the base of the euchelicerate stem group such as *H. optata* from the Cambrian Burgess Shale (see Fig. 1). Crownward of *Habelia*, another Burgess taxon *M. plenovenatrix* has three overlapping lamellate exopod flaps on its trunk appendages, compatible with book gill lamellae being derived from the exopod and suggesting a stage in book gill evolution before the appearance of the operculum (Aria and Caron, 2019). Developmental studies indicate that the opisthosomal appendages of scorpions, including the appendicular component of book lungs (i.e. the opercula) incorporate the telopodite of their ancestral serial homolog (Sharma, 2017; Di et al., 2018). As such, book lung opercula, at least in scorpions, are serially homologous with telopodal structures (e.g. walking legs and presumably endopods). These complementary studies therefore reveal the homology of book gill and book lung lamellae and opercula with the rami of biramous appendages of stem-group euchelicerates.

A derived position of Xiphosura within the arachnids (e.g. as sister group to Arachnopulmonata, e.g. Noah et al., 2020) potentially suggests that book lung-bearing arachnopulmonates evolved and terrestrialized from book gill-bearing ancestors separately to

apulmonate groups (Acari, Palpigradi, Pseudoscorpiones, Ricinulei, Opiliones, Solifugae). Therefore, apulmonate groups may have terrestrialized with tracheal respiration via the replacement or transformation of book gills, without the gill to lung transition that is exemplified by eurypterids and pulmonate arachnids (Lamsdell et al., 2020). However, this hypothesis cannot be robustly tested without further understanding of the evolution of the tracheae in apulmonate groups, for which there is a lack of stem-group taxa at present. As such, it is not possible to ascertain whether tracheae appeared after an initial gill–lung transition or not. It is also possible that the book gills in Xiphosura are a reversal from terrestrial ancestors if Xiphosura diverged within the arachnids – a scenario that is difficult to reconcile with the Early Ordovician marine stem lineage of Xiphosura (as for gnathobasic feeding, see 2.1). In molecular phylogenetic studies in which Arachnida is not monophyletic, Xiphosura is sometimes recovered as sister group to Ricinulei (Sharma et al., 2014; Ballesteros and Sharma, 2019; Ballesteros et al., 2019), and sometimes as sister group to Arachnopulmonata (Noah et al., 2020) or a clade resembling but also including Pseudoscorpiones (Matrix B in Lozano-Fernandez et al., 2019). However, Ricinulei and Pseudoscorpiones are both groups of generally small body size, and it is conceivable that miniaturization resulted in considerable within-lineage respiratory change due to increased surface area to volume ratio – with tracheae serving for respiration rather than book lungs (Dunlop, 2019). If book gills and book lungs are accepted as homologues, then the trees presented here showing arachnid monophyly (Figs. 2 and 3) can be interpreted as such in light of Pseudoscorpiones being recovered as sister group to Scorpiones, or in a close relationship with arachnopulmonates. Ricinulei is recovered as the sister group to Solifugae, with Opiliones as outgroup, implying that reduction in body size may have evolved later than the shift from book lungs/gills to tracheae. Regardless of the specific internal branching order, if homology of book gills/lungs is accepted and arachnids are monophyletic, implicitly the common ancestor of arachnids had book lungs/gills, which were subsequently replaced by tracheae among non-pulmonate groups (see Fig. 1).

2.3. The fate of compound eyes

Chelicerates possess two kinds of eyes – median and lateral. Aside from their position on the prosoma (which varies between taxa), the eye groups can be distinguished by their corresponding innervation relative to the brain and optic neuropils – even at deep levels of chelicerate phylogeny (Strausfeld et al., 2016). Across all living chelicerates, and some extinct groups such as Eurypterida and Chasmataspida, the median eyes each comprise a single lens as opposed to multifaceted compound eyes. Compound eyes are uniquely present in Xiphosura among extant Chelicerata, set on ophthalmic ridges laterally on the prosoma. As they are shared by Mandibulata, compound eyes in Xiphosura are classically seen as a sympleisiomorphy relative to the lateral simple eyes of arachnids (Strausfeld et al., 2016). Multifaceted compound eyes have also been described in Cambrian radiodonts (Paterson et al., 2011), and therefore it is clear compound eyes appeared very early in arthropod evolution – preceding arthrodization of the trunk and the development of articulated post-protocerebral appendages (Strausfeld et al., 2016).

Eurypterids, which are generally interpreted as stem-group arachnids (e.g., Kamenz et al., 2011; Lamsdell, 2013; Legg et al., 2013; Aria and Caron, 2019), are the only other crown-group chelicerates that unambiguously have compound lateral eyes. These are each composed of up to a few thousand ommatidia (Anderson et al., 2014; McCoy et al., 2015), and their cornea is thickened as an exocuticular cone as in Xiphosura (Schoenemann et al., 2019). A

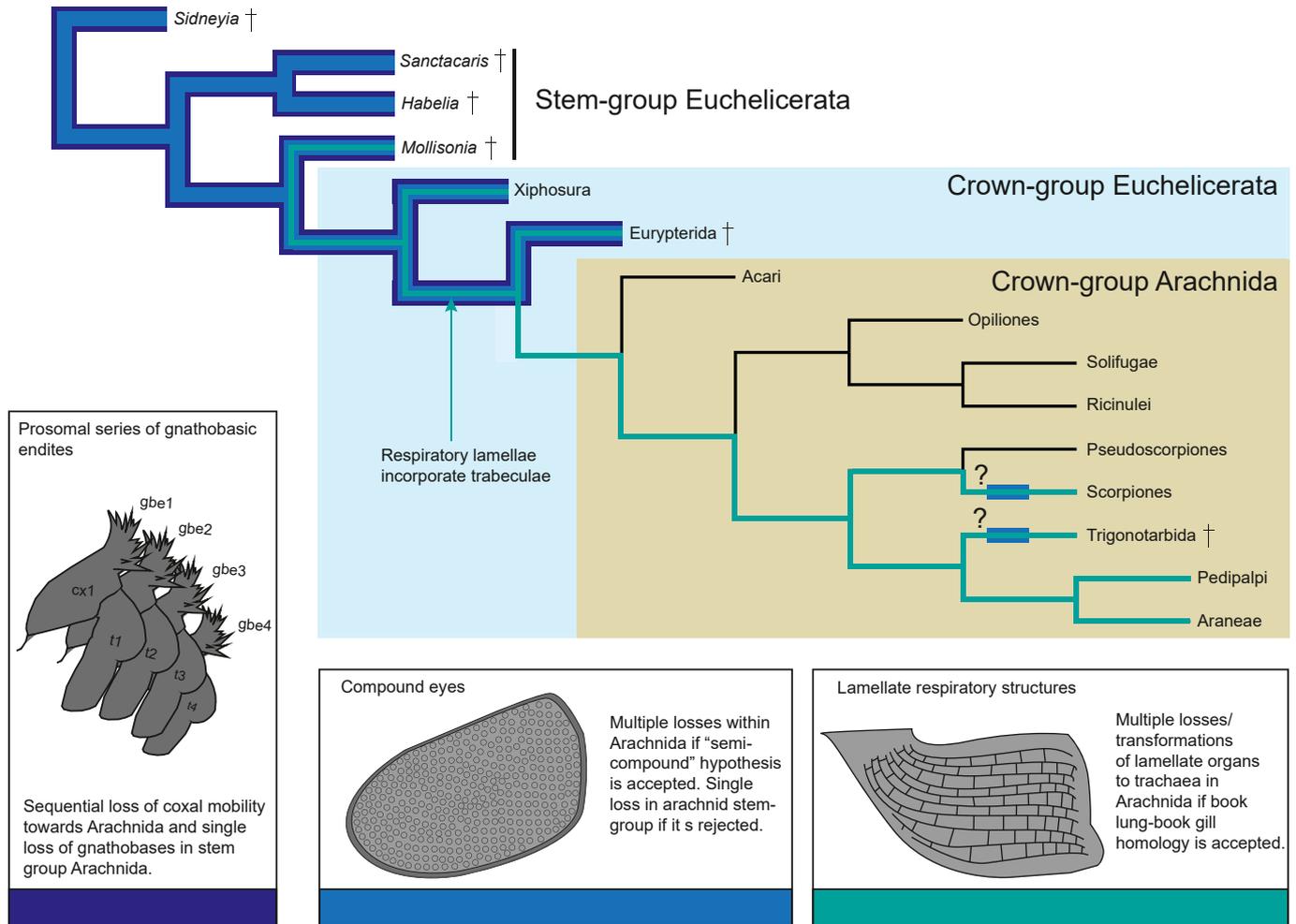


Fig. 1. Topology depicting the consensus of extant chelicerate relationships recovered by this study, with fossil groups added in their best-supported positions. Question marks on the branches leading to Scorpiones and Trigonotarbida represent uncertainty over the “semi-compound” eye hypothesis (see section 2.3 in the main text). Position of trabeculae acquisition according to Lamsdell et al. (2020). Daggers mark fossil taxa. Abbreviations on gnatobase illustration: cx = coxa, t = trochanter, gbe = gnatobasic endite.

survey of the eyes of “synziphosurines” indicates that some taxa lack lateral eyes completely, whereas Palaeozoic representatives of Xiphosura have either elongate, crescentic eyes or reniform eyes like those of extant xiphosurans (Bicknell et al., 2019). Comparative study of these similar ocular structures has, therefore, led palaeontologists to conclude that the lateral compound eye is a plesiomorphic character for Chelicerata (Schoenemen et al., 2019).

In the context of its monophyly, Arachnida had been supported by lateral eyes with a reduced number of simple lenses, with Weygoldt and Paulus (1979) inferring an ancestral state of five or fewer lenses. The fossil record indicates a more complex picture. It has been observed that greater lens numbers are found in various Palaeozoic and Mesozoic arachnids, and even extant arachnids sometimes depict a few more than five lenses (Miether and Dunlop, 2016). Relevant examples include up to ca. 15 lenses in some Trigonotarbida (Shear et al., 1987; Fayers et al., 2005), these eyes having been characterised as semi-compound (Miether and Dunlop, 2016). However, the individual lenses are distinctly separated from their neighbours, neighbouring lenses vary in size and in some cases in shape, and organised rows of lenses are not obvious. In each of these respects, trigonotarbid eyes resemble arachnid simple lateral eyes rather than compound eyes. Compound eyes have been regarded as a typical character of early branching scorpions (Kjellsvig-Waering, 1986), although in most of the cited

instances the evidence consists of large lateral eye tubercles rather than preservation of a compound visual surface. *Proscorpius obsborni*, a species argued to depict compound eyes, does not allow for a distinction between lenses and the texture of the surrounding cuticle (Dunlop et al., 2008, Plate 1, text-Figure 5). Other early scorpions, such as *Paraioscorpio venator* (Wendruff et al., 2020), have comparably large lateral eye tubercles, but lenses are likewise not preserved. Nevertheless, the best-preserved fossil scorpion eyes demonstrate unequivocally that at least some early scorpions possessed a field of ca. 30 lenses. This is the case for Triassic species of *Mesophonus*, which have 28–35 lenses on the surface of their eye tubercles, and the Carboniferous *Kronoscorpio danielsi*, which has 25–29 lenses (Miether and Dunlop, 2016).

While the “five or fewer” scenario for the last common ancestor of Arachnida is clearly contradicted by this evidence, neither the trigonotarbid nor fossil scorpion examples provide a strong case for being compound eyes. The fossils do not show a clear patterning of the lenses into organised rows as typifies the row-by-row mode of eye growth that extant Xiphosura share with trilobites and myriapods (Harzsch et al., 2006). Although *L. polyphemus* shows a measure of deviation from exact hexagonal dense packing of neighbouring ommatidia (Harzsch et al., 2006), its overall patterning is highly ordered into rows that reflect the addition of new ommatidia at the margins of the visual field. Trigonotarbids

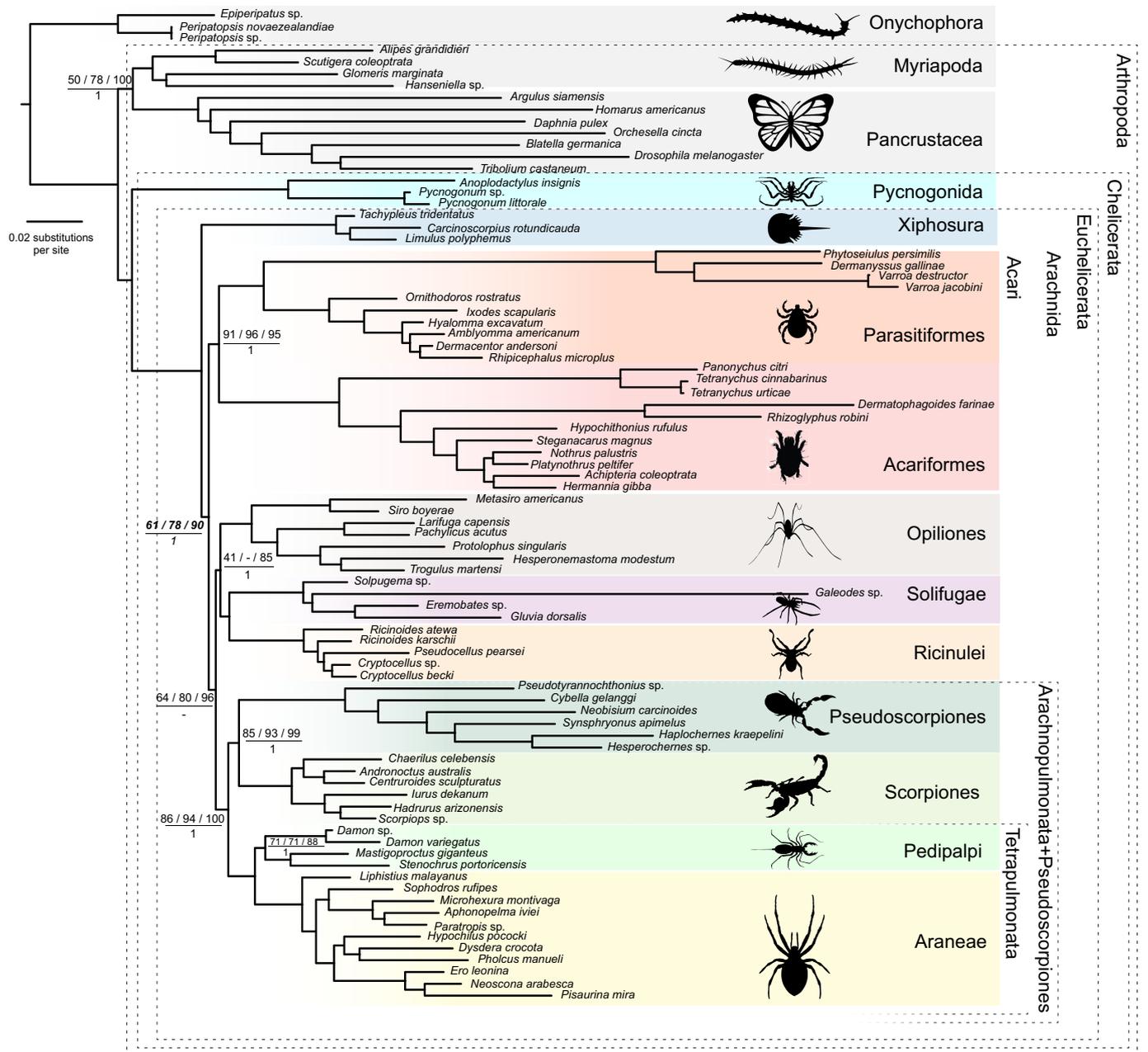


Fig. 2. Phylogenetic tree derived from the LG + C20 + R5 analysis of the 200 slow-evolving loci. Support values represent the UFB of 3 different ML analyses with increasingly better fit in the upper part (LG + F + Γ , LG + R5 and LG + C20 + R5; from left to right) and the posterior probabilities (PP) for the CAT – Poisson analysis below. Support values are just presented for those nodes leading to orders or higher ranks, but most internal splits present UFB or PP greater than 90 and 1, respectively. Convergence statistics of PhyloBayes analysis: Burn in = 2,500; Total Cycles = 10,000; Subsampling frequency = 10; Maxdiff = 0.29; Minimal effective size = 203.

and fossil scorpions have a few dozen lenses at most, unlike the hundreds to thousands in extant xiphosurans and in eurypterids and cannot confidently be distinguished from the lateral eyes of most centipedes and millipedes, which are organised as clusters of simple lens eyes.

These fossils therefore illustrate a picture of either repeated loss of lateral compound eyes among the terrestrial arachnids, with mesoscorpion and palaeocharnid trigonotarbid eyes as transitional examples; or that the common ancestor of arachnids lacked compound eyes, and the multi-lens clusters of these fossils are autapomorphies. Numerous losses of lateral compound eyes within a monophyletic Arachnida (see Fig. 1) are inferred if this transitional

hypothesis is accepted. This extent of homoplasy, combined with morphological arguments against “semi-compound” eyes in fossil arachnids noted above, are challenges for the transitional hypothesis - but it is noted that multiple losses of eye types have been documented in crustaceans (Henze and Oakley, 2015). The inclusion of Xiphosura within Arachnida affects this reasoning, as it is implicit that the common ancestor of arachnids may have had typical aquatic chelicerate compound eyes with thousands of lenses, and reduction was therefore more extreme in each arachnid group. By contrast, a monophyletic Arachnida could imply a rudimentary or reduced lateral compound eye was possessed by the ancestral arachnid, which was subsequently reduced repeatedly

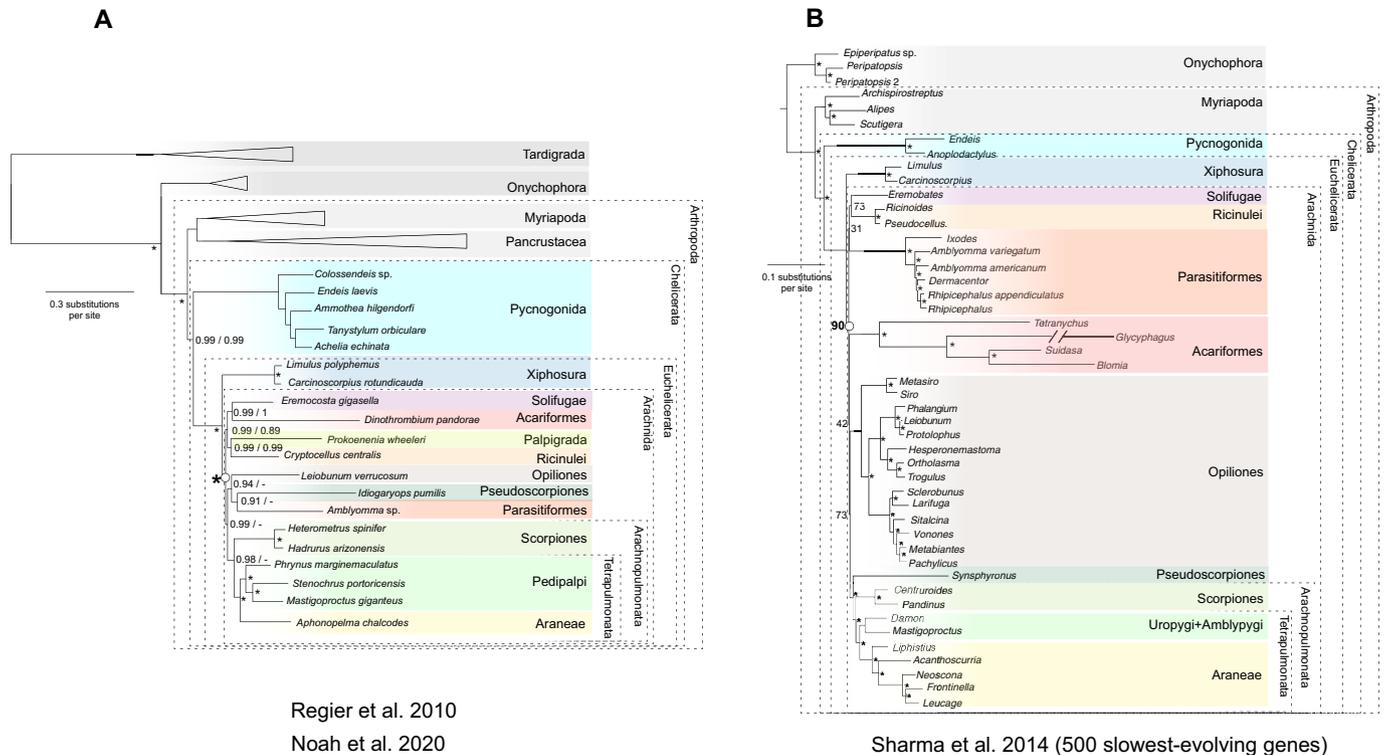


Fig. 3. Reanalysis using site-heterogeneous models of the following matrices: a) Regier et al. (2010) and Noah et al. (2020) using CAT – GTR + Γ in PhyloBayes. Support values represent PP, asterisk (*) represents full support of 1. b) 500 slow evolving loci dataset of Sharma et al. (2014) using LG + C20 + F + Γ in IQTREE. Support values represent UFB, asterisk represents support >95. Outgroups are collapsed in both trees. Convergence statistics of PhyloBayes analyses: For Regier et al. (2010) Burn in = 2,000; Total Cycles = 10,000; Subsampling frequency = 10; Maxdiff = 0.13; Minimal effective size = 53. For Noah et al. (2020) Burn in = 2,000; Total Cycles = 10,000; Subsampling frequency = 10; Maxdiff = 0.18; Minimal effective size = 69.

within derived lineages. Finally, if the “semi-compound” eye hypothesis is rejected, it is inferred the common ancestor of monophyletic Arachnida had only simple lens lateral eyes.

2.4. The stem-group scorpion conundrum - marine or terrestrial ancestry?

A confounding argument against a single arachnid terrestrialization event is that of a potentially plesiomorphic aquatic lifestyle for the scorpion total-group. Scorpions have an extensive Palaeozoic stem lineage - summarised recently in Howard et al. (2019), and new Palaeozoic species have since been described by Wendruff et al. (2020) and Martine et al. (2020). Despite typically lacking obvious adaptations to an aquatic mode of life, Silurian and Early Devonian scorpions are mostly recovered from marine deposits, and it has been inferred that scorpions are ancestrally aquatic (Selden and Jeram, 1989; Dunlop and Webster, 1999), which has been interpreted as support for the non-monophyly of Arachnida (Noah et al., 2020; Nolan et al., 2020). The existence of aquatic basal stem group scorpions would suggest that scorpions originated in the water, which was amplified by their proposed sister group relationship with the Eurypterida in the past (Selden and Jeram, 1989; Dunlop and Webster, 1999; Dunlop and Braddy, 2001). It now appears clear from diverse kinds of data that scorpions are the sister group to tetrapulmonates (Scholtz and Kamenz, 2006; Regier et al., 2010; Garwood and Dunlop, 2014a; Sharma et al., 2014; Klüßmann-Fricke and Wirkner, 2016; Leite et al., 2018; Ballesteros and Sharma, 2019; Lozano-Fernandez et al., 2019; Ballesteros et al., 2019; Noah et al., 2020). It remains, however, that if scorpions originated in the water, as some Palaeozoic scorpion fossil taxa suggest they may have, then scorpion terrestrialization is

presumed to have been independent of other arachnid lineages - regardless of the position of Xiphosura. For example, even if Arachnida is monophyletic, aquatic ancestry for scorpions could imply they colonised the land independently of both tetrapulmonate and non-pulmonate arachnids. This hypothesis also predicts the convergent evolution of book lungs in scorpions and tetrapulmonates, despite their highly detailed similarity (Scholtz and Kamenz, 2006), and very strong molecular arguments that they are each other's closest relatives.

However, it is questionable that scorpions are ancestrally aquatic (Dunlop et al., 2008; Poschmann et al., 2008; Kühl et al., 2012; Dunlop and Selden, 2013; Waddington et al., 2015; Wendruff et al., 2020). No known scorpion fossil is both: 1) attributed to a deposit that unequivocally implies an aquatic lifestyle for the scorpion, and 2) exhibiting adaptations for an aquatic lifestyle without caveat. Three taxa key to this criticism are *Waeringoscorpio* (Størmer, 1970), *Palaeophonus nuncius* (Thorell and Lindström, 1885), and *Allopalaeophonus caledonicus* (Hunter, 1886). These are the only scorpion fossil taxa that show morphological evidence for an aquatic lifestyle, but in spite of that it is not clear that they support the hypothesis of an ancestrally aquatic scorpion total-group. *Waeringoscorpio* from the Lower Devonian of Germany possesses externally projecting filamentous gills consistent in segmental attribution with scorpion book lungs, which led authors to conclude, considering the fluvial/deltaic palaeoenvironment, that it was living a benthic life in a freshwater-brackish environment (Poschmann et al., 2008). Crucially however, these gills are unique among all chelicerates. Poschmann et al. (2008) regarded these gills as convergent with freshwater insect gills, and autapomorphic to the genus *Waeringoscorpio*. As such, Poschmann et al. (2008) did not consider *Waeringoscorpio* an ancestrally aquatic scorpion, but rather a secondarily aquatic one with gills derived

from book lungs. No other fossil scorpion exhibits any kind of gill. Therefore, without corroborating fossils of stem group scorpions illustrating a shift of gills to lungs from stemward to crownward scorpions, *Waeringoscorpio* does not support an aquatic lifestyle as plesiomorphic for scorpions. *Waeringoscorpio* is an outlier, with a unique adaptation to living in rivers and estuaries.

Palaeophonus and *Allopalaeophonus*, from the Silurian of Sweden and Scotland, respectively, exhibit “crab-like” legs lacking paired tarsal claws according to the reconstructions of Thorell and Lindström (1885), Pocock (1901) and Kjellesvig-Waering (1986) – distinguishing them from all other fossil and recent scorpions. This has been interpreted as evidence that these species were aquatic (Kjellesvig-Waering, 1986) – despite crabs being capable of terrestrial locomotion (see Dunlop et al. (2008) and Waddington et al. (2015) for more discussion of tarsal characters in early scorpions). More convincing evidence for an aquatic lifestyle comes from the depositional setting in *Palaeophonus* at least, the locality of which is better (though still poorly) constrained. *Palaeophonus* is believed to come from a fully-marine carbonate setting below storm wave base, which lacks terrestrial plants and arthropods as allochthonous transported components – which are known to occur alongside other early scorpion taxa (Kühl et al., 2012; Dunlop and Selden, 2013). Additionally, *Palaeophonus* and *Allopalaeophonus* lack the derived coxosternal region of living scorpions and have been considered phylogenetically basal (Jeram, 1998). Therefore, it is tempting to view them as more reflective of the ancestral scorpion. However, paired tarsal claws are widespread among arachnids. Assuming homology between scorpion tarsal claws and those of other arachnid groups, it is more parsimonious that tarsal claws were present in the ancestral scorpion, given that molecular phylogenetic studies are at a consensus regarding the position of scorpions in Arachnospulmonata. As such, if the crab-like clawless legs of *Palaeophonus* and *Allopalaeophonus* are truly indicative of an aquatic lifestyle, then like *Waeringoscorpio*, it is more likely for this to be an autapomorphy of aquatic adaptation, despite their purportedly basal phylogenetic position within scorpions (see Jeram, 1998). Based on the Cambrian–Ordovician divergence times recovered for Arachnida in their study (and in this study, see Fig. 4), Lozano-Fernandez et al. (2020) hypothesised that there is a considerable gap in the fossil record of terrestrial arachnids due to the low proportion of terrestrial early Palaeozoic sediments exposed today, as is similarly predicted for land plants (Kenrick et al., 2012). A shift to a marine lifestyle in the lineage of *Palaeophonus* and *Allopalaeophonus* in the Silurian could explain why these scorpions are present in fully-marine deposits at a time when terrestrial arachnid fossils are unknown, but predicted to exist by molecular clocks.

The geologically oldest fossil attributed to Scorpiones, the Llandovery *Parioscorpio venator* Wendruff et al. (2020), preserves structures described as pulmo-pericardial sinuses, which in extant scorpions connect the book lungs with the circulatory system. While this could conceivably also be the case in a stem-group scorpion that had book gills, the identity of respiratory structures in a Silurian and modern scorpions is suggestive of terrestrial respiration in the fossil species.

3. Molecular systematics

3.1. Previous research on arachnid systematics

As noted above, morphological systematics of Chelicerata has generally recovered arachnid monophyly (Weygoldt and Paulus, 1979; Shultz, 2007; Garwood and Dunlop, 2014a; Legg et al., 2013; Aria and Caron, 2017, 2019), albeit with the relationships between arachnid orders largely in discordance apart from

monophyly of Tetrapulmontata. Early attempts at combining morphological characters and a few molecular markers resulted in mixed hypotheses, either supporting Arachnida (Wheeler and Hayashi, 1998), or instead retrieving Acariformes as the earliest diverging clade of Euchelicerata, followed by Xiphosura (Giribet et al., 2002). The first attempt at using a large targeted Sanger sequenced dataset was in the context of a global arthropod phylogeny, comprising 62 nuclear protein-coding genes (Regier et al., 2010). At the nucleotide level, the authors implemented multiple analyses to counteract sources of systematic error, as well as analysing their dataset at the amino acid level, with most of their results yielding moderate support for arachnid monophyly. An updated reanalysis of that matrix correcting some errors in the gene alignments and implementing a new codon degeneration strategy recovered instead Xiphosura as the sister lineage of lung-bearing arachnids (Noah et al., 2020).

The first chelicerate-focussed phylogenomic study (Sharma et al., 2014) analysed amino acid sequences of >3,600 protein-coding loci. The authors found consistent support for an alliance between lung-bearing arachnids, the Arachnospulmonata. This result was anticipated by Regier et al. (2010) as well as some morphologists (e.g. Firstman, 1973), which have been largely corroborated in further studies based on diverse types of data, including anatomy, gene expression and genome duplications (Klußmann-Fricke and Wirkner, 2016; Leite et al., 2018; Nolan et al., 2020). Most analyses in Sharma et al. (2014) yielded non-monophyly of Arachnida, a result deemed to be the product of long branch attraction (LBA) artefacts caused by fast-evolving orders such as Acariformes, Parasitiformes and Pseudoscorpiones. When genes were concatenated based on their rate of molecular evolution, a certain support for arachnid monophyly was restricted to the 1,000th slowest evolving genes, with full support just being found when the 500 and 600 slowest evolving loci were analysed together. Further analyses of that 500 slow-evolving loci matrix, applying the best fitting model per gene partition instead of the same model for all, instead recovered arachnid non-monophyly (Ballesteros and Sharma, 2019). Genes with low rates of evolution are less likely to exhibit homoplasy and therefore less prone to systematic error and are seen as good candidates to be used in phylogenies with old divergences.

Some more recent phylogenomic studies with broader taxon sampling, thousands of genes and more complete sets of analyses did not find support for arachnid monophyly in a single instance, apart from when cherry-picking just those genes favouring that particular hypothesis. The authors of these studies proposed that horseshoe crabs are aquatic arachnids, and that they may be the sister group of Ricinulei (Ballesteros and Sharma, 2019; Ballesteros et al., 2019). On the contrary, Lozano-Fernandez et al. (2019) recovered arachnid monophyly in one of the two phylogenomic matrices they analysed containing 89 arthropod taxa (with 70 chelicerates). That matrix, named “Matrix A”, was composed of 233 protein coding genes that had been previously used in other animal phylogenies, mostly selected because they were actively expressed in most tissues and present in a large array of taxa. The authors also analysed a second dataset of similar final size, named “Matrix B”, composed by 3,982 loci that were concatenated and then stringently trimmed from poorly aligned positions. All analyses of Matrix B retrieved non-monophyly of Arachnida, although the rest of the topology was mostly congruent with the analyses of Matrix A.

3.2. Phylogenetic analyses

In order to test the signal for Arachnida in different datasets, we inferred phylogenies from three independently generated matrices, with two of them not recovering arachnid monophyly in previous

studies under certain parameters. These include a dataset using a small subset of slowly evolving genes retrieved from Matrix B in [Lozano-Fernandez et al. \(2019\)](#); the matrix of [Noah et al. \(2020\)](#) which is derived from [Regier et al. \(2010\)](#) and did not yield arachnid monophyly; and the 500 slowest evolving loci dataset presented in [Sharma et al. \(2014\)](#) that also did not retrieve arachnid monophyly in another subsequent study ([Ballesteros and Sharma, 2019](#)). A summary with the software and models of molecular evolution used to analyse each supermatrix, as well as some matrix features, can be found in [Table S2](#).

Based on the presented results and previous studies, we hypothesize that a) a phylogenetically diverse taxon sampling is needed to infer arachnid relationships using b) a considerably large number of high-occupancy loci, and that the phylogenetic signal is most likely to be retained in (c) genes that are less prone to be affected by systematic biases, such as the slowly-evolving ones. As the splits of the main chelicerate orders date back to the Cambrian and Ordovician (see section 3.3) we suggest that the signal is eroded in the fast evolving loci. Conversely, the slowly evolving genes, although lacking many informative sites, are more likely to preserve genuine phylogenetic signal in their differing sites. It is beyond the scope of this manuscript to fully dissect the molecular signal for Arachnida. Nonetheless, the presented results show how its support increases when a) better fitting models of evolution are used in a slow-evolving loci matrix, and b) complex mixture models are employed in matrices that originally retrieved arachnid paraphyly under simpler models.

3.2.1. 200 slowly evolving loci matrix

We attempted to build a highly complete chelicerate molecular matrix with a wide taxon sampling and comprising genes with slow rates of molecular evolution. Our aim is to assess chelicerate relationships using increasingly complex models of evolution; therefore, we restricted the size of the supermatrix to 200 loci, being within the largest size range allowing the use of the computationally demanding CAT models in Bayesian framework. The dataset is based on Matrix B from [Lozano-Fernandez et al. \(2019\)](#), in which we improve the design by removing some poor transcriptomes, replacing them by high-quality Illumina ones ([Table S1](#)). This aims to reduce the levels of missing data, which has been shown to exacerbate systematic errors in phylogenomics ([Roure et al., 2013](#)). In order to reach an even representation of Arachnida at the ordinal level, we have included more internal diversity from some orders (such as Pseudoscorpiones), while reducing the number of species of others (Araneae and Scorpiones), and including a member of Schizomida. Furthermore, we have followed several of the suggestions of [Ballesteros et al. \(2019\)](#) and improved the previous matrix design by a) effectively removing any gene with less than 35% representation among the taxa; b) trimming poorly aligned positions at the gene level; c) removing genes from the gene matrices with unusually long branches, these being six standard deviations from the rest using the script LBRem.py [<https://github.com/pnatsi/LBRem>]; d) an extra sanitization step to remove sequences containing less than 25% of the size of the alignment, and alignments with a total length below 50 amino

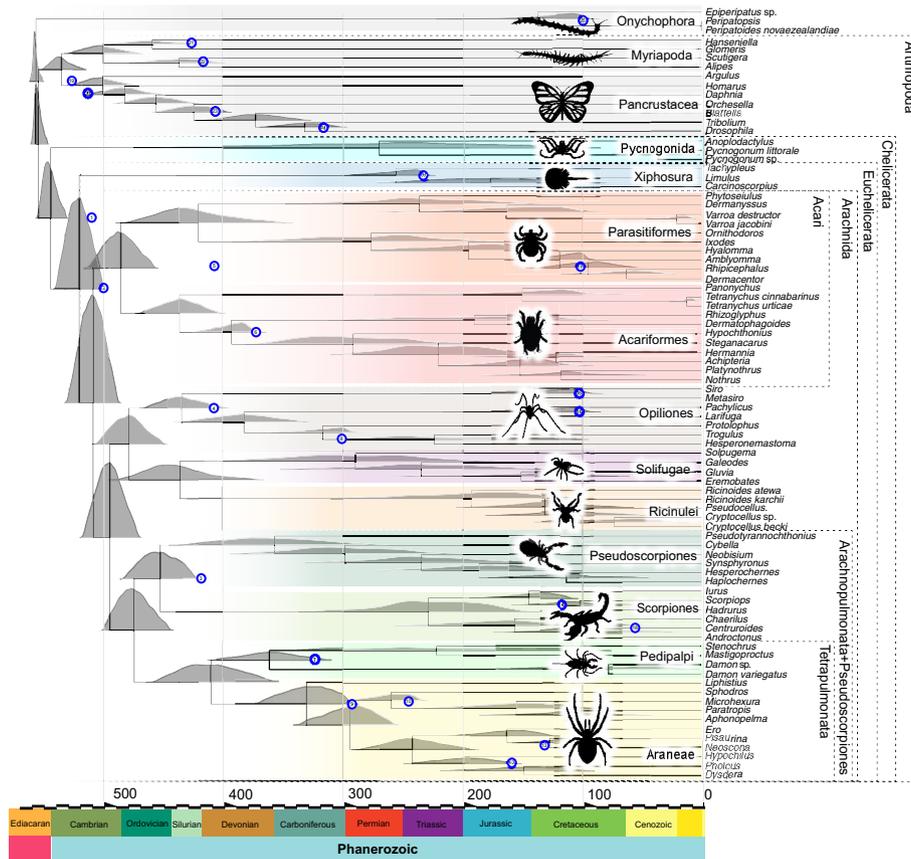


Fig. 4. Chelicerate divergence times derived from the analysis of 200 slow evolving loci. Divergence times shown are obtained under the uncorrelated (IGR), relaxed clock model in MCMCtree. Nodes in the tree represent average divergence times estimated. The density plots represent the posterior distributions for the node that they calibrate. The numbered blue circles represent the age of the fossil calibrations and are located at a height corresponding to the node that they calibrate (see [Table S3](#)). Timescale on the X axis = millions of years before present. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

acids using the script *Al2Phylo.py* script (Ballesteros and Hormiga, 2016). This step reduced the original dataset to 3,948 loci. We then inferred gene trees using the LG + F + Γ model in IQ-TREE (Nguyen et al., 2015) and e) ordered the loci by evolutionary rate based on dividing the total length of the trees by the number of taxa. Finally, f) we removed from the final analysis 10 loci with a calculated evolutionary rate <0.01, that were composed mostly of sequences with 100% identity amongst species that did not yield useful phylogenetic information. The complete dataset consisted of 3,938 loci trimmed from poorly aligned positions, removed from excessively long branches, and sanitized from incomplete sequences. We concatenated the 200 slowest-evolving genes, resulting in a molecular supermatrix with 50,468 amino acid positions, a taxon occupancy of 91% (i.e., every gene present on average in ~91% of the taxa) and completeness of 83.9% (thus 16.1% missing data). Compared with the 233-gene Matrix A presented in Lozano-Fernandez et al. (2019), both matrices present an overlap of 27% of the loci. Additionally, we compared the saturation levels of these 200 slowly evolving genes against the complete dataset and found that they are substantially less saturated (included as a Fig. S1 together with the evolutionary rate comparison). The level of saturation was estimated at the gene level instead of being based on the entire supermatrix (as in Lozano-Fernandez et al., 2019), and was calculated as one minus the slope of the regression of patristic distances versus p-distances (Nosenko et al., 2013).

We analysed the 200-loci matrix under different models of molecular evolution. Phylogenetic trees were inferred using Maximum Likelihood (ML) in IQ-TREE (Nguyen et al., 2015) under three different models of evolution and using a Bayesian framework in PhyloBayes MPI v.4.1 (Lartillot et al., 2013) under the site-heterogeneous CAT – Poisson model of amino acid substitution (Lartillot and Philippe, 2004). For the PhyloBayes analysis, convergence was assessed by running two independent Markov chains and using the *bpcomp* and *tracecomp* tools from PhyloBayes to monitor the maximum discrepancy in clade support (maxdiff), the effective sample size (effsize), and the relative difference in posterior mean estimates (rel_diff) for several key parameters and summary statistics of the model. We ran the analysis for 10,000 cycles and discarded the first 2,000 generations as ‘burn in’. When analysed with the site-heterogeneous CAT – Poisson model in PhyloBayes, arachnid monophyly received full support (Posterior Probability = 1). Results under LG + F + Γ model on IQ-TREE retrieved Arachnida with a weak support value of 61, expressed as Ultrafast Bootstrap (UFBoot - Hoang et al., 2018). Analysis using the best-fitting model according to ModelFinder, LG + R5, resulted in an increase of support (UFBoot 73). Applying more realistic site-heterogeneous mixture models using the posterior mean site frequency (PMSF) approach (Wang et al., 2018) under the LG + C20 + R5 model, the support value increased to 90. We compared on that matrix the fit of those models in IQ-TREE and found that the best fitting model according to the Bayesian information criterion was the site-heterogeneous mixture model (log-likelihood values in Table S2). All four topologies were largely congruent (Fig. 2), retrieving an alliance between Pseudoscorpiones and Scorpiones, with this clade as sister to Tetrapulmonata. This alliance, or in a lesser degree pseudoscorpions as the closest relatives of arachnopulmonates, has been found recently in several phylogenomic studies, mostly in analyses of slowly evolving genes (Sharma et al., 2014; Ballesteros and Sharma, 2019; Lozano-Fernandez et al., 2019; Ballesteros et al., 2019). A Pseudoscorpiones–Scorpiones group has also been found recently in several morphological phylogenies, but not in a close relationship with tetrapulmonates (Pepato et al., 2018; Garwood and Dunlop, 2014a; Wang et al., 2018; Huang et al., 2018). Under these scenarios, a likely hypothesis is that pseudoscorpions are

members of Arachnopulmonata that could have lost book lungs in their course of evolution, concomitantly with their miniaturization process (Dunlop, 2019). Another commonality is the alliance between Parasitiformes and Acariformes (=Acari), as previously found in other phylogenomic analyses (Lozano-Fernandez et al., 2019). The ML analyses just differed on the position of Opiliones, being either the sister group of Ricinulei and Solifugae, or as the sister group to the rest of the arachnids except Acari. The CAT model employed in Bayesian inference found the arachnopulmonates + Pseudoscorpiones clade as the sister group of the remaining arachnids (Fig. 2).

3.2.2. Regier et al. (2010) and Noah et al. (2020) matrices

We reanalysed the original arthropod matrix of Regier et al. (2010), and the updated version presented by Noah et al. (2020). The Regier et al. (2010) matrix has been reanalysed in a few previous studies (Regier and Zwick, 2011; Rota-Stabelli et al., 2013). Rota-Stabelli et al. (2013), focussing on Pancrustacea relationships, established that the dataset was affected by saturation and compositional biases. Furthermore, the authors found an inconsistency between the analysis of amino acids and nucleotides, concluding that neither of the two types of data seems to bring enough genuine phylogenetic information to robustly resolve their relationships. One strategy to reduce the effects of saturation in molecular phylogenies is using amino acid sequence data instead of nucleotides. Amino acids are less informative, but they are also less susceptible to homoplasy. A second strategy is to use more realistic models of evolution, such as the site-heterogeneous CAT models, which provide a better fit to phylogenomic datasets and alleviate tree reconstruction artefacts. The small (compared to current standards) size of the 62-gene matrix allowed us to test the relationships based on those matrices using amino acids and the CAT – GTR + Γ model in PhyloBayes, a computationally demanding complex model that has been proven convenient for overcoming long-branch attraction (LBA) artefacts (Lartillot et al., 2007). Despite the aforementioned issues with this dataset, the Bayesian chains converged on a monophyletic Arachnida in both matrices with full support, with a similar internal topology within chelicerates (Fig. 3a).

3.2.3. 500 slowest-evolving loci matrix from Sharma et al. (2014)

This molecular matrix originally recovered arachnid monophyly with full support when using the same model, LG4X + F, per gene partition in a ML framework (Sharma et al., 2014). However, a subsequent reanalysis using the best fitting substitution and rate model per partition retrieved a different topology, in which Parasitiformes was the earliest split within Euchelicerata (Ballesteros and Sharma, 2019). We have reanalysed this matrix using ML in IQ-TREE (Nguyen et al., 2015) applying a complex mixture model, which allows rates to be modelled as heterogeneous across sites. Using as a starting tree a topology in which arachnids are not monophyletic, results from an unpartitioned analysis under the LG + F + Γ model, the site-heterogeneous LG + C20 + F + Γ model instead retrieves arachnid monophyly with a support value of 90 (UFB) (Fig. 3b). Other relationships are poorly supported, with extremely short branches in deeper nodes, but are largely in agreement with the 200-loci dataset presented above, with pseudoscorpions closely related to arachnopulmonates and ricinuleids in a sister group relationship with solifugids. The different results retrieved from this 500-locus dataset are model-dependent, suggesting poor phylogenetic signal. Nonetheless, arachnid monophyly is retrieved as the best supported hypothesis when complex mixture models of evolution are used.

3.3. Divergence time estimation

Molecular dating for chelicerates has recovered an early origin of arachnids and of their main diversifications, with dates bracketed between the Ediacaran (Ballesteros and Sharma, 2019) and Cambrian to Ordovician (Lozano-Fernandez et al., 2020). Therefore, there are significant differences in the ages inferred for terrestrial lineages between molecular dating and fossils, the latter appearing first in the Silurian. Using the topology retrieved from the site-heterogeneous C20 model in IQ-TREE (see previous section) we here estimate the divergence time of chelicerates according to the 200 slow-evolving loci matrix, in order to compare with ages obtained from “Matrix A” (Lozano-Fernandez et al., 2019), which was used to estimate divergence times in Lozano-Fernandez et al. (2020). We used a carefully selected set of 26 fossil constraints (Table S3), employing the most up to date stratigraphic information available across the tree. Minimum and maximum constraints are based on Lozano-Fernandez et al. (2020), or retrieved from Wolfe et al. (2016) when available - taking into account recent fossil spider reinterpretations by Magalhaes et al. (2019).

Divergence times were estimated in the program MCMCtree in PAML (Yang, 2007). Each time unit represents 100 million years. The birth-death prior for the analysis was set using a $\lambda = 1$, $\mu = 1$, and $\rho = 0$ to produce a uniform, diffuse prior on node times. In the tree, branch rates varied as set by an Independent Gamma Rate (IGR) model, in which rates are sampled from an i.i.d log-normal distribution (Lepage et al., 2007). The mean (μ) and variance (σ^2 , log rate) of the log-normal distribution were sampled from a Dirichlet prior. The prior for the mean substitution rate was estimated using the average pairwise distance per site between two exemplar taxa (*Epiperipatus* and *Pisaurina*) divided by the putative age of their common ancestor (528 million years). This produces an estimated substitution rate of 6.6×10^{-10} substitution per site per year. In MCMCtree, this rate modelled as a gamma distribution with a shape of 2 and scale of 30. The prior variance σ^2 was modelled as gamma distribution with a shape of 1 and scale of 10. We set the LG model (Le and Gascuel, 2008) on the large dataset with 50,468 amino acid sites for 84 taxa, and, given its size, we employed an approximate likelihood approach to analyse the data (dos Reis and Yang, 2011) in MCMCtree. Time priors were constructed from 26 soft-bounded calibration points, each with a Cauchy distribution allowing a 2.5% chance of the estimate exceeding the upper and lower boundaries. The exception was the root node, which instead employed a uniform distribution at 559 million years ago (Ma), but remained 2.5% soft-bounded. Analyses were also run under the priors to evaluate the joint priors induced by our choice of calibrations and root maxima (Fig. S2).

Our analysis retrieved an age estimate of the crown group of Chelicerata with a 95% credibility interval spanning 556–533 Ma, crossing the boundary between the terminal Ediacaran and early Cambrian (Fig. 4). This estimate is slightly more ancient than the estimate obtained from Matrix A in Lozano-Fernandez et al. (2020), which had an interval of 540–527 Ma - only just stepping over into the terminal Ediacaran. More ancient dates are also recovered for both Euchelicerata and Arachnida compared to Lozano-Fernandez et al. (2020), with the estimates of both nodes within the Cambrian, rather than spanning the Cambrian – Ordovician boundary. Crown group Xiphosura is similarly estimated to be older here, with an interval spanning from the Cisuarulian (Permian) to Late Triassic, crossing the P-Tr extinction, whereas Lozano-Fernandez et al. (2020) estimated a wholly Mesozoic interval from Middle Triassic to Early Jurassic. This pattern of more ancient

estimates compared to Matrix A (Lozano-Fernandez et al., 2020) was also recovered for Acariformes and Pseudoscorpiones, the latter possibly impacted by our more diverse taxon sampling of the group in this study, with ages in greater agreement with those presented in Benavides et al. (2019). We found the opposite case for more recent diversifications, with a pattern in which clades such as Pycnogonida, Ricinulei, Solifugae, Scorpiones and Araneae present younger estimates compared to Lozano-Fernandez et al. (2020).

Finally, we also assessed the impact of using hard bounds in PhyloBayes using the 200-loci matrix. For that, we applied similar settings to those used in Lozano-Fernandez et al. (2020), namely the CIR autocorrelated model (Lepage et al., 2007) and CAT – GTR + Γ as the amino acid substitution model to estimate branch lengths. The analysis differed in the molecular matrix used, a modified set of calibrations and the use of hard-bounds instead of soft-bounds. This analysis resulted in a precise, but highly unlikely, narrow credibility intervals (1 Ma for some Cambrian divergences) that mostly agree with the presented results here (Fig. S3), except for notable disagreements regarding the age of Pycnogonida, Ricinulei and Scorpiones. We have included a table (Table S4) comparing the mean age and 95% credibility intervals retrieved in Lozano-Fernandez et al. (2020) and herein. Overall, major disagreements are not found between using different molecular datasets or different software (PhyloBayes and MCMCtree) but a greater effect is observed by treating the calibrations as either soft or hard bounds.

4. Conclusions

All phylogenomic placements of Xiphosura within a paraphyletic Arachnida have put Xiphosura in a derived position, for example as sister clade to Ricinulei or Arachnoplumonata. As such, there can be two interpretations of the morphological characters exhibited by Xiphosura that are shared by fossil marine total-group chelicerates but not by arachnids - such as the spinose prosomal gnathobase series, compound eyes and non-trabeculate book gills (see sections 2.1–2.3). One scenario is that xiphosurans have retained these features whilst other arachnid lineages lost them independently as they each colonised the land. The other, is that xiphosurans are secondarily marine arachnids that reacquired these morphological features associated with more basal marine chelicerates. The first scenario is considered to be more plausible, because the fossil record shows great structural similarity between the gnathobasic endites, compound eyes and book gills of xiphosurans with aquatic fossil groups (e.g. Bicknell et al., 2018b; Schoenemen et al., 2019; Braddy et al., 1999) - and so their homology appears likely. Furthermore, the fossil record of Xiphosura demonstrates that a return to the water from terrestrial arachnid ancestors would have to be constrained to an implausibly early stratigraphic age (i.e. Early Ordovician or older, which is within the interval Arachnida is estimated to have diverged in molecular dating studies).

The phylogenetic relationships among chelicerates have been particularly challenging to resolve in molecular systematic studies and will continue to be interrogated. We have shown that the monophyly of Arachnida is retrieved from three independent molecular matrices, including two that previously yielded arachnid non-monophyly. Divergence time estimation in the context of arachnid monophyly suggests that the origin of arachnids and of their main splits took place early in the group's history (i.e. during an interval in the Cambrian–Ordovician), well preceding the first fossil occurrences. We hypothesize that this rapid and ancient diversification is confounding phylogenetic signal, and contributes

to the difficulty in accurately inferring arachnid relationships from molecular sequences.

If Arachnida is monophyletic, it can be inferred that the arachnid crown node probably lacked a prosomal series of spinose gnathobasic endites, mobility at the body coxa joint, and compound lateral eyes, as no fossil or extant crown-group arachnid unequivocally possesses them. This crown node ancestor may have had book lungs, which evolved from book gills, or possessed book gills with trabeculae as in eurypterids. As such, only a single loss of gnathobasic feeding and compound eyes is required, and possibly only one transition from book gills to book lungs (though this is complicated by the poor understanding of the origins of tracheae in apulmonate groups). If instead xiphosurans are arachnids, then there had to have been multiple evolutionary losses of gnathobasic feeding, compound lateral eyes and trabeculate book gills among the arachnid orders. The fossil record does show there may have been multiple losses of compound lateral eyes if the “semi-compound” hypothesis is accepted (see section 2.3). It remains, however, that the fossil record does not reveal anything about any gradual or repeated losses of a gnathobasic endite series and book gills within Arachnida as it may for compound eyes, and the most reasonable explanation is that these features did not occur in the common ancestor of the crown group. Furthermore, loss of compound eyes is obviously not essential for a terrestrial lifestyle (e.g., insects), whereas gills without trabeculae and feeding by crushing and shredding food along a “conveyor belt” of medially opposed coxal gnathobases would be ineffective in air rather than water. Indeed, the multiple losses of compound lateral eyes is somewhat explained by the wide range of alternate terrestrially-adapted sensory systems in place across arachnid diversity (e.g. trichobothria, pectines, malleoli) that surely evolved post-land colonisation (Miether and Dunlop, 2016).

Finally, we argue that the hypothesis that the scorpion total group is ancestrally aquatic is poorly supported. Only a handful of scorpion taxa show any morphological evidence of an aquatic lifestyle, and each instance is subject to considerable caveat (see 2.4). It is clear that there were aquatic scorpions, but they are likely to be secondarily adapted outliers rather than necessarily reflective of the group’s origin. Therefore, we propose that a single land colonisation for crown group arachnids remains a possibility, if it is accepted that arachnid monophyly is a reasonable ground on which to infer this, and that eurypterids are amphibious stem-group arachnids. We concede that this is currently tenuous due to the lack of fossils illustrating the evolution of non-pulmonate arachnid respiratory systems, but we are optimistic that the phylogenomic resolution of Arachnida is increasing rapidly - which will provide a more robust scaffold for future palaeontological discoveries.

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Research data

Data matrices, gene trees and alignments, output files, and custom scripts can be found in the Figshare data repository at https://figshare.com/projects/Arachnida_ASD/84527.

Author’s contribution

Richard J. Howard: Conceptualization; Investigation; Methodology; Visualization; Writing - original draft; Writing - review & editing; **Mark N. Puttick:** Formal analysis; Methodology; Software; Writing - original draft; **Gregory D. Edgecombe:** Conceptualization; Investigation; Supervision; Writing - original draft; Writing - review & editing; **Jesus Lozano-Fernandez:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing - original draft; Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.asd.2020.100997>.

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