Evolution and morphology of lycophyte root systems

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Thesis submitted for the degree of Doctor of Philosophy

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Abstract

The evolution of plant roots transformed the Earth's surface, engineering new ecosystems and environments, and providing the nutrient and water uptake, as well as anchorage necessary to support the first trees. The lycophytes (clubmosses) were the first group of plants to evolve roots and the study of their morphology and evolution has been a major goal for evolutionary scientists working on both extant and extinct lycopsids. The aim of the research described in this thesis is to increase our understanding of both the morphology and evolution of lycopsid roots. This is achieved by presenting three papers on the theme of root morphology and evolution. First, I report the discovery of root hairs on extinct stigmarian rootlets, highlighting the conserved morphology of all Isoetalean rootlets. Second, in my discovery of the oldest fossilized root meristem, I illustrate how the interpretation of exceptionally well preserved fossils can change the way we think about the evolution of development of living plant roots. Third, I identify that the rootlets of *Isoetes echinospora* and roots of *Selaginella moellendorffii* have similar gene expression profiles. The new results reported in this thesis taken together with a review of the literature of extant and extinct lycopsid rooting structures, enabled me to identify two contrasting evolutionary patterns: conservatism of lycopsid roots, and huge disparity in the structures to which roots are attached. The highly conserved nature of lycopsid roots, supported by the new data presented in this thesis, is consistent with the hypothesis that all lycopsid roots are homologous (described as the lycopsid root hypothesis). In recognising the homology of lycopsid roots, and the two contrasting patterns of rooting structure evolution, the research presented in this thesis makes a significant contribution to our understanding of the morphology and evolution of lycopsid roots.

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Finally, to my family and friends without whom none of this would have been possible. To my Mum and Dad for constant inspiration and last of all to Jo for her support throughout, not to mention her keen eyes for spotting *Isoetes histrix* and being happy to wait in the car while I waded around in numerous Scottish lochs looking for *Isoetes*!

My thanks to you all,

Sandy

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Abbreviations

bp	Base pair
ddH_2O	Double distilled water
DE	Differentially expressed
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
kb	Kilo base pair
LCA	Last common ancestor
LRH	Lycopsid root hypothesis
Mya	Million years ago
MSH	Modified shoot hypothesis
nRPM	Normalised reads per million
OG	Orthogroup
PRR	Putative rootlet regulator
PRDR	Putative rootlet developmental regulator
RBRS	Rhizoid based rooting system
RPM	Reads per million
SEM	Scanning electron micrograph

Chapter 1: General introduction

1.1 Introduction

The lycophytes (clubmosses) are the most ancient living group of vascular plants (Kenrick & Crane, 1997; Qiu *et al.*, 2006; Wickett *et al.*, 2014). They have a rich evolutionary history and encompass a remarkable diversity of extant and extinct species (Fig. 1.01) (Bower, 1908; Eames, 1936; Foster & Gifford, 1959; Niklas *et al.*, 1983; Hueber, 1992; Phillips & DiMichele, 1992; Stewart & Rothwell, 1993; Kenrick & Crane, 1997; Bateman *et al.*, 1998; Gensel & Berry, 2001; Taylor *et al.*, 2009; Xue, 2011). Lycophytes dominated late Silurian and early Devonian plant communities (Fig. 1.01a, b) (Hueber, 1992; Gensel & Berry, 2001; Gensel & Edwards, 2001; Raymond *et al.*, 2006), were the first colossal >50 m trees during the Carboniferous period (Fig. 1.01b-d, f) (Thomas & Watson, 1976; Phillips & DiMichele, 1992; Stewart & Rothwell, 1993) and living species range from jungle epiphytes (Fig. 1.01g), desiccation-tolerant resurrection plants, to aquatic species adapted to use Crassulacean Acid Metabolism (CAM) photosynthesis (Fig. 1.01m, n) (Eames, 1936; Foster & Gifford, 1959; Kenrick & Crane, 1997; Keeley, 2014). This long evolutionary history, rich fossil record (Fig. 1.01a-f) and extant diversity (Fig. 1.01g-n) has made studies of lycophytes central to plant evolutionary biology.

Figure 1.01 The large diversity of both extant and extinct lycopsids. The oldest fossilized remains of lycopsids, the late Silurian genus *Baragwanathia* (a). Characteristic scale-like microphyll (leaf) scars on the fossilized trunk of the Carboniferous *Lepidodendron aculeatum* (b). The giant rooting systems of the Carboniferous arborescent lycopsids named *Stigmaria ficoides*: an isolated rhizomorph with rootlets weathering out of an outcrop close to the Rock and Spindle (Fife, UK) (c), giant rhizomorph axes radiating from the base of a trunk (University of Manchester, Manchester Museum (MANCH), UK) (d). The leafy shoots of the Devonian lycopsid *Archaeosigillaria vanuxemi* (e) and Carboniferous lycopsid *Lepidodendron acutum* (f). An extant epiphytic member of the Lycopodiales (g) (Royal Botanic Gardens (RBG) Edinburgh). The microphylls and sporangia of *Phlegmariurus squarrosus* (h). Lycopodiales growing in their natural environments (Sutherland, UK) *Huperzia selago* (i) and *Lycopodium clavatum* (j). Microphylls on fronds of *Selaginella martensii* (k) (RBG Edinburgh). The terrestrial *Isoetes* species *Isoetes histrix* in growth position (Cornwall, UK) (l). Aquatic *Isoetes echinospora* developing a highly branched network of rootlets from its base (m). The young sporophyte of *Isoetes echinospora* grown in axenic culture (n) and used to examine the gene expression profile of both rootlets and microphylls in Chapter 4. Fossil collections from the University of Manchester, Manchester Museum (MANCH) (UK): LL. 15952.5 (a); EM 197821 (e). University of Oxford Museum of Natural History (OUMNH) (UK): E. 3634 (b). Bolton Library and Museum Services (UK): INV:1583 (f). All images taken by A.J.H. except (d) taken by Kate Sherburn (University of Manchester, Manchester Museum (MANCH)).

A major goal of plant evolutionary biology is to understand the origin, and subsequent diversification, of key plant organs such as roots. Plant roots were one of a suite of key innovations (Bateman *et al.*, 1998; Donoghue, 2005; Doyle, 2013) that evolved during the Silurian–Devonian primary radiation of vascular plants allowing plants to hugely increase in size and break from their physiological dependence on moist habitats (Bateman *et al.*, 1998). Plant roots provide the primary interface between vascular plants and the terrestrial surface, and their evolution had lasting impacts on the Earth system. Roots transformed the physical environment by limiting erosion and changing fluvial processes (through both the channelling of water flow on the terrestrial surface and by increasing the transpiration stream from the sediment, through plants, to the atmosphere), while at the same time increasing the chemical weathering of silicate rocks – the dominant sink for atmospheric CO_2 in the long-term carbon cycle (Algeo & Scheckler, 1998; Berner, 1998; Berner & Kothavala, 2001; Bergman *et al.*, 2004; Gibling & Davies, 2012; Jasechko *et al.*, 2013; Gibling *et al.*, 2013). For plants, the evolution of roots provided a new capacity to mine into the substrate for deeper reserves of water and nutrients, as well as the anchorage necessary to support the first trees. Characterising how roots evolved is thus crucial for understanding how plants conquered the land. Lycophytes are critical for understanding root evolution as roots are not homologous in vascular plants but, in fact, had at least two separate origins: once in lycophytes and independently in the euphyllophytes (the group of plants with true leaves, such as ferns, gymnosperms and angiosperms) (Raven & Edwards, 2001; Boyce, 2005; Kenrick, 2013; Kenrick & Strullu-Derrien, 2014). The evidence supporting the independent origins of roots comes from both extant and extinct plants.

1.2 The independent origins of roots in lycophytes and euphyllophytes is supported by evidence from anatomy and development of extant roots

Morphological evidence from extant plants supports the hypothesis that roots independently evolved. The roots of lycophytes and euphyllophytes are morphologically distinct – most strikingly in their modes of branching. Lycophyte roots branch dichotomously through the splitting of the apex – a process which is termed exogenous branching. This pattern of branching contrasts with the lateral root branching in euphyllophytes, where branching occurs at a distance from the apex with the development of new lateral roots from internal tissues of the parent root (from the endodermis in monilophytes and the pericycle in seed plants (Schneider *et al.*, 2009) in a process termed endogenous branching (Bower, 1908; Foster & Gifford, 1959)). This fundamental difference in branching provides morphological evidence in support of the hypothesis that roots independently evolved in lycophytes and euphyllophytes.

1.3 The independent origins of roots in lycophytes and euphyllophytes is supported by evidence from the fossils of extinct plants and the rock record

Alongside the evidence from morphology and development of extant plants, the multiple origins of roots are also supported by the fossils of extinct plants and the rock record. Three pieces of evidence from the fossil and rock record taken together support the hypothesis that roots independently evolved.

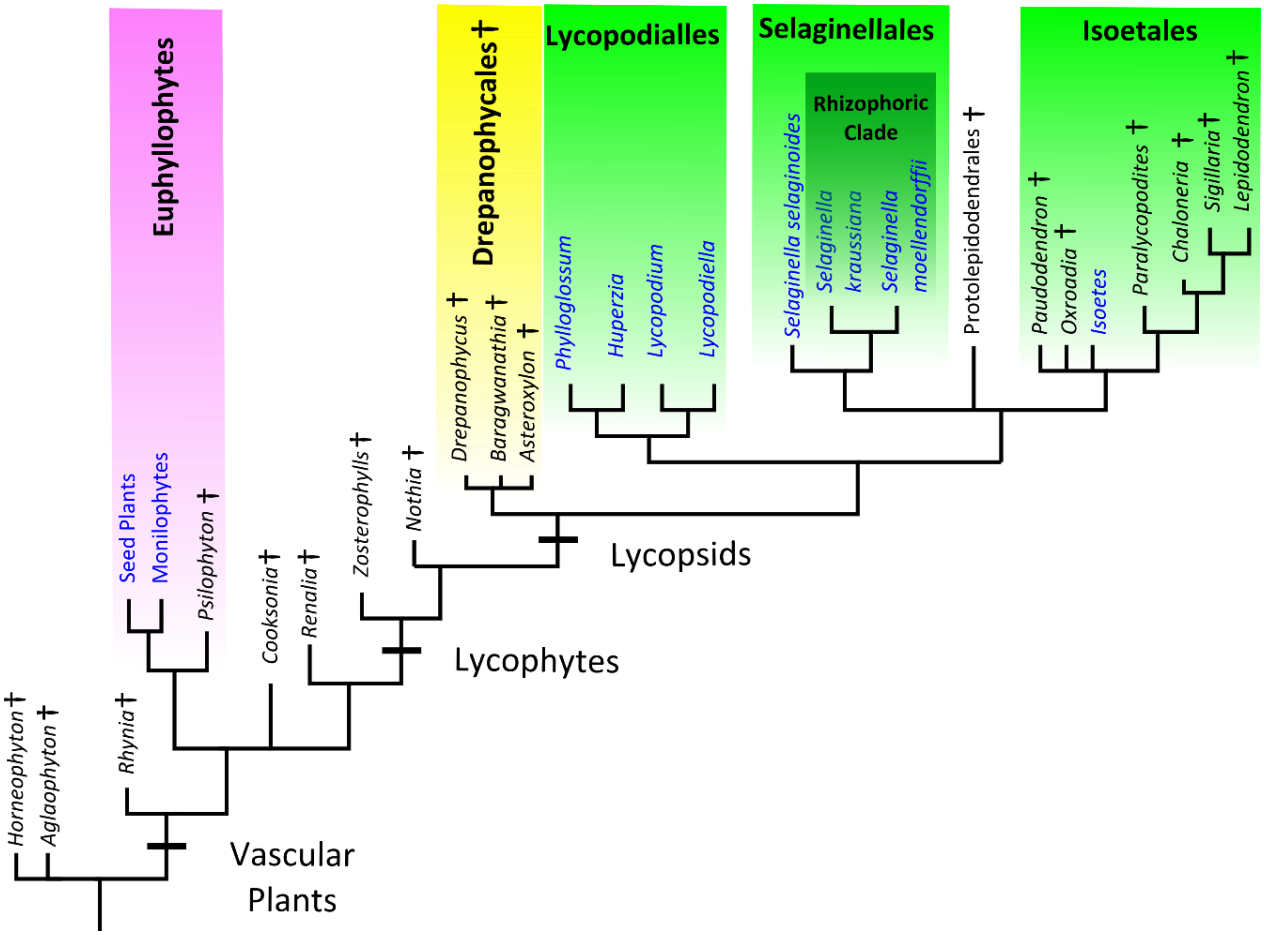

Figure 1.02 Land plant cladogram. A schematic phylogeny of living (blue text) and extinct (black text and †) plants showing the divergence of euphyllophytes and lycophytes. The extant and extinct clades of the lycopsids have been highlighted in green and yellow respectively. *Isoetes* is shown unresolved at the base of the Isoetales (Bateman, 1992; Xue, 2011), despite its similarities to *Chaloneria* (DiMichele & Bateman, 1996). The phylogeny is based on; the analysis by Kenrick & Crane, 1997, however to increase the number of representatives in the the lycophytes the lycopodialles were expanded based on Field *et al.*, 2016; the Selaginellaes based on Korall & Kenrick, 2002 and the Isoetales based on Bateman, 1992; DiMichele & Bateman, 1996; Xue, 2011.

1.3.1 The last common ancestor (LCA) of vascular plants was rootless

Based on cladistics analyses (Kenrick & Crane, 1997) of the exceptionally well preserved plants of the 407.1±2.2 million-year-old (Mark *et al.*, 2011) Rhynie chert and slightly younger Windyfield chert (such as; *Aglaophyton major*, *Rhynia gwynne-vaughanii*, and *Horneophyton lignieri*) (Fig. 1.02) (Kidston & Lang, 1917, 1920a, 1921, Edwards, 1986, 2004; Kenrick & Crane, 1997), as well as cosmopolitan relatives of these plants (e.g. *Stockmansella remyi* (Schultka & Hass, 1997) we have a clear understanding of the rooting

systems of plants spanning the origin of the vascular plant lineage. These plants lacked a specialised rooting axis but instead were united by the presence of rhizoid-based rooting-systems (RBRSs) (Kenrick & Strullu-Derrien, 2014) (Fig. 1.03). This supports the hypothesis that the plesiomorphic rooting state in vascular plants was RBRSs, making the last common ancestor (LCA) of vascular plants rootless.

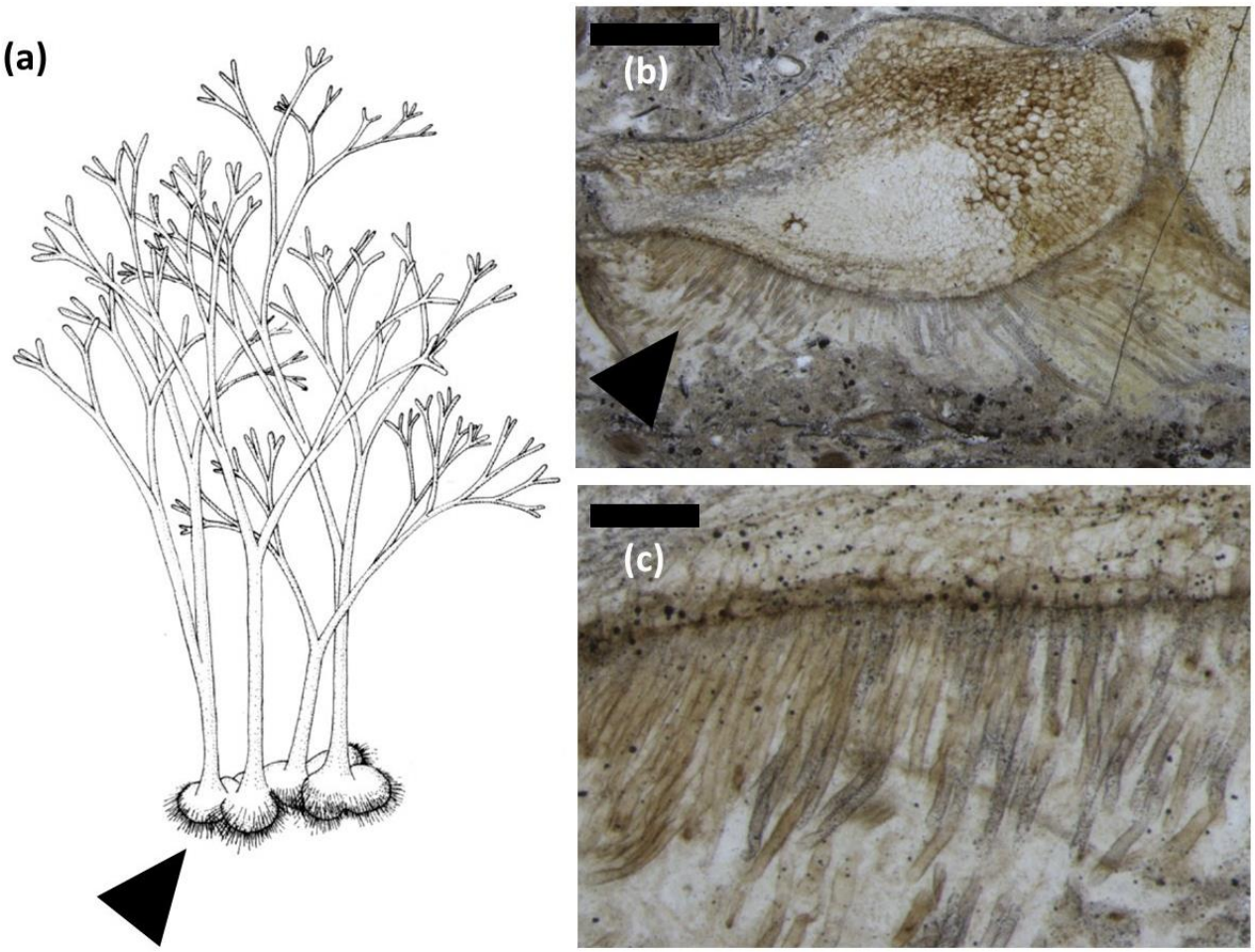

Figure 1.03 Rhizoid based rooting system (RBRS) of *Horneophyton lignieri*. Reconstruction of the 407-million-year old plant *H. lignieri* from the Rhynie Chert (a) (reproduced from Edwards, 2004), rhizoid based rooting system (RBRS) developing from the lobed tuberous rhizome which anchored the plant (black arrow head). Thin section showing a transverse section through the rhizome of *H. lignieri* with rhizoids developing from the base (black arrowhead) (b). Higher magnification image of the rhizome in (b) showing the development of large number of rhizoids from the superficial cell layer of the rhizome (c). Scale bars: 1 mm (b), 200 µm (c). Thin section W. Hemingway No. 371.78, School of Biology, University of St Andrews (courtesy of Dr Iain Matthews) (b, c).

1.3.2 The lycophytes and euphyllophytes diverged from this rootless ancestor and both maintained rootless taxa at the base of their lineages

If the LCA of the lycophytes and euphyllophytes was also rootless this would indicate that roots evolved independently in both lineages. In order to determine the rooting structure of the LCA of lycophytes and euphyllophytes it is necessary to examine early diverging vascular plants spanning the stem lineages and total groups of both lycophytes and euphyllophytes. This examination provides support for the hypothesis that the LCA of lycophytes and euphyllophytes was rootless and that roots had at least two separate origins. The evidence for rootless taxa in the stem lineages of both the lycophytes and euphyllophytes comes from the earliest land plant body fossils – the paraphyletic eutracheophyte genus *Cooksonia* (Kenrick & Crane, 1997; Gonez & Gerrienne, 2010) (Fig. 1.02). *Cooksonia* species lacked a specialised rooting axis distinguishable from their sporangium-bearing shoot axes. This suggests that plants of the genus *Cooksonia* were rootless and their rooting function was carried out by RBRSs developing on either their sporophyte or gametophyte (Boyce, 2008; Gerrienne & Gonez, 2011). Evidence from the genus *Cooksonia* is consistent with the hypothesis that the LCA of lycophytes and euphyllophytes was rootless.

Rootless plants have also been described within the early diverging lineages of both lycophytes and euphyllophytes, further supporting the hypothesis that roots evolved independently in both lineages. The lycophytes: *Nothia aphylla* (Kenrick & Crane, 1997; Kerp *et al.*, 2001; Edwards, 2004), *Ventarura lyonii* (Powell *et al.*, 2000; Edwards, 2004) and potentially *Trichopherophyton teuchansii* (Lyon & Edwards, 1991; Edwards, 2004) are all rootless and their rooting function is carried out by RBRSs. Additionally, many early diverging lycophytes (e.g. *Zosterophyllum decucuum* (Gerrienne, 1988) lack distinguishable rooting axes, suggesting they were also rootless. However, the fossilised

remains of the majority of early diverging lycophytes lack the necessary preservation to score for the presence of RBRSs, or defining root characters such as root hairs and root caps. Rootless taxa are also present in the earliest diverging lineages of the euphyllophytes such as the genus *Psilophyton* (Kenrick & Crane, 1997) (Fig. 1.02). Adventitious root-like branches were reported by both Banks *et al.* (1975) and Doran (1980), in their descriptions of *Psilophyton dawsonii* and *Psilophyton crenulatum* respectively. However, unlike the roots of almost all extant euphyllophytes (Bower, 1908; Foster & Gifford, 1959), these root-like structures developed and branched exogenously (Banks *et al.*, 1975). Endogenous origin and endogenous lateral root branching are defining features of extant euphyllophyte roots indicating that the root-like structures of *Psilophyton* are not roots, and that *Psilophyton* was rootless (early reconstructions of *Psilophyton* with roots (e.g. Dawson, (1859) should be ignored as they were later dismissed by Hueber & Banks (1967) as artefacts of fossilisation). Taken together, first the evidence that the LCA of vascular plants was rootless, and second that rootless taxa were present in both stem and total group lycophytes and euphyllophytes, supports the hypothesis that the LCA of lycophytes and euphyllophytes was rootless. This, therefore, indicates that roots had at least two origins in vascular plants. Moreover, the fossil and rock record provides an additional line of evidence for the independent origin of roots.

1.3.3 Distinct temporal origins of roots in lycophytes and euphyllophytes

The temporal origin of roots also supports their independent origin in lycophytes and euphyllophytes. If roots are homologous and had a single origin two further predictions would be expected: first, roots must have appeared very early in the geological record at the time of the LCA of lycophytes and euphyllophytes; second, that we would expect to find roots contemporaneously in lycophytes and euphyllophytes. The fossil and rock

record, combined with evidence from molecular clocks, provides the opportunity to test both of these hypotheses.

If roots had a single origin they must have been present in the LCA of lycophytes and euphyllophytes. Combining evidence from molecular clocks and the fossil record allow us to make a prediction for the timing of this LCA, and provides us with a prediction of when we should discover the first root fossils. The minimum age for the divergence of total group Lycopsida is the late Silurian to early Devonian (Ludlow Series), based on the finding of Zosterophyllum species and the genus *Baragwanathia* (a basal lycopsid) (Garratt *et al.*, 1984; Kotyk *et al.*, 2002; Raymond *et al.*, 2006) (Fig. 1.01a, 1.02). This indicates that the LCA of the lycophytes and euphyllophytes must have predated these fossils, and based on molecular clock studies must have evolved earlier in the Silurian or in the late Ordovician (Clarke *et al.*, 2011; Kenrick *et al.*, 2012). If a root-developing ancestor of both lycophytes and euphyllophytes was present in the late Ordovician to early Silurian we would expect to find a body of fossil evidence for roots in both lycophytes and euphyllophytes during this time period, which we do not. Although this lack of fossils may be attributed to poor preservation we may expect to find independent proxies for roots during the late Ordovician to early Silurian.

The evolution of roots led to significant geomorphological changes to the terrestrial surface (Gibling & Davies, 2012; Gibling *et al.*, 2013), including the transformation of riverine systems from dominantly braided to meandering river channels. If roots had a single origin predating the divergence of lycophytes and euphyllophytes we would expect to find evidence of significant changes to riverine systems towards the end of the Ordovician and throughout the Silurian. By contrast, if roots independently evolved we would expect the geomorphological changes associated with the origins of roots to occur later, after the divergence of euphyllophytes (by the late Silurian and Early Devonian). A

detailed investigation of the timeframe of geomorphological evidence associated with roots suggests that major changes to riverine systems only occurred at the end of the Silurian (Gibling & Davies, 2012; Gibling *et al.*, 2013), consistent with the independent origins of roots in lycophytes and euphyllophytes.

If roots did have an ancient origin in the LCA of lycophytes and euphyllophytes we would expect to find evidence of roots concurrently in lycophytes and euphyllophytes. However, examination of the fossil record indicates that roots of lycophytes and euphyllophytes have distinct temporal origins. There is roughly a 15-million-year gap between the first lycophyte roots and the first euphyllophyte roots. A diversity of rooting structures is found in lycophytes in the early Devonian (Gensel *et al.*, 2001), the earliest described dates from 413 million years ago (mya) (Hao *et al.*, 2010); and roots have been described in lycopsids dating from the Early Devonian (Matsunaga & Tomescu, 2016; Xue *et al.*, 2016). Euphyllophytes are found in comparable strata in the lower Devonian, such as the genus *Psilophyton* (Banks *et al.*, 1975; Doran, 1980). However, as discussed previously these taxa lacked roots and it was not until the mid-Devonian 385–398 mya that clear euphyllophyte roots are found in the relatives of the ferns and gymnosperms (Fairon-Demaret & Li, 1993; Mintz *et al.*, 2010; Stein *et al.*, 2012; Giesen & Berry, 2013; Morris *et al.*, 2015). The lack of evidence for roots at the estimated divergence time of lycophytes and euphyllophytes, combined with the *c*. 15-million-year time difference between the first lycophyte and euphyllophyte roots supports the hypothesis that roots had at least two separate origins.

Taken together these multiple lines of evidence from both extant morphology and from the fossil record support the independent origin of roots in the lycophytes and euphyllophytes. Lycophytes were, therefore, the first group of plants with roots and their roots have a separate evolutionary history to those of euphyllophytes. However, our

understanding of the evolution of roots has been shaped almost exclusively by studies of euphyllophytes (in particular seed plants such as model flowering plant species). These studies only shed light on one 'half' of root evolution and, as such, fail to account for the convergent evolution of roots, and the complex and diverse root forms and functions in the lycophytes. Increasing our knowledge of the evolution of both independent origins of roots is central to understanding the evolution of life on the terrestrial surface, and the way that roots have altered the Earth system. Moreover, it will help to refine evolutionary theory by generating a better understanding of the remarkable process of convergent evolution. For a research area of such importance it may therefore be surprising that lycophyte root morphology and evolution is still a highly debated topic and has been so for over 150 years (Nägeli & Leitgeb, 1868; Goebel, 1905; Bower, 1908) with major unanswered questions such as; how many origins of roots were there within lycophytes (Rothwell & Erwin, 1985; Gensel *et al.*, 2001), and which parts of lycophyte rooting systems can be deemed homologous (Rothwell & Erwin, 1985). Part of this debate stems from four issues:

1. The poorly preserved fossils and poorly supported phylogenies of early diverging lycophytes have limited our understanding of early lycophyte root evolution.
2. Previously there has been a lack of unifying terminology for discussing the huge diversity of both extinct and extant lycopsid rooting structures.
3. There has previously been a lack of key defining rooting characters described in fossil material, limiting the ability to confidently identify roots in the fossil record.
4. Previous studies have lacked integration of molecular data across a range of lycophyte species.

The aim of the research described in this thesis is to overcome a number of the shortcomings of previous investigations by tackling the four points listed. However, before

describing the research carried out, it is first necessary to outline each of these four points to justify the rational of this study.

1.4 Poorly preserved fossils and poorly supported phylogenies of early diverging lycophytes have limited our understanding of early lycophyte root evolution

Total group lycophytes (collectively all living and extinct lycophytes) are a very large taxonomic group encompassing numerous extinct plants (often with limited fossil evidence) which makes constructing robust phylogenies and mapping character state evolution difficult (Kenrick & Crane, 1997). Defining the precise origin and early evolution of roots within total group lycophytes has therefore proved very challenging, especially as many of the key characters which define roots are rarely preserved in fossil material (Gensel *et al.*, 2001). This bottom-up (from the oldest fossil evidence of roots to extant lycopsid roots) approach to studying root evolution is hugely important as these early fossils provide the earliest evidence for root-like structures in vascular plants. However, without the necessary preservation of key anatomical characters, such as root caps and root hairs, these structures are very difficult to integrate into theories about root evolution. Put simply, it is not possible to be certain that root-like structures really are roots without the necessary preservation (Gensel *et al.*, 2001). To bypass the problematic question of when roots evolved in total group lycophytes it is useful to focus solely on the evolution of rooting structures in the lycopsid clade, which includes all living lycopsids and their extinct relatives. Studying root evolution within the lycopsids has three key advantages. First, the fossil record and phylogeny of the lycopsids are more extensive and more robust than for many of the earlier diverging lycophyte groups (such as the

paraphyletic Zosterophylls (Kenrick & Crane, 1997)). Second, the lycopsids, unlike earlier diverging lineages of the lycophytes, have more characters allowing the distinction between roots and shoots (such as the evolution of more diverse branching patterns and microphyll leaves (Matsunaga & Tomescu, 2016)). Third, studying root evolution in the lycopsids has the additional advantage that fossils can be interpreted in light of the anatomy and development of roots in living species which lack the preservation biases of fossil material. **The aim of the research described in this thesis is to: examine root evolution solely in the lycopsids.**

1.5 Previously there has been a lack of unifying terminology for discussing the huge diversity of both extinct and extant lycopsid rooting structures

The second reason why lycophyte root evolution is such a controversial area is the lack of a unified theory, or even terminology, for describing the rooting structures of different lycopsids. This is, in part, because lycopsids display a huge diversity (both in terms of number and dissimilarity (disparity)) of rooting structures, many of which challenge classic concepts of plant morphology (Goebel, 1905) (in a similar fashion to the way that the genus *Utricularia* does for flowering plants (Rutishauser & Isler, 2001)). The anatomy and evolution of these disparate structures has perplexed botanists and palaeobotanists for well over a century. Although there is great diversity in rooting structures there is also great conservatism. The conserved parts of extant lycopsid rooting structures are termed roots (Nägeli & Leitgeb, 1868; Harvey-Gibson, 1902; Bower, 1908; Saxelby, 1908; Uphof, 1920; Foster & Gifford, 1959; Paolillo, 1963), however the term ‘root’ is often used in the evolutionary literature to collectively refer to a number of diverse rooting structures (Stewart & Rothwell, 1993; Gensel *et al.*, 2001; Hao *et al.*, 2010). This

discrepancy in the use of the term ‘root’ makes it difficult to directly compare the rooting structures of extinct and extant lycopsids. The research in this thesis, therefore, aims to start by reviewing the diversity of rooting structures in extant lycopsids. Extant lycopsids lack the preservational biases of fossil plants and therefore the study aims to use these to identify major trends and a unified terminology for describing the diversity of lycopsid rooting structures. **The aim of the research described in this thesis is to: identify major trends in lycopsid root evolution from extant species, and test if the roots of extinct lycopsids also display the same patterns.** The research described in the study recognises two patterns in the evolution of extant lycopsid rooting structures. The first is the conservatism – the occurrence of the same morphologies in related taxa indicating that the traits have not changed since they diverged from an ancestral state – of lycopsid roots. The second is disparity – the presence of diverse morphological types among a group of related taxa indicating that the trait has changed considerably since they diverged from an ancestral state – of the structures from which lycopsid roots develop.

1.5.1 Conservatism: roots comprise isotomous branching axes in extant lycopsids

All three clades of extant lycopsids – Lycopodiales, Selaginellales and Isoetales (Kenrick & Crane, 1997; Korall & Kenrick, 2002; Field *et al.*, 2016) (Fig. 2.01) – develop roots: indeterminate axes typically characterised by a root cap, root hairs, and the lack of lateral, determinate appendages) (Nägeli & Leitgeb, 1868; Harvey-Gibson, 1902; Bower, 1908; Saxelby, 1908; Uphof, 1920; Foster & Gifford, 1959; Paolillo, 1963) (Fig. 1.04). Although extant lycopsids are a diverse group of plants adapted to a range of environmental niches, the anatomy and architecture of their roots are remarkably conserved (Fig. 1.04). The meristem at the apex of lycopsid roots forms a cap distally (Fig. 2a), and differentiated cells and tissues develop proximally with root hairs developing from cells in the surface

cell layer (Fig. 1.04a, b). Root hairs of lycopsids develop from the smaller epidermal cells produced by asymmetric cell divisions (termed type 2 epidermal patterning) (Leavitt, 1904; Dolan, 1996). Growing roots branch dichotomously through the splitting of the apex – a process termed exogenous branching (Fig. 1.04c, d). Branching is almost exclusively isotomous, where the two branches that form by dichotomy of the parent axis are roughly the same dimensions (length and width) (Fig. 1.04e–h). Lycopsid roots lack a vascular cambium and organogenic properties outside the apical meristem: apart from branching to produce daughter roots they do not develop other organs such as shoots (with the exception of root buds in *L. cernuum and L. ramulosum* (Holloway, 1916)) or leaves. By contrast, both shoots and leaves are features known to develop from euphyllophyte roots (Goebel, 1905; Richards *et al.*, 1983; Groff & Kaplan, 1988; Koi & Kato, 2003)). Although lycopsid roots are structurally uniform, there is variation in the organisation of cells in root apical meristems (reviewed by (Hetherington *et al.*, 2016b) in Chapter 3) and the structure of vascular tissues. In general, the Lycopodiales develop an exarch xylem (but this development is from multiple poles making the roots range from monarch, diarch, triarch, tetrarch or even polyarch (Wigglesworth, 1907; Saxelby, 1908)) which forms a horseshoe shaped strand (Wigglesworth, 1907; Saxelby, 1908). Phloem forms in the middle of this horseshoe shaped crescent (Pixley, 1968; Bierhorst, 1971). In the Selaginellales the xylem is typically monarch with exarch maturation, with phloem surrounding the xylem strand (Harvey-Gibson, 1902; Uphof, 1920). The roots of the Isoetales develop a monarch bundle (Scott & Hill, 1900; Stewart, 1947; Bhambie, 1963), the phloem is located adjacent (collateral) to the metaxylem and is centripetal (Stewart, 1947). The vascular strand of all three groups is also composed of a pericyle. In the Lycopodiales and Selaginellales the pericyle surrounds both the xylem and phloem (Harvey-Gibson, 1902; Wigglesworth, 1907; Uphof, 1920). By contrast, in the Isoetales

the pericycle just surrounds the protoxylem pole and does not extend around the phloem (Scott & Hill, 1900). An endodermis with distinct Casparian bands forms in both the Isoetales and Selaginellales (Scott & Hill, 1900; Harvey-Gibson, 1902; Bhambie, 1963; Krautrachue & Evert, 1978), but is not found in the lycopodiales (although cortical cell walls in a position analogous to the endodermis do fluoresce intensely when stained with berberine hemisulfate (Brundrett *et al.*, 1988), there are no clear Casparian bands (Damus *et al.*, 1997)). The final major distinguishing feature between the internal anatomy of the three groups in the development of an air lacuna in the roots of the Isoetales, a feature common in wetland plants (Peterson, 1992; Seago *et al.*, 2005), and also present in some members of the lycopodiales (Saxelby, 1908; Ben Hill, 1919; Bierhorst, 1971; Bruce, 1976) and Selaginellales (Duthie, 1922). Despite there being some differences in the meristem organisations and internal anatomy of extant lycopsid roots, their overall morphology is remarkably similar forming isotomously branched systems covered in root hairs developing from a root meristem covered by a protective cap (Fig. 1.04). This conservatism is striking considering extant lycopsids grow in diverse ecological niches.

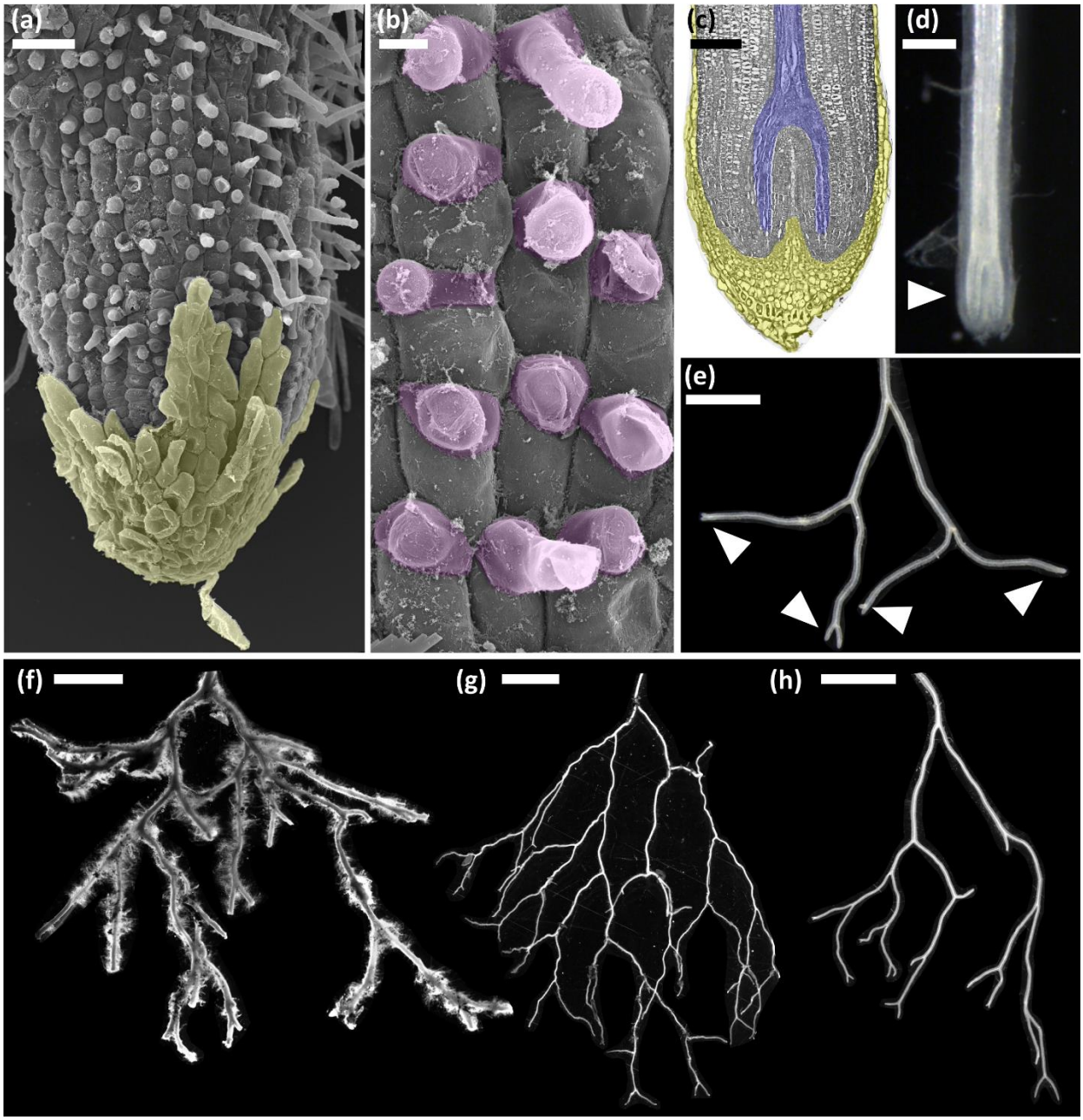

Figure 1.04. Conservatism of roots in extant lycopsids. All extant lycopsid roots develop a protective root cap that is highlighted in yellow on a scanning electron micrograph (SEM) of a root apex of *Phlegmariurus squarrosus* (member of the Lycopodiales (Field *et al.*, 2016)) (a). The epidermis of extant lycopsid roots develops root hairs – highlighted in purple on SEM of root epidermis of *P. squarrosus* (b). All lycopsid roots branch exogenously and dichotomously (producing two daughter roots of similar dimensions) through the splitting of the parent meristem; developmental series of exogenous root branching from the micro (c, d) to the macro scale (e) in roots of *Isoetes echinospora*. False colour yellow and blue highlight the root cap and procambium respectively in (c). White arrowheads indicate the positions of branching apices in (d, e). Repeated dichotomous divisions leads to the formation of similar root branching architectures in all three clades of extant lycopsids – Lycopodiales (*Phlegmariurus phlegmaria* (f)), Selaginellales (*Selaginella kraussiana* (g)) and Isoetales (*Isoetes histrix* (h)). Scale bars; 100 µm (a, c); 20 µm (b); 500 µm (d); 2 mm (e); 5 mm (f–h).

1.5.2 Disparity of root producing structures in extant lycopsids

While roots are conserved among the extant lycopsids, the structures from which these roots develop display remarkable disparity. The structures which 'serve as bases of attachment for roots' (Bower, 1908) in living lycopsids encompass a variety of organs and meristems including shoots, embryos and bulbils (Bower, 1908; Saxelby, 1908; Benca, 2014), and unique organs such as the rhizophores of the rhizophoric clade of extant Selaginalleles (Fig. 1.02) (Imaichi & Kato, 1989; Lu & Jernstedt, 1996; Korall & Kenrick, 2002), the basal swellings of *Selaginella selaginoides* (Karrfalt, 1981), rhizomorphs of Isoetales (Paolillo, 1963; Rothwell & Erwin, 1985; Yi & Kato, 2001) (rhizomorph anatomy also differs between species, in the number and types of lobes (Karrfalt & Eggert, 1977; Rury, 1978) and the presence of branching (Karrfalt & Eggert, 1977; Karrfalt, 1984)) and tubers or protocorms of the Lycopodiales (Treub, 1890; Bower, 1908). These root producing organs are not themselves roots (as defined above) –they lack a typical root meristem, root cap and root hairs. Unlike roots, which are formed throughout development, some of these root-bearing organs are produced only once early in development (e.g. the rhizomorph, basal swelling and protocorms (Bower, 1908; Paolillo, 1963). While these structures are not roots, they may play anchorage roles that are also carried out by roots. Two such structures with anchorage roles are the rhizomorph of Isoetales (Paolillo, 1963; Rothwell & Erwin, 1985) and the underground shoots (runners) of some Lycopodiales (Benca, 2014). Furthermore, not only are these structures disparate but the point of origin of roots from these structures varies. The first embryonic roots of *Isoetes* species (Yi & Kato, 2001); and a number of species of the Lycopodiales (such as *Huperzia selago* (Bower, 1908) develop from superficial cells. Superficial root development also occurs from the protocorms and tubers of species such as *Lycopodium cernuum* (Treub, 1890) and *Phylloglossum drummondii* (Bower, 1908). Roots of the

Selaginellales develop from internal tissues, either the cell layers beneath the rhizophore tip (in the rhizophoric clade (Fig. 1.02)) or from internal cell layers of the basal swelling in *Selaginella selaginoides* (Fig. 1.02) (Karrfalt, 1981; Imaichi & Kato, 1989; Lu & Jernstedt, 1996; Korall & Kenrick, 2002). Roots of *Isoetes* species (with the exception of the embryonic first root) develop from within the external derivatives of the basal root producing meristem of the rhizomorph (Paolillo, 1963; Yi & Kato, 2001). Roots of the Lycopodiales which do not develop from superficial cells develop from the pericycle of the shoot or internal cell layers of protocorms (as in *Lycopodium inundatum*) (Goebel, 1905; Bower, 1908; Saxelby, 1908; Foster & Gifford, 1959). In summary, although roots appear to be remarkably conserved among extant lycopsids the organs to which they are attached and the tissues of these organs from which roots originate are, by contrast, very disparate.

The thesis aims to examine lycopsid root evolution in light of these two contrasting patterns found in the root structures of living lycopsids: conservatism and disparity. Given that the conserved part of the rooting structures has been given less attention in evolutionary literature, characterising the evolutionary history of the conserved part of the rooting system will be a primary focus of the thesis.

1.6 There has previously been a lack of key defining rooting characters described in fossil material, limiting the ability to confidently identify roots in the fossil record

The third factor limiting our understanding of lycopsid root evolution is the lack of key defining root characters in fossil material (Gensel *et al.*, 2001; Hao *et al.*, 2010; Matsunaga & Tomescu, 2016). As described above, the roots of living lycopsids are defined by their

strictly isotomously branching architecture, the presence of a root cap, and root hairs. Although the presence of isotomous branching is easily identified in compression fossils (Williamson, 1887; Li & Edwards, 1995; Matsunaga & Tomescu, 2016) there is currently only one record of a fossil lycopsid with root hairs ((McLoughlin & Bomfleur, 2016); published after the work described in this thesis) and no examples of fossil lycopsid root meristems. Both of these structures are minute and require exceptional cellular preservation to be fossilised, meaning they are rarely preserved (Gensel *et al.*, 2001; Matsunaga & Tomescu, 2016). However, given that they are defining features of roots it is crucial to discover both structures to increase our understanding of root evolution. **The aim of the research described in this thesis is to: identify and describe key rooting characters in the fossil record, such as root hairs and root meristems.** The work described in this thesis will achieve this by making use of permineralised Carboniferous fossils called coal balls.

Coal balls are nodules of permineralised peat which formed during, or just after, peat formation in the wetland coal swamp forests that dominated lowland regions during the Carboniferous period, entombing the plants within the peat with exception cellular preservation (Fig. 1.05). Coal balls were first described in 1855 by Hooker & Binney, who interpreted them as limestone nodules and noted the exceptional preservation of the plant tissues preserved within them. Stopes and Watson (1909) provided the most extensive early description of coal balls, describing their *in-situ* formation (which included the first description of a fossilised root apex) and discussion of their possible origin (which they believed resulted from the interactions of the coal swamp peat with marine water). Although the precise method of formation of coal balls is still currently unknown, as is the reason these deposits are not found after the Permian period (Scott & Rex, 1985; Scott *et al.*, 1996), their importance for palaeobotany cannot be understated. After the discovery of

the first coal balls within the Lancashire and Yorkshire coal fields of Great Britain, coal balls were discovered across Europe and America (Galtier, 1997). As new coal-ball deposits were being discovered techniques to cut and produce thin sections, and subsequently acetate peels (Joy *et al.*, 1956), were creating vast palaeobotanical resources (such was the demand for fossil thin sections that at the turn of the 20th Century there was at least one company specialising in selling palaeobotanical slides the 'Lomax Palaeobotanical Company' (Howell, 2005)) (Fig. 1.05b). The exceptional preservation of plant material within coal balls has provided the opportunity to examine development and cellular anatomy of plants growing over 300 mya. This includes the fossilised remains of shoot (Good & Taylor, 1972) and leaf apices (Bower, 1923), key developmental stages such as germinating spores (Scott, 1904); pollen tubes (Rothwell, 1972) and even subcellular structures, including starch grains (Baxter, 1964), and nuclei (Millay & Eggert, 1974). This window onto development of plants over 300 mya has been invaluable for the study of plant evolution and still has huge potential for our understanding of root evolution. Given that coal balls preserve *in-situ* soils they commonly preserve lycopsid root material (e.g. stigmarian rootlets discussed in detail in Chapter 2 are described as being some of the most ubiquitous structures found in coal balls (Weiss, 1933)), the interactions of roots with other organs (e.g. Fungi – (Krings *et al.*, 2011; Strullu-Derrien *et al.*, 2011)), and even have the potential to preserved key structures such as root hairs and root apices (Osborn, 1909; Stopes & Watson, 1909; Stopes, 1917; Halket, 1930, 1932). The thesis therefore aims to use coal balls as a resource to further our understanding about lycopsid root evolution, and look for evidence of key defining features such as root hairs and root apices.

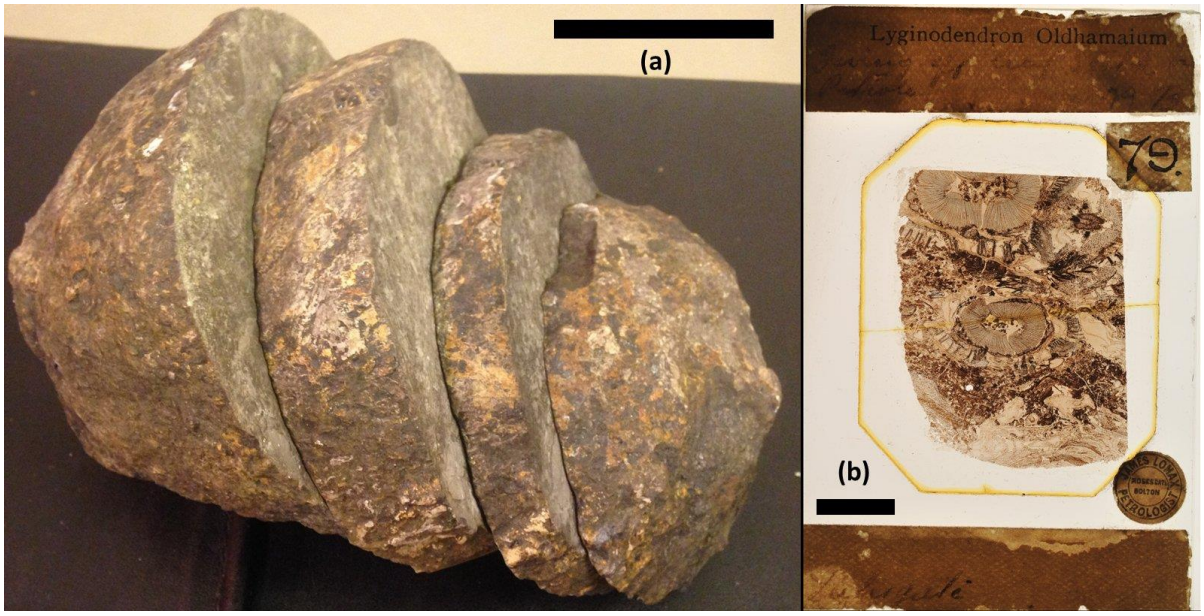

Figure 1.05 Coal balls provide exceptional preservation of fossil plants. Carboniferous coal ball (nodule of permineralised fossil peat), which has been cut into slithers exposing fossil plant material on the cut faces (a). Thin sections made from coal balls show the remarkable preservation of plant material within (b). Scale bars; 5cm (a), 5mm (b). LL. 15952. 1592 collection of the University of Manchester, Manchester Museum (MANCH) (a). Thin section 79, made by the Lomax Palaeobotanical Company, Oxford University Herbaria (OXF) (b).

1.7 Previous studies have lacked integration of molecular data across a range of lycophyte species.

The fourth and final reason for the continued debate about lycopsid root evolution is that there has only been a limited number of studies investigating the genetic basis of lycopsid roots, these studies have been exclusively with the genus *Selaginella*, and two of the three studies disagree in their conclusions. During the last two decades the field of plant evolutionary developmental biology has aimed to better characterize the genetic changes associated with the evolution of development. Integrating molecular data into theories about plant evolution has been very successful especially in shedding new light on hypotheses about the origins and evolution of leaves (Harrison *et al.*, 2005; Floyd & Bowman, 2006, 2010; Tomescu, 2009; Vasco *et al.*, 2016). Although studies focussing on leaf evolution have proved very successful, there has been a lack of equivalent studies with

roots. Examination of the molecular basis of the diversity of root forms in land plants, especially in the lycopsids, has great potential to provide new insights into root evolution. Although these studies would potentially be very informative there is a paucity of studies investigating the molecular basis of lycopsid root evolution, and those studies have been carried out solely with the genus *Selaginella*.

An example of how the integration of molecular data can be hugely successful for studying lycopsid root evolution was the study by Kawai *et al.*, (2010) who used molecular data to investigate the *Selaginella* rhizophore. Kawai *et al.*, (2010), investigated the expression of Class 1 KNOTTED1-LIKE HOMEOBOX (Knox) genes in the rhizophore of *Selaginella uncinata*. The rhizophore, as already described, is a unique root bearing organ characteristic of the rhizophoric clade of extant Selaginalleles (Fig. 1.02) (Imaichi & Kato, 1989; Lu & Jernstedt, 1996; Korall & Kenrick, 2002). The rhizophore shares similarities with both roots and shoots and the longstanding debate about the organ concerns whether it should be classified as either a root or a unique root bearing organ (Nägeli & Leitgeb, 1868; Harvey-Gibson, 1902; Goebel, 1905; Bower, 1908; Uphof, 1920; Wochok & Sussex, 1974; Jernstedt & Mansfield, 1985; Imaichi & Kato, 1989, 1991; Webster, 1992; Jernstedt *et al.*, 1992; Lu & Jernstedt, 1996; Banks, 2009). Although Class 1 Knox genes had an ancient origin in land plants (as demonstrated by Catarino & Hetherington *et. al.*, 2016 *Molecular Biology and Evolution* (Appendix 1)) they are hugely useful for the study of the evolution of plant development as they are markers for 'shoot identity' in euphyllophytes. Class 1 Knox genes have been shown to be crucial for the function and development of both the shoot meristems and leaves but lack expression in the roots of euphyllophytes (Jackson *et al.*, 1994; Lincoln *et al.*, 1994; Schneeberger *et al.*, 1998; Byrne *et al.*, 2000; Hofer *et al.*, 2001). Moreover, it was shown that Class 1 Knox expression is also conserved in the shoots of the lycophyte *Selaginella kraussiana*

(Harrison *et al.*, 2005), indicating that Class 1 Knox expression is highly conserved in the shoots of vascular plants. The study by Kawai *et al.,* (2010) discovered that Class 1 Knox gene expression is found in the shoot apex and rhizophore, but not in the root of *Selaginella uncinata*. The authors took these finding to support the concept that the rhizophore cannot be described as a root (as some authors have argued (Wochok & Sussex, 1974)), but instead support the hypothesis that it is a unique root bearing organ. The findings of the study by Kawai *et al.,* (2010) demonstrated the use of examining gene expression data to add further lines of evidence to help classify the disparity of rooting structures found in the lycopsids. Although their study demonstrated the potential of examining Class 1 Knox genes as makers for the disparate parts of lycopsid rooting structures no further studies have been carried out in other members of the lycopsids. This illustrates the need in the future to extend studies investigating the molecular basis of lycopsid rooting structures into other lineages of the lycopsids.

Alongside the study of Kawai *et al.,* (2010), investigating the rhizophore of *Selaginella*, molecular studies have also been used to investigate the genetic basis of lycopsid roots. However, an additional reason for the continued debate about lycopsid root evolution is that two recent studies (both published after the start of the research described in this thesis) have drawn contrasting conclusions from similar datasets. The first study, with the genus *Selaginella*, was carried out by Huang and Scheifelbein, (2015). The aim of the study was to investigate the similarity in gene expression profiles of a set of phylogenetically distinct taxa from angiosperms and the lycopsid *Selaginella moellendorffii*. The authors utilised next generation sequencing technologies to compare the gene expression profile of the root of *S. moellendorffii* with six angiosperm species: three eudicots (mouse-ear cress [*Arabidopsis thaliana*], tomato [*Solanum lycopersicum*], soybean [*Glycine max*], and cucumber [*Cucumis sativus*]) and two monocots (rice [*Oryza*

sativa] and maize [*Zea mays*]). Comparison of the root expression profiles of the meristem, elongation, and differentiation zones of these plants identified high degrees of similarity between *S. moellendorffii* and the angiosperm species. Huang & Scheifelbeing, (2015) took the similarities in root gene expression profiles to support the hypothesis that roots may not have independently evolved in lycophytes and euphyllophytes, or alternatively that the gene expression patterns in roots have evolved through convergence and co-option of pre-existing genetic networks. The authors took their interpretation to be at odds from the findings of the fossil record and to support only a single origin of roots.

The second study, was carried out by Coudert *et al.*, (2013). The aim of the study was like Huang & Scheifelbeing, (2015) to evaluate the evidence for the single or multiple origin of roots. Unlike the study by Huang & Scheifelbein, (2015) who found striking similarity between lycophyte and euphyllophyte roots, the study by Coudert *et al.*, (2013) found evidence pointing towards the independent origin of roots in lycophytes and euphyllophytes. Auxin has been indicated in the development of both lycophyte (Williams, 1937; Wochok & Sussex, 1974; Sanders, 2007; Sanders & Langdale, 2013) and euphyllophyte roots (Overvoorde *et al.*, 2010). In euphyllophyte roots the auxin developmental pathway is dependent on ASL (asymmetric leaves-2-like)/LBD (LOBdomain) transcription factors (TFs) (Coudert *et al.*, 2013). Although LOBdomain TFs play diverse roles in plant development (Chanderbali *et al.*, 2015) (and LBD TF's had an ancient origin in the aquatic streptophytes ancestors of land plants (Catarino *et al.*, 2016) (Appendix 1)), the phylogenetic analysis carried out by Coudert and colleagues (2013) identified one subclass (Class 1b) which is almost exclusively expressed in the roots of euphyllophytes. By contrast, the only lycophyte included in their study *S. moellenderfii* entirely lacked Class 1b proteins. The authors took the lack of Class 1b proteins in *S. moellenderfii* to indicate that the auxin developmental pathways present in

roots of lycophytes and euphyllophytes is distinct, supporting the independent origin of roots in lycophytes and euphyllophytes.

Taken together these two studies using the genus *Selaginella* examined comparable datasets but came to different conclusions about the single or multiple origins of roots. Both illustrate the continued debate about the origin and evolution of lycopsid roots. However, both studies provide valuable resources for the research described in this thesis. They offer a framework for examining the genetic basis for lycopsid roots. The thesis intends to build on this existing framework (*based on Selaginella*) and generate an additional gene expression dataset for the lycopsid *Isoetes echinospora* in order to compare lycopsid root gene expression profiles between more lycopsid taxa to test hypotheses about lycopsid root evolution. **The aim of the research described in this thesis is to: define a molecular mechanism controlling root development in the genus *Isoetes*.**

1.8 Thesis plan

The goal of the study is to further our understanding of lycopsid root morphology and evolution. The research described in this thesis will achieve this goal by tackling four aims:

- Examine root evolution solely in the lycopsids
- Identify major trends in lycopsid root evolution from extant species, and test if the roots of extinct lycopsids also display the same patterns
- Identify and describe key rooting characters in the fossil record, such as root hairs and root meristems
- Define a molecular mechanism controlling root development in the genus *Isoetes*

First, it aims to examine rooting structure evolution solely in the lycopsids, to avoid the inclusion of problematic early-diverging lycophytes that lack the necessary preservation to correctly characterise their rooting systems. Second, the aim of the research is to examine extant lycopsids to identify patterns of rooting structures, and to test if the roots of extinct lycopsids also display the same patterns. By examining the rooting structures of extant lycopsids (Chapter 1) it is clear that roots of extant lycopsids display two contrasting patterns: conservatism and disparity. The aim of the research is to test if the roots of extinct lycopsids also display these patterns, especially focussing on the evolution of the conserved parts 'roots' (Chapter 2). Third, it aims to look for evidence of illusive root hairs and root meristems of the fossil lycopsids (Chapters 2 and 3). Finally, the research described in this thesis aims to add, and evaluate, new root gene expression data from the lycopsid *Isoetes echinospora* to what is currently known from the genus *Selaginella* to generate a better understanding of the genetic basis of lycopsid roots (Chapter 4). The thesis will achieve these four aims by presenting three papers on the shared theme of root evolution, before presenting a general discussion of the findings in Chapter 5.

Chapter 2: Networks of highly branched stigmarian rootlets developed on the first giant trees

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Original PNAS formatted paper Appendix 2

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Author Contributions

A.J.H. and L.D. designed research; A.J.H. performed research; A.J.H. analyzed data; A.J.H. and L.D. wrote the paper; and C.M.B. provided the first coal ball peels that were found to contain root hairs on stigmarian rootlets and provided advice on fossil material throughout the project.

2.2 Abstract

Lycophyte trees, up to 50 m in height, were the tallest in the Carboniferous coal swamp forests. The similarity in their shoot and root morphology led to the hypothesis that their rooting (stigmarian) systems were modified leafy shoot systems, distinct from the roots of all other plants. Each consists of a branching main axis covered on all sides by lateral structures in a phyllotactic arrangement; unbranched microphylls developed from shoot axes and largely unbranched stigmarian rootlets developed from rhizomorphs axes. Here we re-examined the morphology of extinct stigmarian systems preserved as compression fossils and in coal balls from the Carboniferous period. Contrary to the long-standing view of stigmarian systems, where shoot-like rhizomorph axes developed largely unbranched, root hairless rootlets, here we report that stigmarian rootlets were highly branched, developed at a density of approximately 25,600 terminal rootlets per metre of rhizomorph and were covered in root hairs. Furthermore we show that this architecture is conserved among their only extant relatives, herbaceous plants in *Isoetes*. Therefore despite the difference in stature and the time that has elapsed, we conclude that both extant and extinct rhizomorphic lycopsids have the same rootlet system architecture.

2.3 Significance Statement

Coal swamps were the carbon burial factories of the Carboniferous period forming huge coal deposits and driving climate cooling. The Carboniferous forests were also home to the first giant (>50 m) trees to grow on the planet. These trees were anchored by a unique structure termed a stigmarian system, which is hypothesised to represent a leafy shoot modified to function as a root. Here we report the discovery of the complex, highly branched rootlet structure of these trees. Our findings demonstrate that rootlet architecture

is conserved from the giant extinct trees of the Carboniferous to the small extant herbs of today's flora.

2.4 Introduction

The spread of the first wetland forests with tall trees during the Carboniferous period (359-300 million years ago) had a dramatic impact on the carbon cycle by burying large amounts of organic carbon in the form of peat in coal swamps (Algeo & Scheckler, 1998; Berner, 1998). Lycophyte trees up to 50 m in height (Thomas & Watson, 1976; Stewart & Rothwell, 1993) were dominant components of coal swamp forests (Phillips & DiMichele, 1992; DiMichele & Phillips, 1994). They were key components of coal forming environments throughout the Carboniferous period but dominated in the lower–middle Pennsylvanian (Namurian – Wetsphalian) where they typically contribute between 60–95% of the biomass in buried peat (Phillips *et al.*, 1985; Collinson & Scott, 1987; Rex & Scott, 1987; Lapo & Drozdova, 1989; Cross & Phillips, 1990; Phillips & DiMichele, 1990; Collinson *et al.*, 1994). The preserved remains of lycophyte trees form some of the most extensive fossil plant deposits of any geological period. This is in part because of their size and ecological dominance but also the result of the high probability of preservation in the waterlogged conditions in which these trees grew (Stewart & Rothwell, 1993). Detailed descriptions of the morphology of these plants on a range of scales – from entire *in-situ* tree lycophyte forests (Macgregor & Walton, 1948; DiMichele & Demaris, 1987) to cellular descriptions of developing spores (Phillips, 1979) – have made these trees some of the best understood fossil plants of the Carboniferous coal swamps.

The rooting system of the arborescent lycopsids – stigmarian systems – consist of large shoot like axes (rhizomorphs) that develop lateral organs called rootlets (Williamson, 1887; Frankenberg & Eggert, 1969; Eggert, 1972; Stewart & Rothwell, 1993; Taylor *et al.*, 2009). Rootlets, which have been described as largely unbranched and root hairless (Williamson, 1887; Schoute, 1938; Stewart, 1947; Lemoigne, 1963; Frankenberg & Eggert, 1969; Eggert, 1972; Phillips & DiMichele, 1992; Stewart & Rothwell, 1993; DiMichele & Bateman, 1996; Taylor *et al.*, 2009), are arranged in a characteristic pattern or rhizotaxy on the rhizomorph (Charlton & Watson, 1982). It is the arrangement of these largely unbranched leaf-like rootlets on a shoot like axis which first led to the theory that stigmarian systems were modified leafy shoots (Schimper, 1872; Renault, 1882; Solms-Laubach, 1891). The modified shoot hypothesis got more support towards the end of the twentieth century. The discovery of fossilized embryos showing that the shoot and root axes were derived from a branching event during embryogenesis (Phillips, 1979; Stubblefield & Rothwell, 1981), the documentation that rootlet abscission resembled foliar abscission (Frankenberg & Eggert, 1969; Eggert, 1972), observations on well preserved fossil rhizomorph apices (Rothwell, 1984; Rothwell & Erwin, 1985; Rothwell & Pryor, 1991) and their interpretation within a phylogenetic context (Rothwell & Erwin, 1985; Bateman *et al.*, 1992) led to a complete revival of Schimper's (Schimper, 1872) modified shoot hypothesis.

The only living members of the Isoetales (the group containing these Carboniferous giant trees) are small herbaceous plants in the genus *Isoetes* (Bateman *et al.*, 1992; DiMichele & Bateman, 1996; Kenrick & Crane, 1997; Xue, 2011). The rooting system of *Isoetes* also consists of a rhizomorph meristem which develops rootlets in a regular rhizotaxy (Paolillo, 1963, 1982; Rothwell & Erwin, 1985). Aside from the reduction and modification of the rhizomorph, the rooting systems of *Isoetes* and the tree lycopsids are

morphologically similar (Williamson, 1887; Lang, 1915, 1923; Stewart, 1947; Karrfalt, 1980; Rothwell & Erwin, 1985; Stewart & Rothwell, 1993). However, current models suggest that rootlet architecture is different in extant *Isoetes* and extinct stigmarian rootlets. *Isoetes* rootlets form dense, highly branched networks of rootlets covered in root hairs (Scott & Hill, 1900; West & Takeda, 1915) while stigmarian rootlets are thought to be largely unbranched and root hairless (Williamson, 1887; Schoute, 1938; Stewart, 1947; Lemoigne, 1963; Frankenberg & Eggert, 1969; Eggert, 1972; Phillips & DiMichele, 1992; Stewart & Rothwell, 1993; DiMichele & Bateman, 1996; Taylor *et al.*, 2009). This difference is even more puzzling because the cellular anatomy of stigmarian rootlets and *Isoetes* rootlets is almost identical (Stewart, 1947; Karrfalt, 1980).

Given that the architecture of the stigmarian rootlet systems differs markedly from rooting systems of their extant relatives we hypothesized that the rootlet architecture of the stigmarian rooting system may have previously been misinterpreted. Here we report the discovery of the complex structure of stigmarian rootlet systems from quantitative analysis of rootlet branching and multiple lines of geological evidence. The proposed model reveals that the highly branched rootlet architecture has been conserved over the past 300 million years and is found in the closest living relatives of arborescent lycophytes.

2.5 Results

To compare rootlet architecture of the stigmarian systems with their extant relatives we first defined quantitatively rootlet branching in *Isoetes echinospora* Durieu and *Isoetes histrix* Bory (Supplementary Fig. 2.05). *Isoetes* rootlets branch dichotomously along their length and rootlet diameter decreases by approximately 25% at each dichotomy (Fig. 2.01A, B, D, E, H); the average diameter of the rootlet that develops from the rhizomorph

is 0.73 mm (SD ± 0.21 / SE ± 0.02) mm and the average rootlet diameter of the fifth order branch is 0.21 mm (SD ± 0.04 / SE ± 0.002) mm after four rounds of dichotomous branching (X4 in Fig. 2.01E). Rootlet diameter does not decrease between branch points, i.e. the branches do not taper (Fig. 2.01G, H). These data indicate that *Isoetes* rootlets are highly branched – there are up to 5 orders of branching on each rootlet – and decrease in diameter by approximately 25% at each dichotomy but do not taper.

To test the hypothesis that stigmarian rootlets formed branched networks like *Isoetes*, we characterized the branching morphology of rootlets preserved as compression fossils in Carboniferous sediments (see Supplementary information and Supplementary Fig. 2.06). We found rootlets with up to 4 orders of branching (Fig. 2.01C). Furthermore rootlet diameter decreased by approximately 25% with each order of branching (Fig. 2.01D, F, I) with no evidence of tapering (Fig. 2.01G, I). Together these data from rootlets preserved as compression fossils demonstrate that stigmarian rootlets were branched, rootlet diameter decreased by approximately 25% at each branch point and did not taper. This indicates that the pattern of rootlet branching is similar in extinct stigmarian and extant *Isoetes* rootlets.

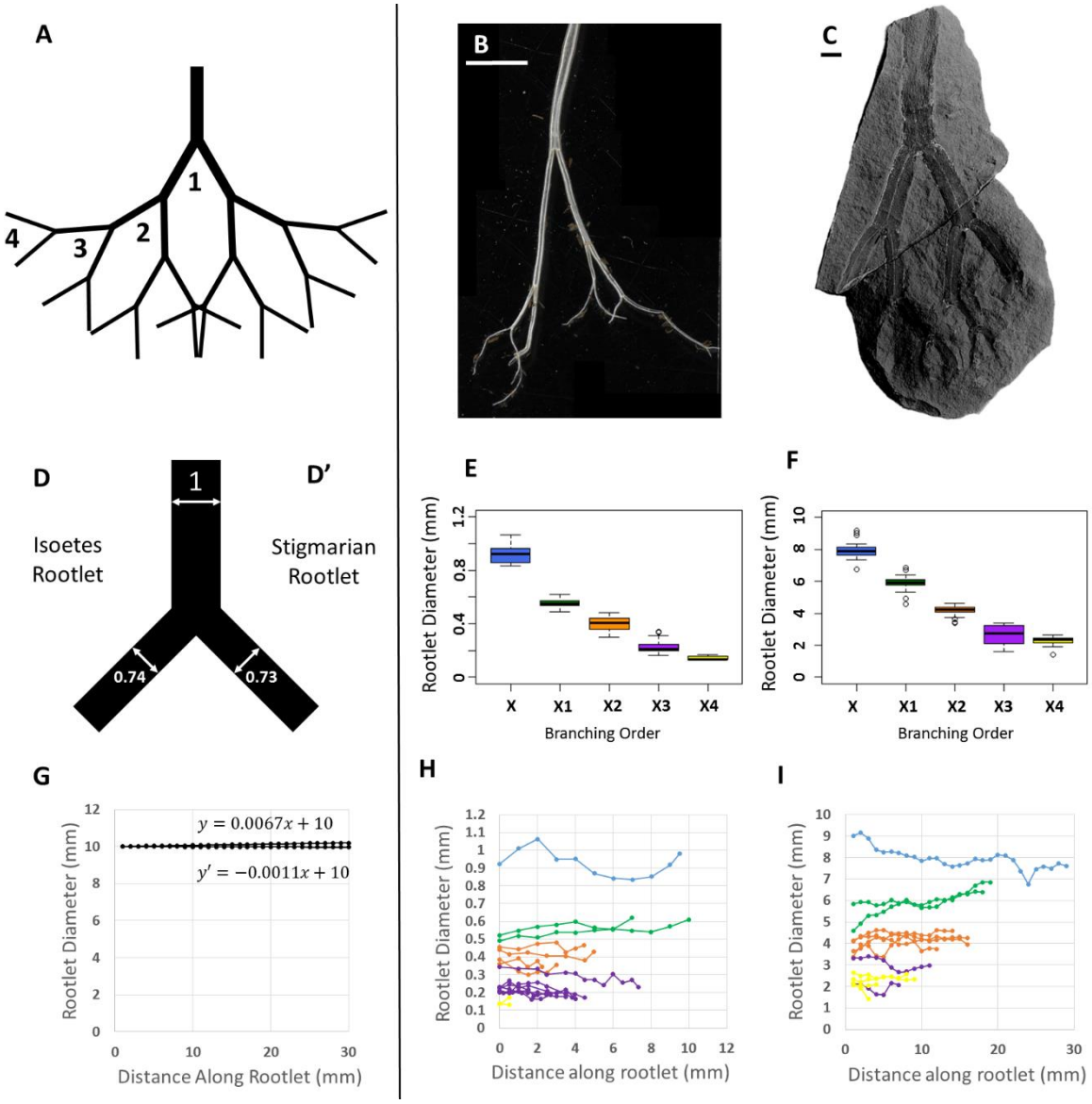

Figure 2.01 Stigmarian and *Isoetes* rootlets do not taper but branch dichotomously, decreasing in diameter in a stepwise manner through multiple orders of branching. (A) Cartoon of branched rootlet showing four orders of dichotomous branching with branching orders labelled. (B) *Isoetes* and (C) stigmarian rootlets branch in a strictly dichotomous manner through multiple orders of branching (scale bar 5 mm). After a branching event, daughter rootlets have an average diameter 74% of the parent rootlet in *Isoetes* (D) and 73% of the parent rootlet in stigmaria (D'). *Isoetes* (E) and stigmarian (F) rootlet diameter decreases in a stepwise manner over four orders of branching (X–X4). (G) Average gradients of the diameter of *Isoetes* (y') and stigmarian (y) rootlet segments indicate that rootlets do not taper between branch points. *Isoetes* (H) and stigmarian (I) rootlets do not taper but decrease in diameter in a stepwise manner through multiple orders of branching; first order branch (blue), second order branch (green), third order branch (orange), fourth order branch (purple) and fifth order branch (yellow). Stigmarian rootlet (C) courtesy of British Geological Survey, Asset number: 687585 [CP15/032]. Photograph taken and fossil determined by Robert Kidson in 1912. Collection locality Ilkeston (United Kingdom).

To test independently the hypothesis that stigmarian rootlets were highly branched we modeled the predicted frequency of rootlet diameters sampled from thin sections of coal balls (Supplementary Fig. 2.07). Coal balls are permineralised peat from the coal swamps in which the anatomical and cellular detail of growing stigmarian rootlets are preserved *in situ* (Stopes & Watson, 1909; Scott & Rex, 1985; Stewart & Rothwell, 1993; Scott *et al.*, 1996). If rootlets branched dichotomously (Fig. 2.02A), as we observed in the compression fossils described above (Fig. 2.01C), we hypothesized that there would be more thin rootlets than thick rootlets in coal ball-preserved stigmarian systems (Fig. 2.02C red). This is because of the geometric increase in the number of progressively smaller terminal rootlets in the dichotomously branching stigmarian rootlet system. However, if rootlets were relatively unbranched, as the long-standing model suggests, and therefore decreased in diameter by tapering (Fig. 2.02B) we would expect to observe equal numbers of small and large diameter rootlets in a sample of roots preserved in coal balls (Fig. 2.02C blue). Our model therefore allows us to determine if stigmarian rootlets preserved in coal balls were unbranched or branched.

We measured the diameter of 785 stigmarian rootlets preserved in 94 coal ball thin sections (Supplementary information) (Supplementary Fig. 2.08). Rootlet diameter was then calculated and the frequency of rootlets in each 0.5 mm diameter class was calculated (Fig. 2.02D). These data demonstrate that there are many more small-diameter rootlets than large diameter rootlets which supports the dichotomous branching rootlet model. To ensure that the distribution of rootlet diameter is not due to variation in local growth conditions we plotted separately the data from 16 individual thin sections with more than 15 stigmarian rootlets and thin sections collected from a variety of collection sites in Central Britain (Yorkshire and Lancashire coal fields (Galtier, 1997)) (Supplementary Fig. 2.09, 2.10). In all cases, the same frequency distribution was observed – there were more

(Supplementary Fig. 2.09, 2.10) small diameter rootlets than large diameter rootlets. This demonstrates that the relationship between rootlet diameter and frequency does not vary from place to place or in different samples. This is consistent with the model in which the stigmarian rootlets are highly branched and this branching pattern did not vary from site to site or from sample to sample.

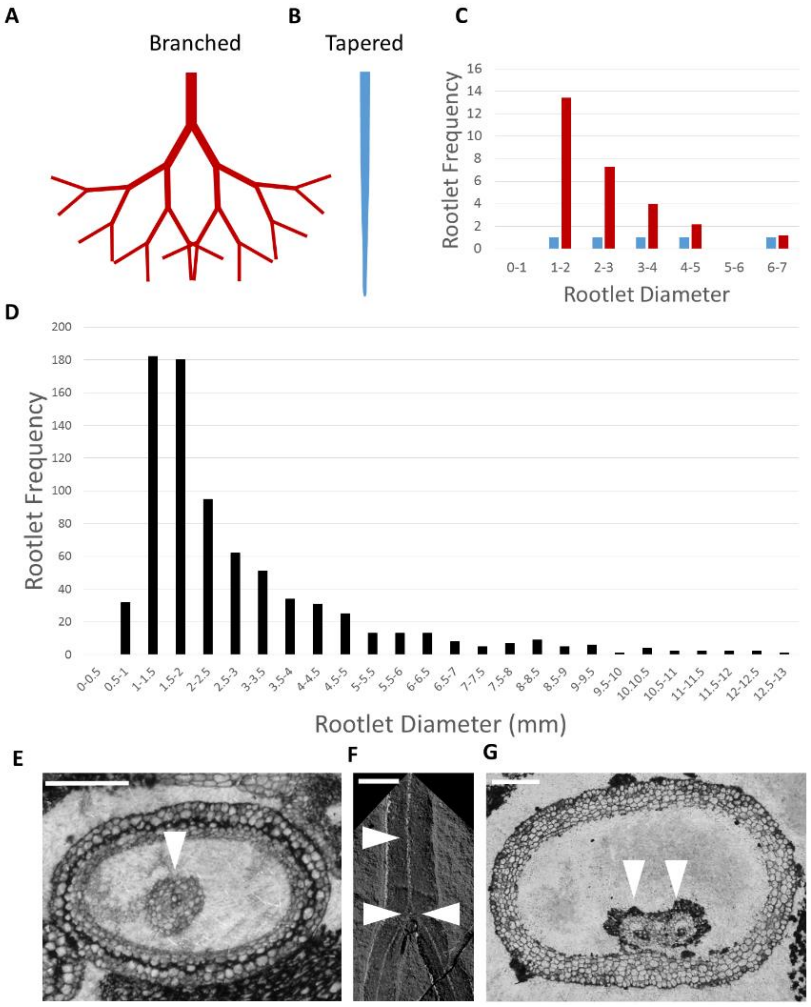

Figure 2.02 Measurement of 785 stigmarian rootlets preserved in coal balls indicates that branching was common. Modelling the predicted frequency of rootlet diameters in coal balls based on a branched (A) and tapered (B) rootlets reveals that the two rootlet types have characteristically different frequency distributions (C; red, branched model; blue, tapered model). (D) Frequency histogram of the diameters of 785 measured stigmarian rootlets preserved in coal balls – note the similarity between the predicted branched rootlet model (red) and the measured results (D). (E) Stigmarian rootlets have a single vascular strand (vascular strand indicated by white arrowheads E–G) except where it bifurcates just before a branching point (F). Twin vascular strands (G) were found in 51 rootlets indicating that rootlets in the coal balls branched frequently. Scale bars (E, G) 0.5 mm, (f) 5 mm. (E, G) Stigmarian rootlets identified in Slide 54 (E) and Slide 25 (G) in the collections of the Oxford University Herbaria (OXF). (F) Magnified image of Fig. 2.01C.

To determine if young, developing rootlets near the rhizomorphs apex could have contributed to the large numbers of thin rootlets in our sample, we measured the diameters of the earliest stages of rootlet development on two preserved apices (Supplementary Fig. 2.12 A, B). Mean rootlet scar diameter within the first 10 cm from the rhizomorph apex was 3.48 mm (SD ± 0.95). The measurements of the rootlet scars on these apices are similar to those observed on other stigmarian apices (Rothwell, 1984; Rothwell & Pryor, 1991). The diameter of these young rootlets were more than twice the diameter of the most abundant, small diameter rootlets that we observed in coal balls (Fig. 2.02D). This demonstrates that the youngest developing rootlets near the apex of the rhizomorph could not account for the large numbers of thin rootlets observed in coal balls. Instead it supports the hypothesis that the thin rootlets were formed through progressive rounds of dichotomous branching of large rootlets.

To verify independently that stigmarian rootlets branched, we searched the coal ball thin sections for rootlets in which there were two vascular strands. Stigmarian rootlets have a single vascular strand (Stewart, 1947; Lemoigne, 1963; Frankenberg & Eggert, 1969; Stewart & Rothwell, 1993) (Fig. 2.03E, white arrowhead); however, above a point of branching the vascular strand locally bifurcates (Fig. 2.03F, white arrowheads). Finding rootlets in coal balls containing two vascular strands therefore indicates that rootlets in coal balls branched (Fig. 2.03G, white arrowheads). Of the 785 samples observed, twin vascular strands were found in 51 rootlets (Fig. 2.03G, white arrowheads), demonstrating that these sections were made just above a branch point (Supplementary Fig. 2.11). Of these the circumference of 42 rootlets could be measured. Twin vascular strands were observed in rootlets with diameters ranging from 1.1 mm to 12.8 mm indicating that rootlets of all size classes branched (Supplementary Fig. 2.11). The frequency distribution of the diameters from the 42 rootlets further supported the branched rootlet model

(Supplementary Fig. 2.11). The observed peak frequency diameter of branching rootlets was 2–2.5 mm (Supplementary Fig. 2.11) indicating that the smallest and most frequent rootlets undergoing dichotomous branching were in the 2–2.5 mm diameter range. We previously showed that daughter rootlet diameter is approximately 73% the diameter of the rootlet from which they formed. According to this measure the peak diameter that we would expect to see produced from the branching of 2–2.5 mm diameter rootlets would be in the 1.5–1.8 mm diameter range. The peak frequency rootlet diameter found in all rootlets examined being between 1–2 mm (Fig. 2.02D). Taken together diameter frequency distributions of rootlets preserved in permineralised coal balls demonstrate that stigmarian rootlets were highly branched.

Root hairs have not previously been found on stigmarian rootlets and their absence led to the suggestion that root hairs did not develop in these plants (Schoute, 1938; Lemoigne, 1963; Phillips & DiMichele, 1992; Taylor *et al.*, 2009). However because root hairs develop on *Isoetes* rootlets (Scott & Hill, 1900; Leavitt, 1904; West & Takeda, 1915) (Fig. 2.03A–C, black arrows) we hypothesized that root hairs would have formed on stigmarian rootlets. A total of 21 root hairs were discovered on 9 stigmarian rootlets on 7 individual thin sections made from different coal balls (Fig. 2.03D and E, see Supplementary information and Supplementary Fig. 2.13). Mean stigmarian root hair diameter was 14.3 µm (SD ± 2.6 / SE ± 0.56 µm) and the root hair highlighted with an arrow in Fig. 3E was 13.9 µm in diameter. Root hair diameter of the two *Isoetes* root hairs shown in Fig. 2.03C were 9.2 and 10.7 µm. These data indicate that root hairs developed on stigmarian rootlets and that they were morphologically similar to the root hairs that develop on extant *Isoetes* species.

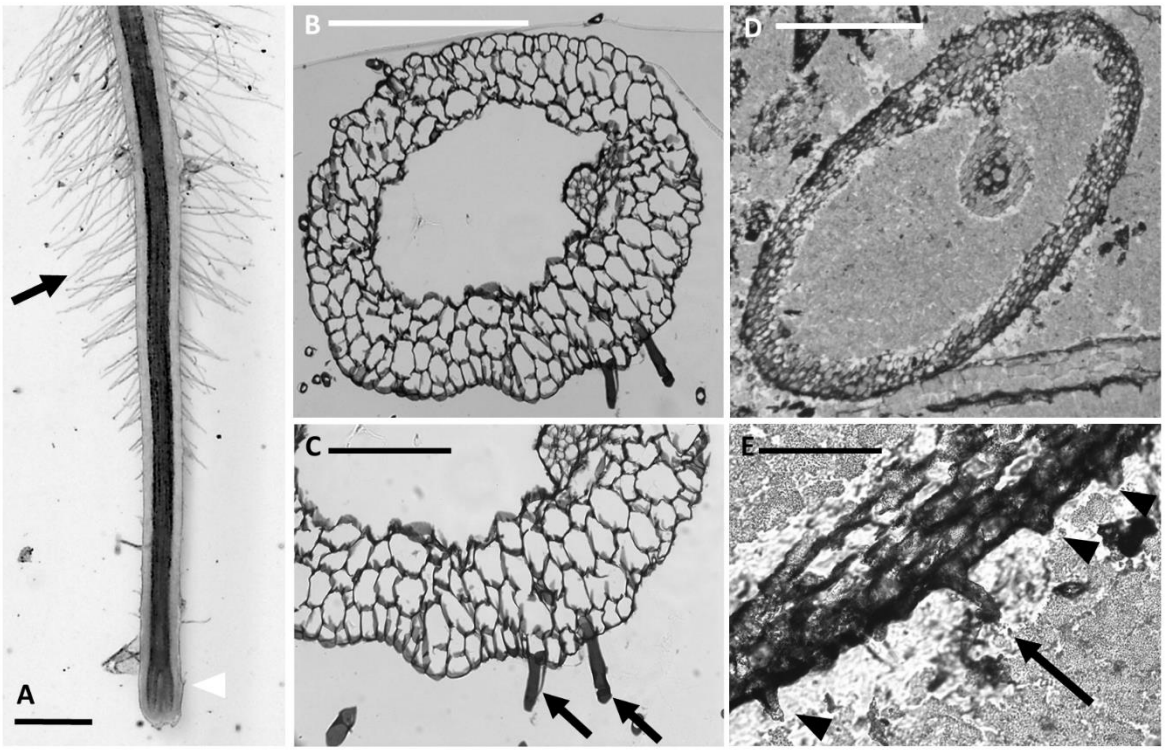

Figure 2.03 Stigmarian rootlets possessed root hairs. (A) *Isoetes* rootlets develop root hairs (black arrows A–E) (note the *Isoetes* rootlet is in the process of branching at the apex – white arrowhead). (B–E) Transverse sections of *Isoetes* (B, enlarged region of B, C) and stigmarian (D, enlarged region of D, E) rootlets showing root hair outgrowths from the epidermis. Black arrowheads indicate the base of root hairs which have been broken off. Scale bars (A, D) 500 µm, (B) 200 µm, (C, E) 100 µm. (D, E) stigmarian rootlet identified in National Museum of Wales . NMW 2016.9G.1.

The lycopsid trees of the British Carboniferous wetland forests comprised both sigillarian and non-sigillarian species (Phillips *et al.*, 1985; Bateman *et al.*, 1992; Galtier, 1997). To determine if the rootlet branching pattern was the same in each, we scored the presence of rootlets with twin vascular strands and determined the distribution of rootlet diameters in both sigillarian and non-sigillarian rootlets. There is a "connective" of cortical tissue between the vascular trace and the outer cortex in sigillarian rootlets (Leclercq, 1930; Stewart, 1947; Frankenberg & Eggert, 1969; Eggert, 1972; Stewart & Rothwell, 1993) (Supplementary Fig. 2.14a). By contrast, there is no connective in the central cavity of the non-sigillarian rootlet and the central vascular trace is free within the rootlet cavity (Stewart, 1947; Frankenberg & Eggert, 1969; Stewart & Rothwell, 1993) (Supplementary

Fig. 2.14b). First we identified twin vascular stands in both sigillarian and non-sigillarian rootlets, which indicates both sigillarian and non-sigillarian rootlets in the coal balls sampled were branching (Supplementary information). Second, the frequency distribution of rootlet diameters is similar for sigillarian and non-sigillarian rootlets (Supplementary Fig. 2.14c) (Supplementary information). These data indicate that both sigillarian and non-sigillarian rootlets branched 3-4 times (Supplementary information). Furthermore, root hairs are present on both sigillarian and non-sigillarian rootlet types (Supplementary information). We conclude that both sigillarian and non-sigillarian rootlets formed similar bifurcating rootlets systems to those found in *Isoetes* today.

Using the quantitative data from this analysis, we constructed a new model for the stigmarian system (Fig. 2.04). Since rootlets developed at densities of approximately 1,600 rootlets m^{-1} rhizomorph (Charlton & Watson, 1982) (this study) and we assumed that each rootlet branched at least four times (this study), we calculated a density of 25,600 terminal rootlets m^{-1} of rhizomorph with a surface area 5.5 times larger than unbranched rootlet systems (assuming that living root hairs are present only on the terminal two orders of branching) (see methods). This model shows a stigmarian system with a densely-packed cylinder of interwoven rootlets around the rhizomorph axes (Fig. 2.04).

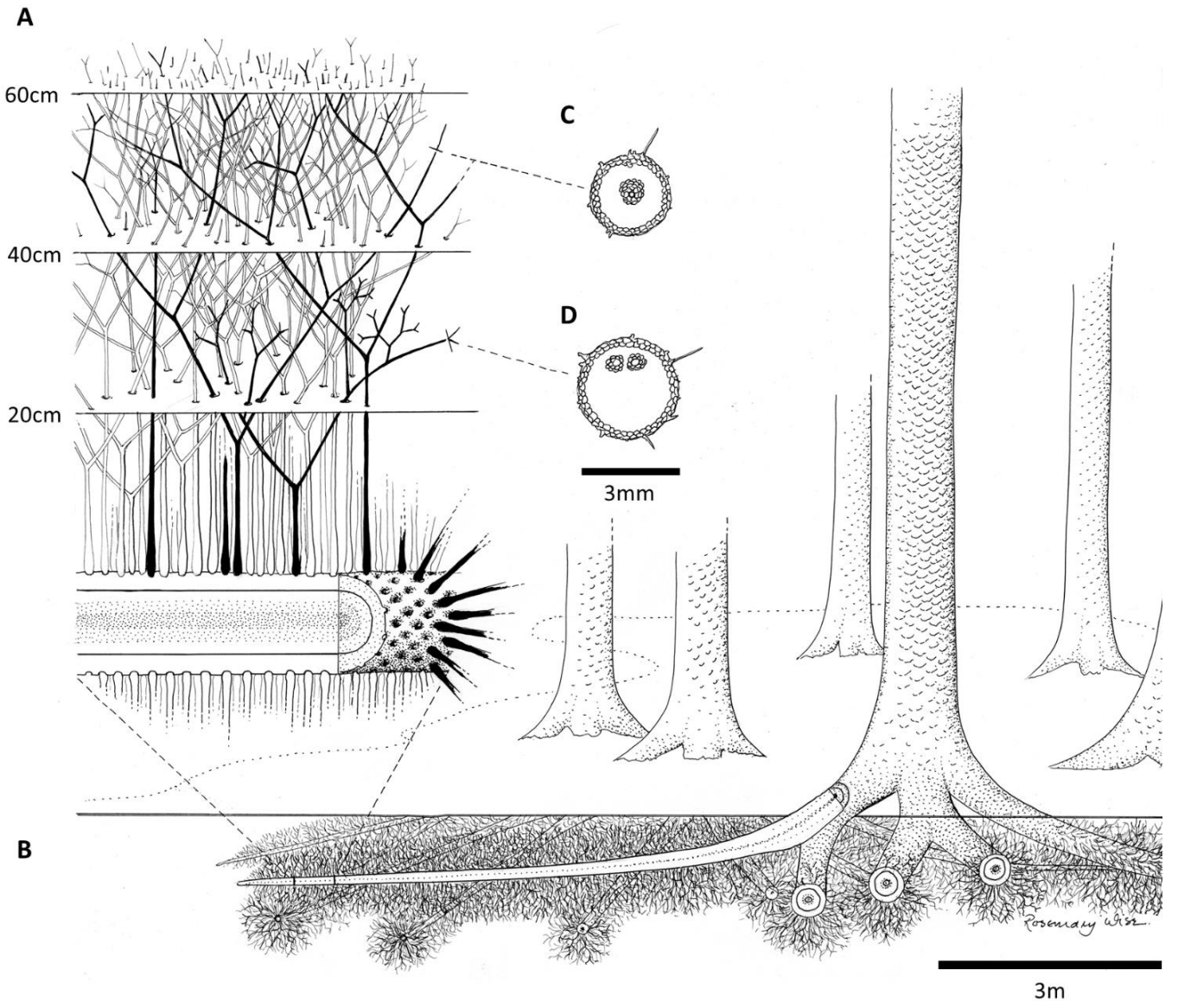

Figure 2.04 Reconstruction of stigmarian root systems with highly branched systems of rootlets. (A) Reconstruction of a population of branching rootlets that could grow to over 90 cm in length. (B) Reconstruction of the root plate that comprised rhizomorph axes covered with rootlets producing approximately 25,600 terminal rootlets per metre. (C, D) Rootlets were covered in root hairs. (C) The single vascular strand characteristic of stigmarian rootlets bifurcated at each branch point (D), and the diameter of the two daughter branches produced was approximately 25% that of the parent rootlet.

2.6 Discussion

We demonstrate that the rootlets of stigmarian systems were highly branched – branching dichotomously up to 5 times – and were covered in root hairs. We verified the highly branched architecture through quantitative analysis of the numbers and diameters of stigmarian rootlets preserved in coal balls. Analysis of the size distribution of stigmarian rootlets in coal balls provided us with the unique opportunity to investigate the entire population of stigmarian rootlets growing *in situ* regardless of either the diameter of the rootlet or the proximity of the rootlet to the rhizomorph axis. This analysis was possible because stigmarian rootlets are ubiquitous in coal balls (Scott, 1900; Weiss, 1933), and can be readily identified because of their unique cellular anatomy composed of three zones of cortex, the middle of which rapidly disintegrates leading to the formation of a large air space containing the inner cortex and central vascular strand (Stewart, 1947; Frankenberg & Eggert, 1969). This anatomical detail allows stigmarian rootlets to be easily distinguished from the rooting structures of other plants that grew in the Coal swamps (Supplementary Fig. 2.08). Furthermore, the exquisite cellular preservation of these *in situ* fossils allowed the visualization of root hairs developing from the rootlet epidermal surface for the first time. Such an extensive branched system would have formed a subterranean network with a large surface area available for nutrient uptake and tethering these giant trees in place.

We suggest that the previous model for stigmarian rooting systems was incomplete because it was based on compression fossils in which the full extent of the rootlet network was obscured. Furthermore, isolated stigmarian rootlets preserved in compression fossils have few features distinguishing them at this coarse level of preservation making them difficult to identify (Binney & Kirkby, 1882). Therefore isolated branched rootlets have not contributed to the construction of the long-standing model of stigmarian rootlet

architecture. This means that previous interpretations of stigmarian systems were biased; reconstructions were based on the proximal portions of the rootlets where they attach to the rhizomorphs and could be identified unequivocally. However, because rootlets can extend for over 90 cm from the rhizomorph surface (Lindley & Hutton, 1831; Logan, 1842; Binney, 1844; Binney & Harkness, 1845) this bias means that the morphology of the distal branched regions of the rootlets remained undescribed. Through quantitative analysis of rootlet architecture in both compression and *in situ* preserved permineralised fossils, we have been able to demonstrate that stigmarian rootlets were highly branched.

Highly branched rootlets would have contributed to the anchoring of these giant trees. Branched root structures are between twice and seven times more resistant to pull-out compared with unbranched structures (Dupuy *et al.*, 2005; Mickovski *et al.*, 2007; Giadrossich *et al.*, 2013) and the discovery of root hairs would not only have increased the surface area but would have further contributed to anchorage (Ennos, 1989). The tree lycophytes would have formed large root plates as individual rhizomorph axes could extend for over 12 m (Williamson, 1887) from the trunks of large trees. Given that tree lycopsids have additionally been reported to grow at high densities (up to 1,769 stems ha^{-1} (DiMichele & Demaris, 1987)) in coal swamp forests (Macgregor & Walton, 1948; DiMichele *et al.*, 1996) root plates would have also interlocked with neighbouring stigmarian systems. Highly branched rootlets would have further consolidated these extensive root plates (Fig. 2.04). It is the ability of root plates to resist movement when the aerial parts of the tree are subjected to lateral force that provides structural support to tall trees (Niklas, 1998). We predict that highly branched stigmarian rootlets would have contributed to the anchorage of these giant trees.

The first giant wetland trees to grow on Earth with their unique stigmarian rooting systems have attracted the attention of scientists for well over 150 years (Steinhauer, 1818;

Binney & Harkness, 1845; Williamson, 1887; Stewart, 1947; Frankenberg & Eggert, 1969; Eggert, 1972; Rothwell & Erwin, 1985; Stewart & Rothwell, 1993). Recent studies have built on this foundation of knowledge and have shed fresh light on physiological mechanism controlling their development, structure and interaction with other organisms (Krings *et al.*, 2011; Sanders *et al.*, 2011; Rothwell *et al.*, 2014; Boyce & DiMichele, 2016). The discovery that stigmarian rootlets were highly branched, developed root hairs and share the same branching architecture as extant *Isoetes* rootlets reveals a remarkable conservatism in rootlet architecture between the first giant trees and their only living herbaceous relatives.

2.7 Methods

2.7.1 Isoetes collection and plant growth

Isoetes histrix was collected in March 2014 on the Lizard Peninsula, Cornwall, UK with the permission of the National Trust and Natural England. *Isoetes echinospora* was collected in September 2013 and 2014 from North West Sutherland, Scotland, UK with the permission of the John Muir Trust and The Scourie Estate. *Isoetes histrix* plants were grown in Levington M2 compost. *Isoetes echinospora* were grown submerged in aquaria in Levington M2 compost topped with coarse gravel. Both were grown at 20^{0}C with a 16 hr photoperiod.

2.7.2 Quantifying *Isoetes* and stigmarian rootlet architecture

Isoetes rootlets were imaged with a Leica M165 FC (Fig. 2.01B, Supplementary Fig. 2.05A–I). *Isoetes* rootlet diameter was measured using Fiji (Schindelin *et al.*, 2012) (Fig. 2.01H, Supplementary Fig. 2.05A'–I'). Standard deviation and Standard error were calculated using (Microsoft Excel 2013). Graphs were plotted using (Microsoft Excel

2013). Box plots were made in RStudio (2013) ('RStudio: Integrated development environment for R (R version 3.0.2)', 2013). To establish whether rootlets taper, the diameter of 167 rootlet segments (only including segments covered by five or more diameter measurements) were plotted against distance along their respective rootlets. A linear trend line was then applied to each data series allowing the gradient of each trend line to be calculated (Microsoft Excel 2013). An average gradient of y' = -0.0011x (Fig. 2.01G) was calculated from the 167 rootlet segments. Decrease in rootlet diameter at each branching point was calculated by comparing the average diameter of 227 daughter rootlets with the average diameter of their parent rootlet. Daughter rootlets had an average diameter of 74% of their parent rootlet. Stigmarian rootlet architecture was quantified using the same method described above for *Isoetes* rootlets (Fig. 2.01C; Supplementary Fig. 2.06A–D). The same method was used to investigate if stigmarian rootlets tapered, this time the average rootlet gradient was calculated from 40 rootlet segments giving an average rootlet gradient of y = 0.0067x (Fig. 2.01G). Average decrease at each branching point was calculated in a similar fashion to *Isoetes* using the measurements of 36 daughter rootlet diameters compared to their parent rootlets. Daughter rootlets had an average diameter of 73% of their parent rootlet.

2.7.2 Modelling the predicted frequency of rootlet diameters in coal balls

The model is based on the principle that the length of a rootlet segment is equal to the frequency of finding that segment in a random rootlet sample from coal balls.

2.7.2.1 The branched rootlet (Fig. 2.02A, Supplementary Fig. 2.07A)

The branched rootlet model undergoes 4 rounds of dichotomous branching, resulting is 16 terminal rootlets. At each dichotomy the diameter of the daughter rootlet is 0.73 that of the parent rootlet (based on the measurements made in this study). After a bifurcation point the daughter rootlet segment is 0.92 the length of the previous segment. This value is based on

measurements of 96 *Isoetes* daughter rootlet segments compared to their parent rootlet segments (only using rootlet segments which started and terminated with a branching point to avoid the bias of using rootlets which had not finished growing or had been broken off).

2.7.2.2 The tapered rootlet (Fig 2.02B, Supplementary Fig. 2.07B)

The tapered rootlet is made up of 5 segments of equal length. Each segment is 0.73 the diameter of the previous segment such that the size decrease (tapering) between the branched rootlet and the tapered rootlet is the same.

2.7.2.3 The model Supplementary Fig. 2.07C

To compare between the two types of rootlets an initial starting diameter (D) and a combined length of the 5 segments (L) was assumed for both rootlets. From this it is possible to calculate the length (frequency) of finding a particular diameter (D) of rootlet segment. In order to determine a realistic value for diameter (D) a starting diameter of 6 mm (Frankenberg & Eggert, 1969; Phillips & DiMichele, 1990; Stewart & Rothwell, 1993; Taylor *et al.*, 2009) was used (Fig, 2.02C). After 4 orders of decreasing in diameter by 0.74 this results in a terminal rootlet diameter of 1.7 mm, a value similar to the terminal rootlets of the compression fossil (Fig. 2.01C) and approaching the smallest sizes of isolated stigmarian rootlets previously reported from coal balls (Leclercq, 1930) interpreted as coming from distal portions of stigmarian systems (Eggert, 1972).

2.7.3 Measuring the diameter of stigmarian rootlets from coal balls

Thin sections prepared from Carboniferous coal balls held in the Oxford University Herbaria (OXF) (97 Slides) and Oxford University Museum of Natural History (OUMNH) (42 Slides) were inspected and stigmarian rootlets identified. All of the available slides were used rather than only those that were made to display stigmarian systems, in order to take an unbiased approach. Images were captured of 785 rootlets from 94 thin sections

with a Leica M165 FC stereo microscope. The circumference of 785 rootlets was measured using Fiji (Schindelin *et al.,* 2012). The rootlets were grouped into 0.5 mm size bins and plotted on a histogram (Fig. 2.03C) (Microsoft Excel 2013).

2.7.4 Measurement of rootlet scar diameter on rhizomorph apices

Two rhizomorph apices were photographed by A.J.H in the collections of The University of Manchester, Manchester Museum (MANCH). The diameter of 36 rootlet scars were measured from the well preserved apex (Collection No: LL. 15952.470, Supplementary Fig. 2.12A), and 12 rootlet scars were measured form the poorly preserved apex (Collection No: LL. 15952.471, Supplementary Fig. 2.12 8B) using Fiji (Schindelin *et al.*, 2012) (Supplementary Fig. 2.12A,B). Average rootlet scar diameter was plotted for each 2 cm interval from the apex (Supplementary Fig. 2.12A',B') (Microsoft Excel 2013).

2.7.5 Isoetes and stigmarian root hairs

The distal portion of a single *Isoetes echinospora* rootlet was imaged using a Leica M165 FC stereo microscope (Fig. 2.03A). Additional rootlets were embedded in paraffin, sectioned and stained with toluidine blue. Slides were imaged with an Olympus BX50 compound microscope using bright field (Fig. 2.03B,C). Stigmarian root hairs were imaged with an Olympus BX50 and root hair diameter was measured with Fiji (Schindelin *et al.*, 2012).

2.7.6 Estimating surface area increase

In order to estimate the increase in surface area due to branching we again used the simplified rootlet models (branched and unbranched Fig. 2.02A and B; Supplementary Fig. S7A–C). Rootlets were assumed to be cylindrical and the surface area of each segment of rootlet was calculated with $SA = \pi dh$. The presence of branching results in the branched rootlet model having a surface area 3.9 times larger than the tapered model. Next we

included an estimate of the increased surface area provided by root hairs. Dittmer (Dittmer, 1937) estimated that the surface area of the rye (*Secale cerale* L.) root systems was 6,875.4 ft^{-2} with root axes contributing 2,554.09 ft^{-2} and the root hairs contributing 4,321.31 ft^{-2}. We therefore assumed that an axis with root hairs has a surface area 1.7 times that of the same axis lacking root hairs. Dittmer (Dittmer, 1937) estimate is applicable for the stigmarian system as 50% of the epidermal cells in *Isoetes* form root hairs(Dolan, 1996) as they do in rye (Clowes, 2000). As a conservative estimate we did not take into account root hairs on the top three branching orders (where they may have been sloughed off in the soil) but estimated that root hairs would contribute an additional 1.7 times the surface area over the final two orders of branching. This resulted in our new branched root model having a surface area 5.5 times that of the tapered model (Stewart, 1947; Lemoigne, 1963; Frankenberg & Eggert, 1969; Stewart & Rothwell, 1993; Taylor *et al.*, 2009).

2.8 Acknowledgements

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2.9 Supplementary Information

2.9.1 Stigmarian rootlets

Eight branched stigmarian rootlets were identified from five compression fossils. Four branched stigmarian rootlets were found in the photographic collections of the British Geological Survey and reproduced with the permission of the British Geological Survey (CP15/032):

Two branched rootlets were identified in Asset number: **P685925 –** Supplementary Fig. 2.06A

Caption: *Stigmaria ficoides* - dichotomous rootlets. Monckton Main Coll, Barnsley. [Fossil plant].

Description: *Stigmaria ficoides* - dichotomous rootlets. Kidston negative number: Kidston 50. Thin section no. Kidston 2600. Magnification x 100. Half plate. Box 1.

Date taken: 01/01/1899

Photographer: Kidston, R.

One branched rootlet was identified in Asset number: **P687428 –** Supplementary Fig. 2.06B

Caption: *Stigmaria ficoides* (Sternberg). Maltby Bore, Rotherham. [Fossil plant].

Description: *Stigmaria ficoides* (Sternberg). Kidston negative number: Kidston 1648. Magnification x 100. Quarter plate. Box 3.

Date taken: 01/01/1911

Photographer: Kidston, R.

One branched rootlet was identified in Asset number: **687585 –** Fig. 2.01C

Caption: *Stigmaria* / Dichotomously divided rootlet. Shipley Clay Pit, Ilkeston. [Fossil plant].

Description: *Stigmaria* / Dichotomously divided rootlet. Kidston negative number: Kidston 1836. Dr Moysey's specimen. Magnification x 100. Quarter plate. Box 4.

Date taken: 01/01/1912

Photographer: Kidston, R.

One branched rootlet was photographed by AJH in the collections of the London Natural History Museum (NHMUK)

Collection No: **V.24929 –** Supplementary Fig. 2.06C

Description: Stigmarian rootlet (branching).

Formation: Coal Measures

Locality: Hensies Boring, nr, MONS. Pit No.1. 786m.

Three branched rootlets were photographed by AJH in the collections of The University of Manchester, Manchester Museum (MANCH)

Collection No: **W.1896 –** Supplementary Fig. 2.06D

Description: *Stigmaria* (dichotomising branches).

Location: Barnsley, 625 yds deep

2.9.2 Coal balls thin sections

All Carboniferous coal ball thin sections from the Oxford University Herbaria (OXF) (97 Slides) and Oxford University Museum of Natural History (OUMNH) (42 Slides) were examined for the presence of stigmarian rootlets. All of the available slides were used rather than only those that were made to display stigmarian systems, in order to take an unbiased approach. All slides examined were believed to come from individual coal balls because there was no evidence of sequential sections on consecutive slides. Most slides lack maker's marks but 38 are marked as being produced by James Lomax (Howell, 2000). Individual coal ball slides thus provide us with individual time points during the swamp ecosystem. The coal balls used were also collected from a variety of collection sites in the Lancashire and Yorkshire coal fields (Galtier, 1997). The carboniferous plants from the Lancashire and Yorkshire coal deposits were the principle material used for the early anatomical characterizations of the stigmarian system (Williamson, 1887; Leclercq 1930;

Weiss 1933; Scott 1900; Binney & Harkness, 1945; Binney, 1862) thus making these slides ideal for a reinvestigation.

2.9.3 Rhizomorph apices

Two rhizomorph apices were photographed by A.J.H in the collections of The University of Manchester, Manchester Museum (MANCH).

Collection No: LL. 15952.470

Description: *Stigmaria ficoides,* Brong. (root contracting to point). Coal Measures. E Coll Williamson.

Collection No: LL. 15952.471

Description: *Stigmaria ficoides,* Brong. (root contracting to point). Coal Measures. E Coll Williamson.

2.9.4 Stigmarian root hairs

The same slides used to investigate stigmarian rootlet diameter were investigated for the presence of root hairs as well as additional collections of acetate peels (Joy, 1956) from the teaching collection in the School of Earth and Ocean Sciences, Cardiff University. Nine rootlets were found with stigmarian root hairs (Fig. 2.03D and E, Supplementary Fig. 2.13A–J). The collection localities of the coal balls containing root hairs are; Oxford University Herbaria (OXF) 78, 94, 95, were from individual coal balls from Dulesgate (Galtier, 1997). Manchester Museum (MANCH) LL15952 unknown locality. The acetate peels from the teaching collection in the School of Earth and Ocean Sciences, Cardiff University are of unknown locality but are believed to be have been made for Prof. A. G. Lyon by his technician from coal balls collected at the Sutcliffe (quarry/mine) Burnley, United Kingdom.

2.9.5 Sigillarian rootlets branched

To test the hypothesis that sigillarian rootlets were highly branched we scored each of the 785 rootlets for the presence of a connective characteristic of sigillarian rootlets. There was a connective in 122, no connective in 464 and 199 rootlets could not be scored unequivocally. This indicates that approximately 20% of the rootlets which could be scored for the connective were sigillarian. The frequency of sigillarian and non-sigillarian rootlet diameters was plotted (Supplementary Fig. 2.14c). The distribution of diameter frequencies of sigillarian and non-sigillarian rootlets were similar: there were few large diameter rootlets and many small diameter rootlets. The observation that there are many more small diameter rootlets than large diameter rootlets in both sigillarian and non-sigillarian rootlets demonstrates that both were highly branched and supports the branched rootlet model reported in Fig. 2.02C.

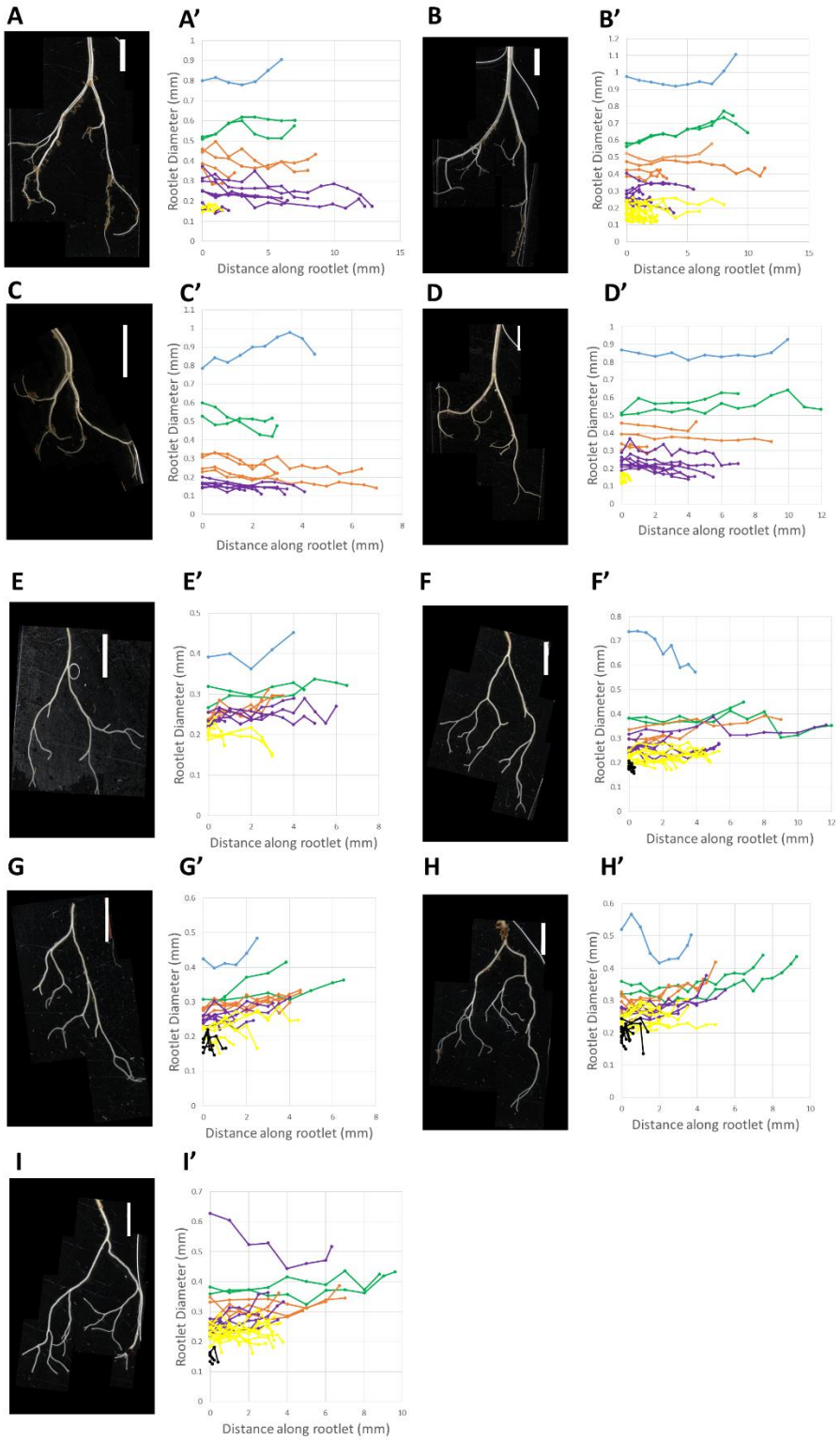
To independently verify that sigillarian rootlets branched we scored for the presence of a connective in the 51 rootlets identified with twin vascular strands. There was a connective in 7 rootlets with twin vascular strands indicating that they were sigillarian. 22 rootlets with twin vascular strands lacked a connective indicating that they were non-sigillarian rootlets. The connective could not be scored in the remaining 22 sectioned rootlets. This presence of twin vascular strands in rootlets with and without connectives indicates that both sigillarian and non-sigillarian rootlets branched.

The maximum diameter of a sigillarian rootlet found in our analysis was 4.1mm. If a typical sigillarian rootlet starts with an initial diameter of 3-4 mm which is consistent with our observed results (Supplementary Fig. 2.14C) it would require three to four orders of branching for the rootlet diameter to decrease to the peak rootlet size of 1-1.5 mm. With the removal of sigillarian rootlets the peak rootlet frequency of non-sigillarian rootlets is 1.5-2 mm in diameter. If a typical non-sigillarian rootlet starting diameter is 5-7mm

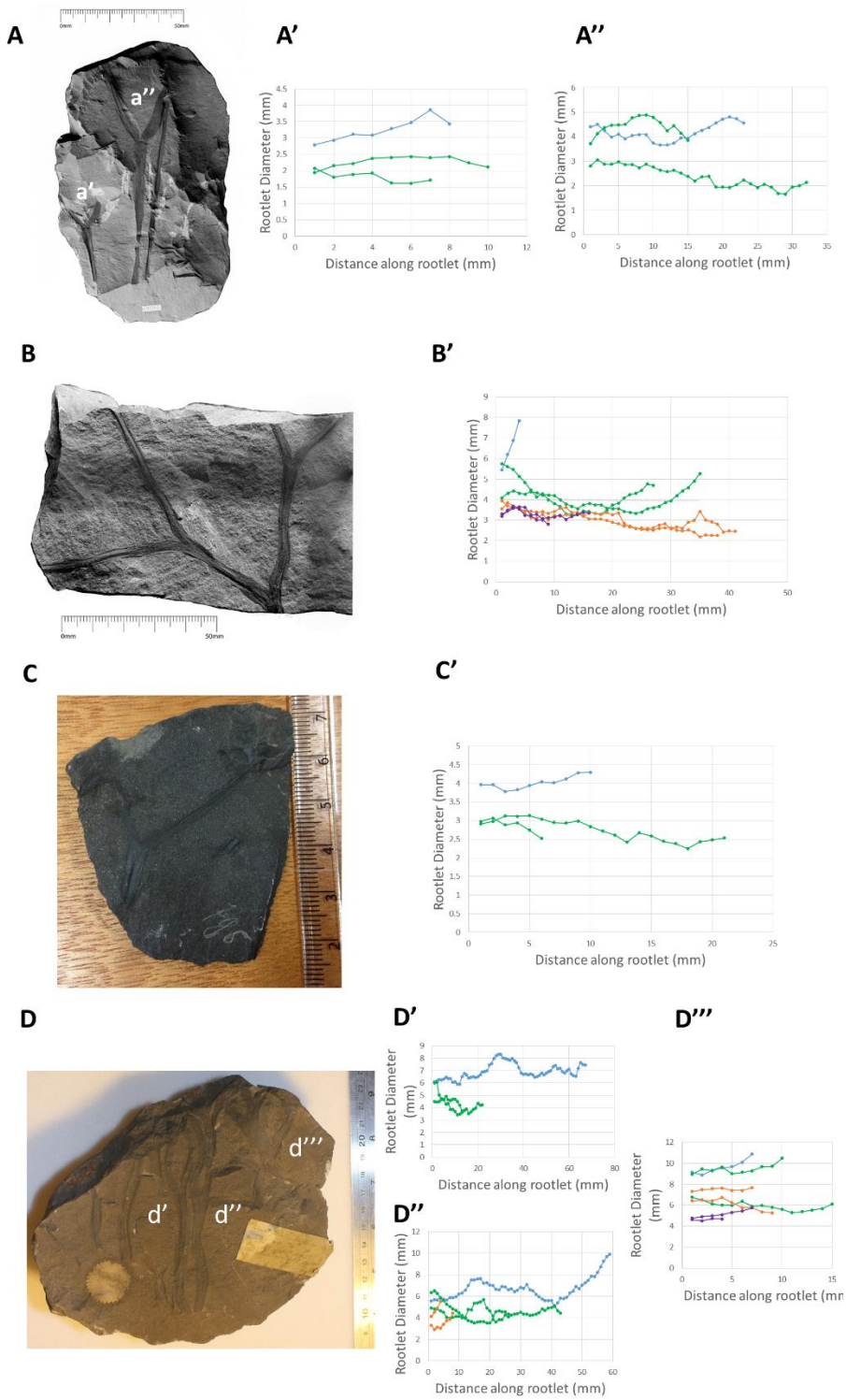
(Stewart & Rothwell, 1993; Frankenberg & Eggert, 1969; Taylor *et. al.*, 2009; Stewart 1947; Lemoigne, 1963) it would decrease in diameter to this peak frequency through three to four orders of branching (four orders for any rootlet starting with a diameter >5.2mm). We conclude that the majority of rootlets –sigillarian and non-sigillarian –underwent approximately 4 orders of branching.

To determine if root hairs developed on both sigillarian and non-sigillarian rootlets we determined if connectives were present in rootlet sections on which root hairs had been identified. Both sigillarian (4 rootlets Fig. 2.03D, Supplementary 2.13 A-E) and non-sigillarian rootlets (2 rootlets Supplementary Fig. 2.13 F, J) developed root hairs. Taken together these data indicate that both sigillarian and non-sigillarian rootlets were highly branched and developed root hairs similar to the rootlets of extant *Isoetes*.

2.9.6 Supplementary figures

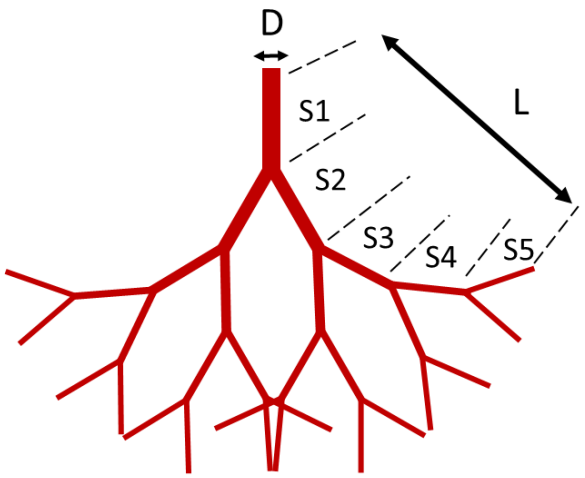


Supplementary Figure 2.05 ***Isoetes*** **rootlet architecture.** Images of *Isoetes echinospora* (A–D) and *Isoetes histrix* (E–I) rootlets and measurements of their respective diameters along the length of each rootlet segment. First order branch (blue), second order branch (green), third order branch (orange), fourth order branch (purple) and fifth order branch (yellow). Scale bar 5 mm.



Supplementary Figure 2.06 **Stigmarian rootlet architecture.** Images of stigmarian (A–D) rootlets and measurements of their respective diameters along the length of each rootlet segment. First order branch (blue), second order branch (green), third order branch (orange), fourth order branch (purple). (A) British Geological Survey Asset number: P685925 [CP15/032]. (B) British Geological Survey Asset number: P687428. (C) [CP15/032]. London Natural History Museum (NHMUK) Collection No: V.24929. (D) The University of Manchester, Manchester Museum (MANCH) Collection No: W.1896.

A



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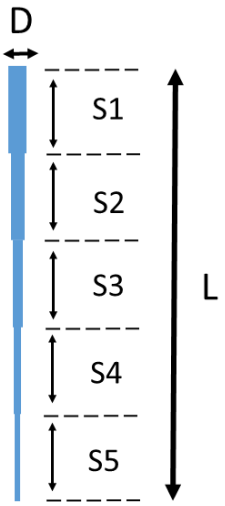


C

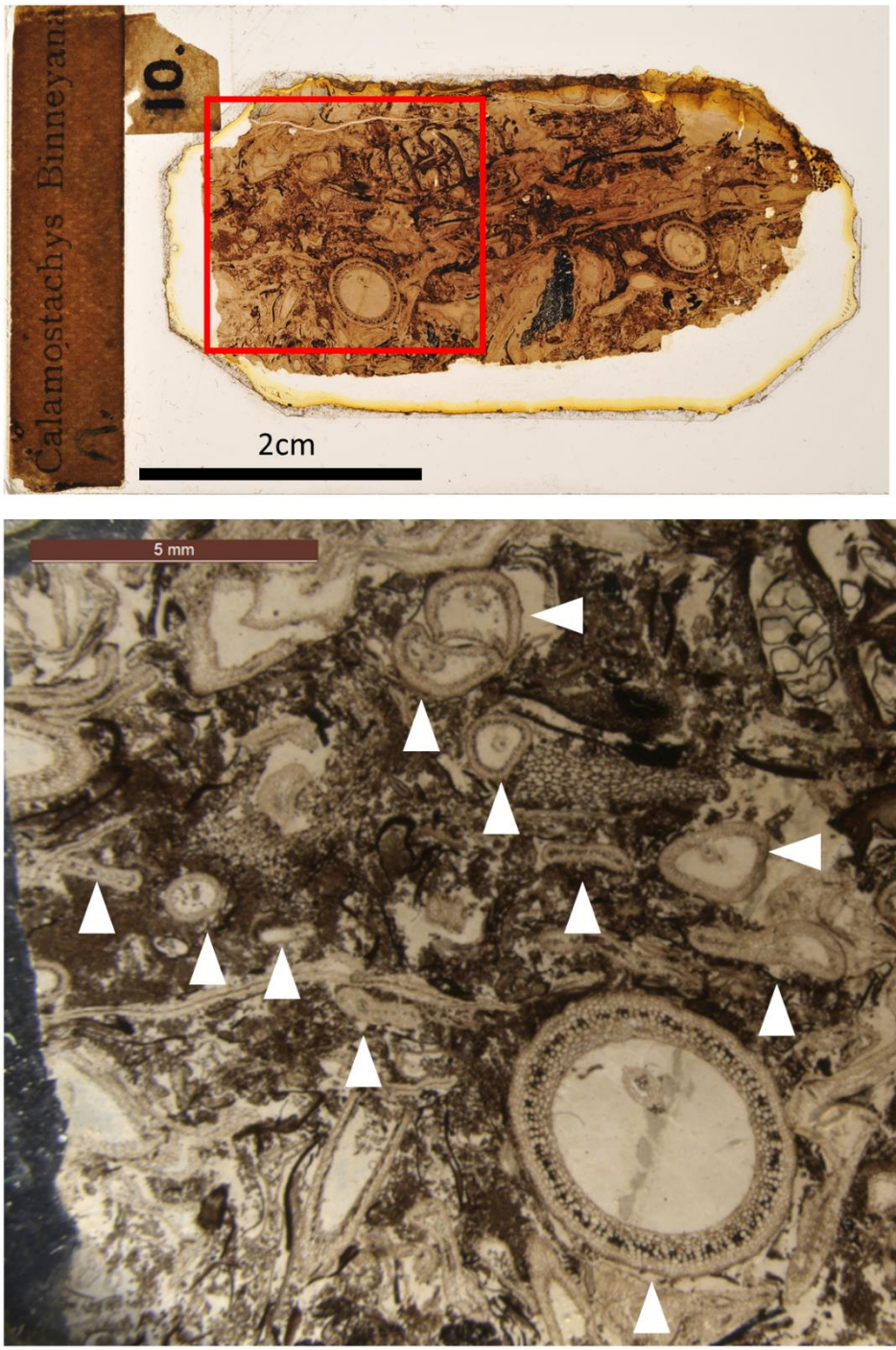
Branched

Segment	Diameter	Diameter	Length	Length	Rootlet number	Length / Frequency
S1	D1=D	1D	x	0.26L	1	0.23L
S2	D2=(D1*0.73)	0.73D	0.92x	0.24L	2	0.43L
S3	D3=(D2*0.73)	0.53D	0.85x	0.22L	4	0.79L
S4	D4=(D3*0.73)	0.39D	0.78x	0.20L	8	1.46L
S5	D5=(D4*0.73)	0.28D	0.72x	0.19L	16	2.69L
		Total Length	4.27x			
		Total Length	4.27x = L			

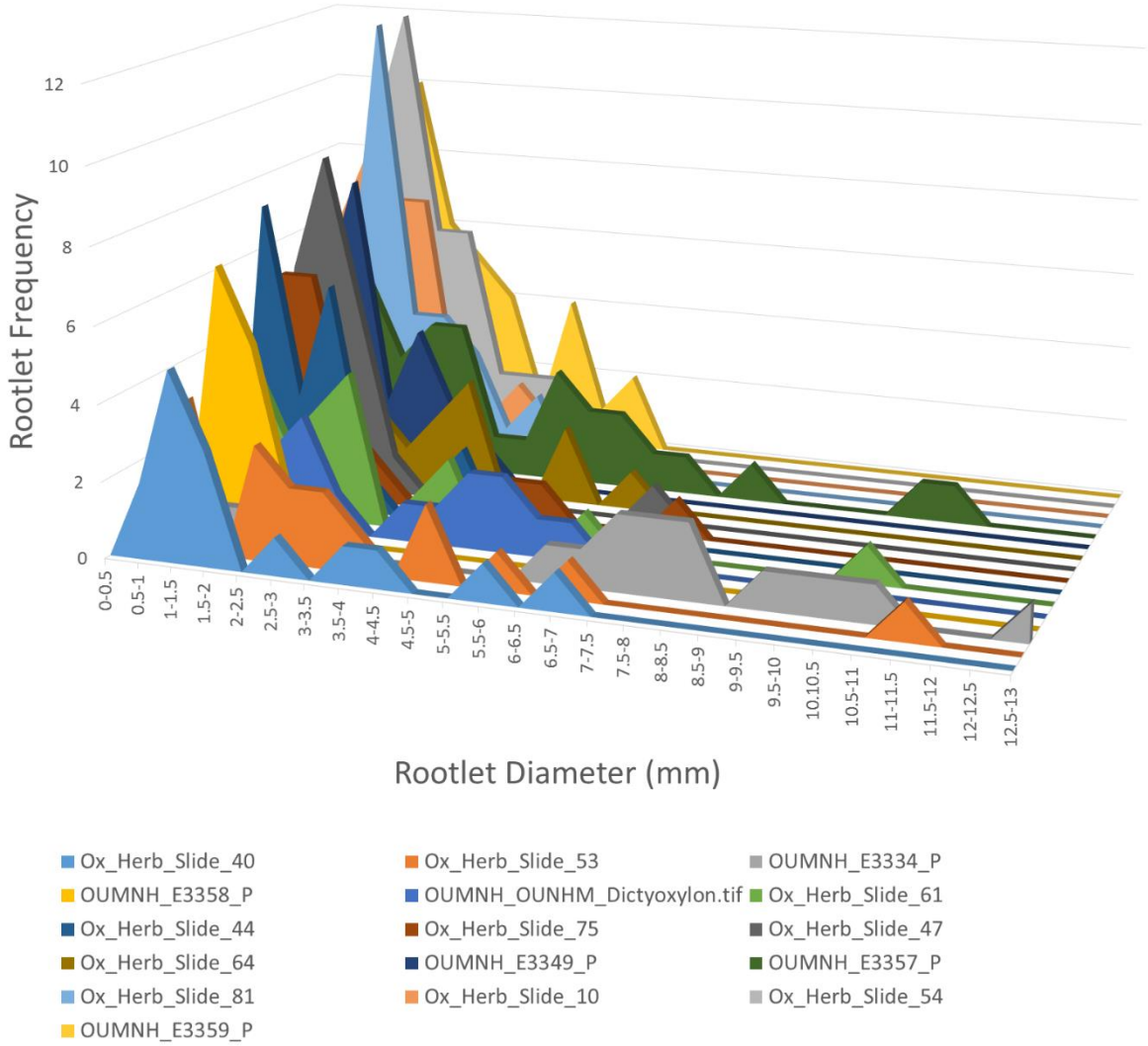
Taper

Segment	Diameter	Diameter	Length/ Frequency
S1	D1=D	1D	0.2L
S2	D2=(D1*0.73)	0.73D	0.2L
S3	D3=(D2*0.73)	0.53D	0.2L
S4	D4=(D3*0.73)	0.39D	0.2L
S5	D5=(D4*0.73)	0.28D	0.2L

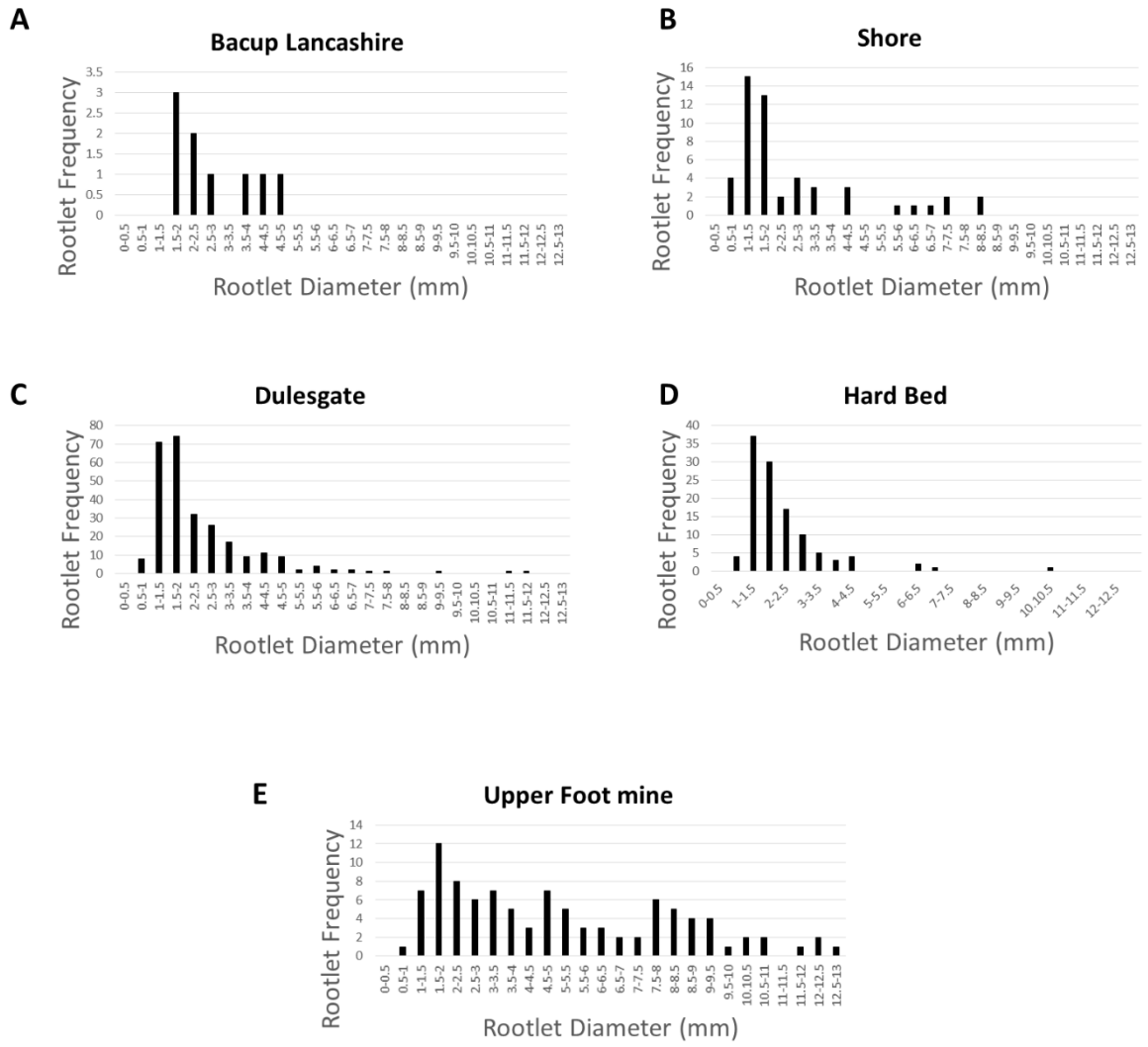
Supplementary Figure 2.07 Modelling the predicted frequency of rootlet diameters in coal balls. Branched (A) and tapered (B) rootlet models indicating the rootlet diameter D, the five segments (S1–S5) and the combined length of the rootlet segment (L). (C) The model of predicted frequency of finding branched or tapered rootlets – based on the frequency (length L) of finding each rootlet segment (S1–S5) and the diameter (D) of each segment.



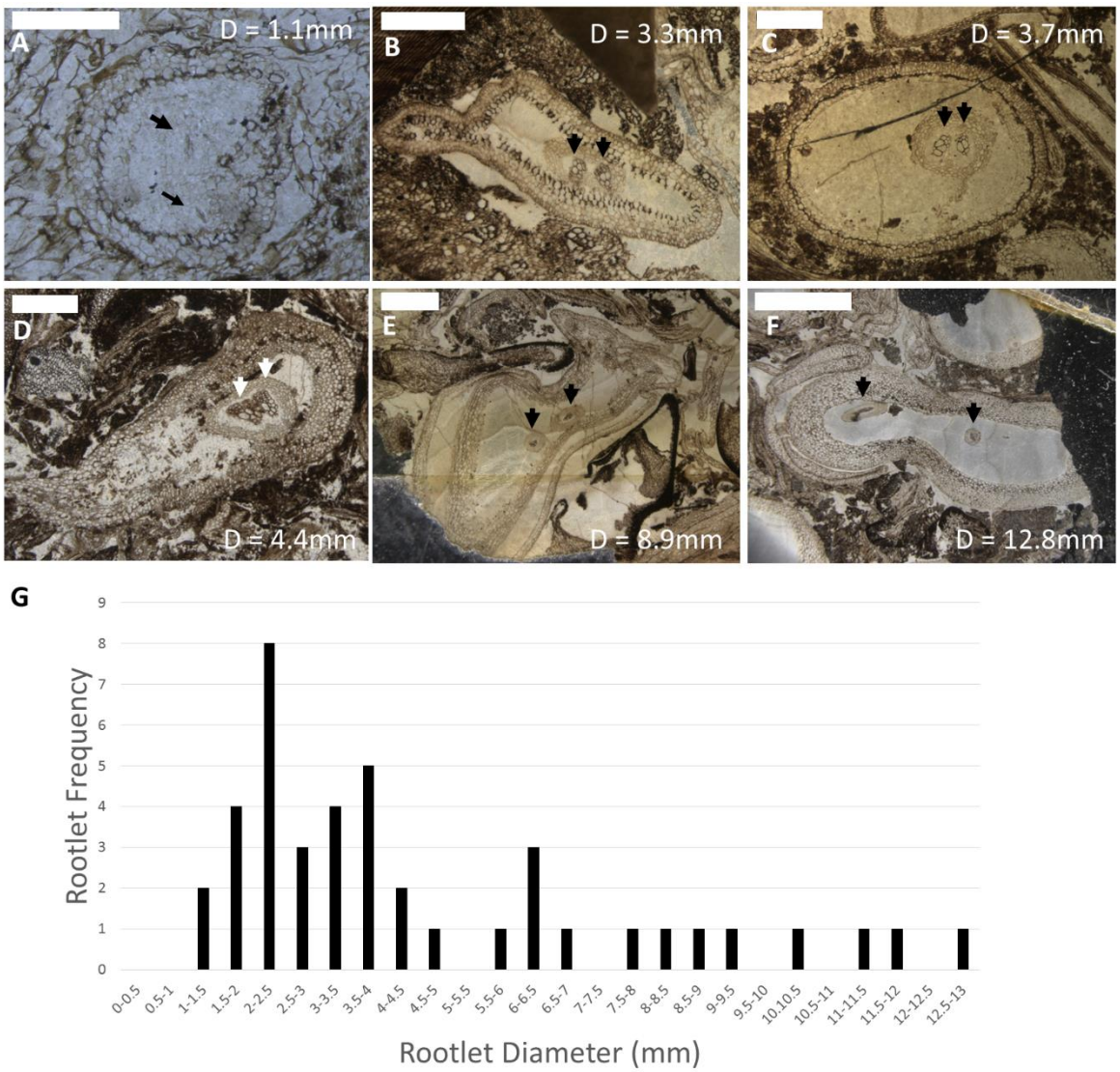
Supplementary Figure 2.08 Stigmarian rootlets in a coal ball thin section. Oxford University Herbaria (OXF) Slide 10. A red box is drawn around the area of the slide which is magnified below. White arrowheads indicate stigmarian rootlets cut in transverse section. Note the range in sizes of rootlets and their high density.



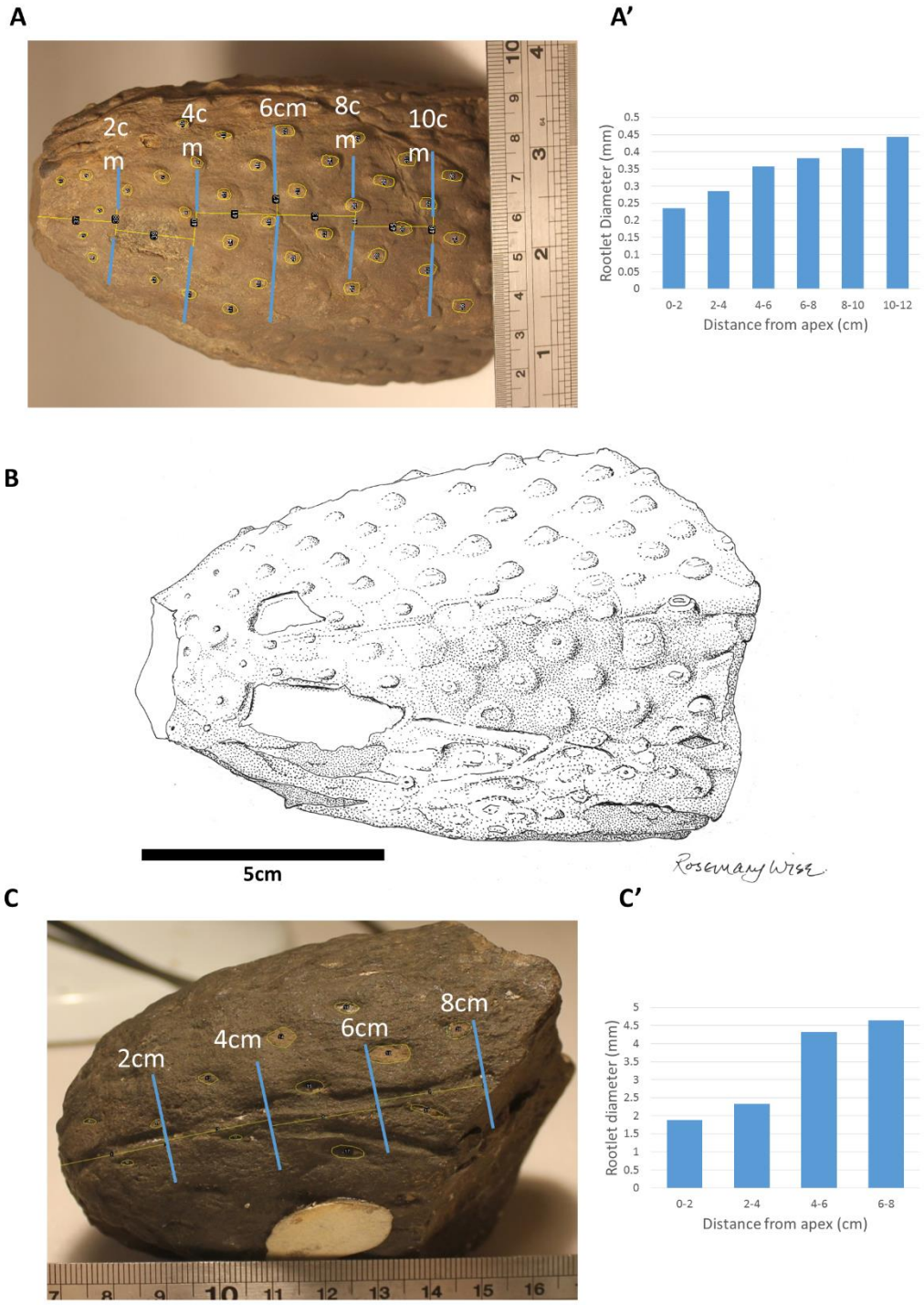
Supplementary Figure 2.09 Frequency of rootlet diameters in 16 individual slides. Frequency histograms showing the distribution of rootlet diameters in 16 individual coal ball slides which have > 15 stigmarian rootlets present. Slide names are labelled below the histogram. Ox_Herb, Oxford University Herbaria (OXF) ; OUMNH, Oxford University Museum of Natural History (OUMNH). Note that almost all individual slides examined have more small rather than large diameter rootlets in support of the branched rootlet model.



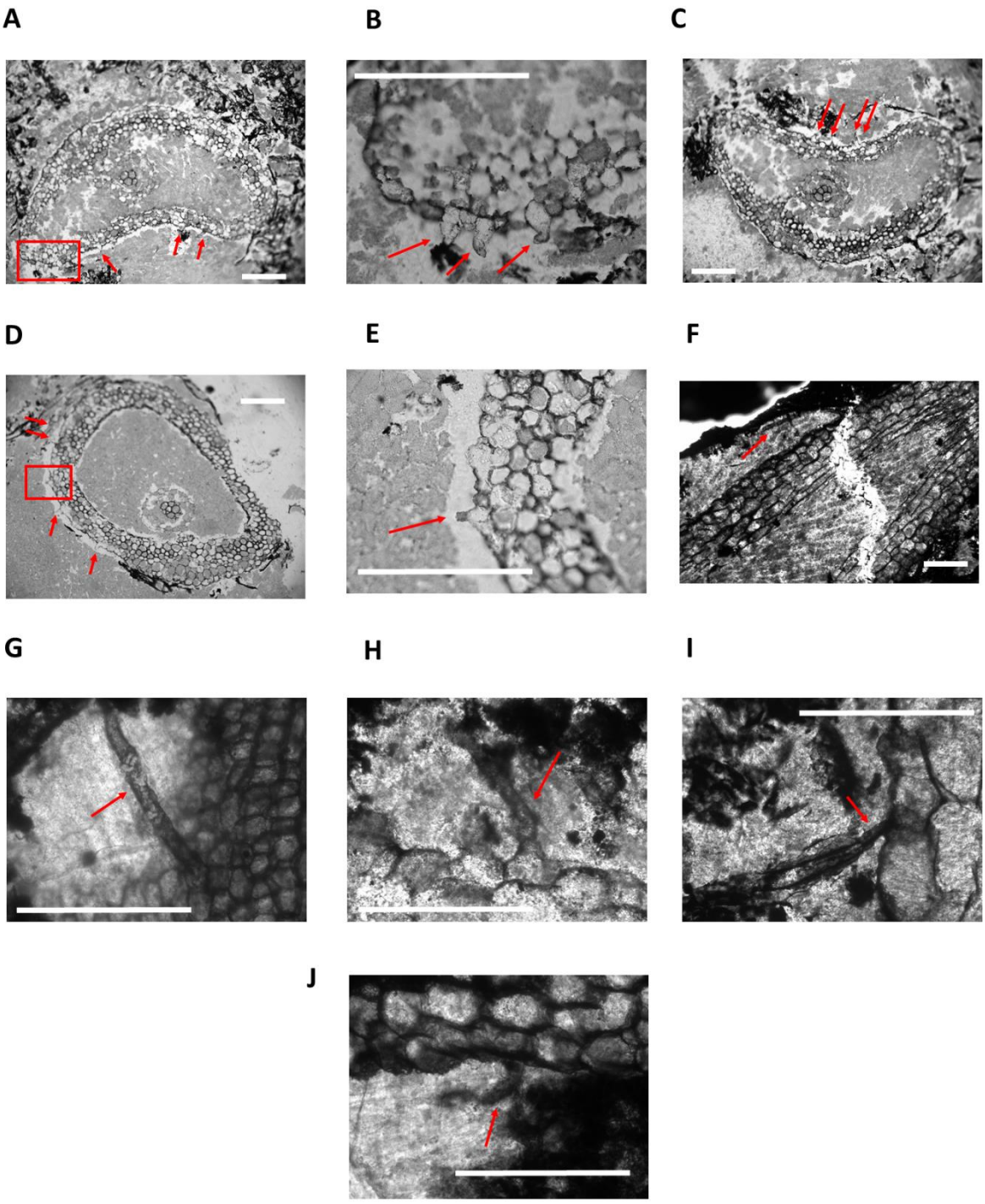
Supplementary Figure 2.10 Frequency of rootlet diameters from five different collection localities. Frequency histograms showing the distribution of rootlet diameters from five different collection localities from the Lancashire and Yorkshire Coal field. (A) Dulesgate. (B) Shore. (C) Bacup Lancashire. (D) Hard Bed. (E) Upper Foot Mine. Note that all collection localities have more small rather than large diameter rootlets in support of the branched rootlet model.



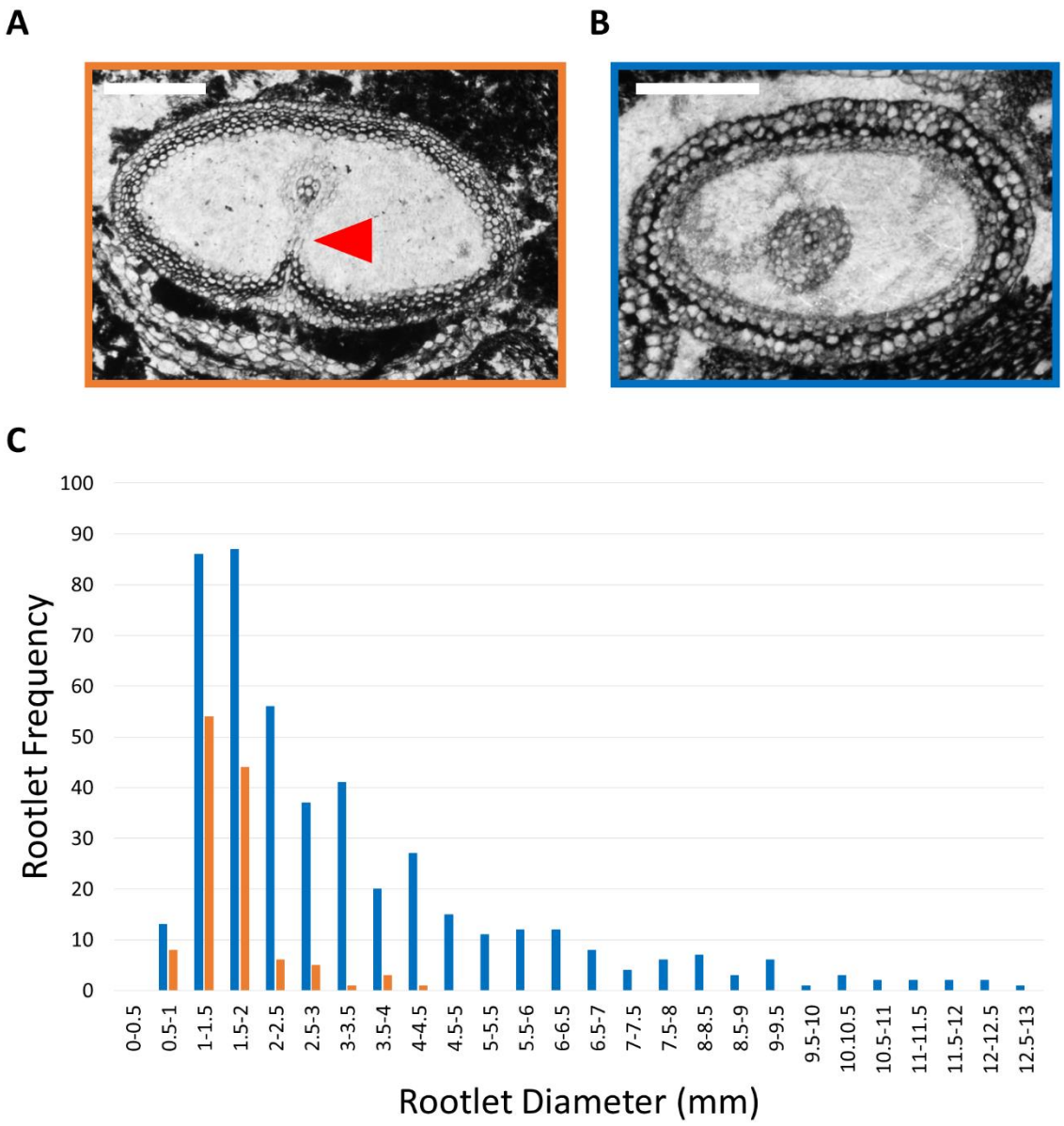
Supplementary Figure 2.11 Branching rootlets preserved in coal ball slides. (A–F) Examples of stigmarian rootlets from coal balls possessing two vascular strands (black or white arrows) indicating that these rootlets were in the process of branching. The diameter of each rootlet is indicated by a D value. Note that branching is present in rootlets from 1.1 mm (A) to 12.8 mm (F). (G) Frequency histogram showing the diameters of the 42 rootlets with two vascular strands where the circumference could be measured. Note that there are more small rather than large diameter rootlets in support of the branched rootlet model. Scale bars (A) 500 µm, (B–D) 1 mm, (E) 2 mm, (F) 5 mm. Images taken from slides: Oxford University Herbaria (OXF) 6(B), 33(E), 44(A), 75(C), Oxford University Museum of Natural History (OUMNH) E3335_P (F), E3340_P (D).



Supplementary Figure 2.12 Characterization of stigmarian rhizomorph apices. Images of the apical portions of two stigmarian rhizomorph apices (A, C) and drawing of A from the opposite perspective (B). Measured rootlet scars are circled in yellow. Blue lines indicate the distance from the apex. (A', C') The average rootlet scar diameter measured as distance from the apex. (A, B) The University of Manchester, Manchester Museum (MANCH) Collection No: LL. 15952.470. (C) The University of Manchester, Manchester Museum (MANCH) Collection No: LL. 15952.471.



Supplementary Figure 2.13 Stigmarian root hair. Stigmarian root hairs (red arrows) preserved on 8 stigmarian rootlets (A–J). (B, E) Magnified images of the red boxes highlighted in (A) and (D), respectively. Scale bars 200 µm. Images taken from slides: Oxford University Herbaria (OXF) 78(F), 94(J), 95(H, I); The University of Manchester, Manchester Museum (MANCH) LL15952 (G); reproduced by kind permission of Amgueddfa Cymru – National Museum Wales NMW 2016.9G.2 (A and B), NMW 2016.9G.3 (C), and NMW 2016.9G.4 (D and E).



Supplementary Figure 2.14 Sigillarian rootlets and non-sigillarian rootlets branched. (A) Sigillarian rootlet, identified by the presence of a connective indicated by red arrowhead. (B) Non-sigillarian rootlet, lacking connective. (C) Frequency histogram of the diameters of 122 sigillarian (orange) and 464 non-sigillarian (blue) rootlets preserved in coal balls. Scale bars (A,B) 500µm. (A,B) Rootlets identified on Slide 75 (A), and Slide 54 (B) of the Oxford University Herbaria (OXF).

Chapter 3: Unique cellular organisation in the oldest root meristem

Hetherington AJ, Dubrovsky JG, Dolan L. 2016. Unique cellular organization in the oldest root meristem. *Current Biology* **26:** 1629–1633.

Original Current Biology formatted paper Appendix 3

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Author Contributions

AJH and LD designed the project. AJH carried out the analyses with assistance from JD. AJH, JD and LD wrote the paper.

3.2 Abstract

Roots and shoots of plant bodies develop from meristems – cell populations that self-renew and produce cells that undergo differentiation – located at apices of axes (Clowes, 1961). The oldest preserved root apices in which cellular anatomy can be imaged are found in nodules of permineralised fossil soils called coal balls (Scott & Rex, 1985), which formed in the Carboniferous coal swamp forests over 300 million years ago (Osborn, 1909; Stopes & Watson, 1909; Weiss, 1913; Halket, 1930, 1932; Dennis, 1969; Ehret & Phillips, 1977). However, no fossil root apices described to date were actively growing at the time of preservation (Osborn, 1909; Stopes & Watson, 1909; Weiss, 1913; Halket, 1930, 1932; Dennis, 1969; Ehret & Phillips, 1977; Strullu-Derrien *et al.*, 2012). Because the cellular organisation of meristems changes when root growth stops, it has been impossible to compare cellular dynamics as stem cells transition to differentiated cells in extinct and extant taxa (Shishkova *et al.*, 2008). We predicted that meristems of actively growing roots would be preserved in coal balls. Here report the discovery of the first fossilized remains of an actively-growing root meristem from permineralised Carboniferous soil with detail of the stem cells and differentiating cells preserved. The cellular organisation of the meristem is unique. The position of the Körper-Kappe boundary, discrete root cap and presence of many anticlinal cell divisions within a broad promeristem distinguish it from all other known root meristems. This discovery is important because it demonstrates that the same general cellular dynamics are conserved between the oldest extinct and extant root meristems. However, its unique cellular organisation demonstrates that extant root meristem organisation and development represents only a subset of the diversity that has existed since roots first evolved.

3.3 Highlights

- The oldest fossilized root meristem is described from >300 million year old soil
- The discovery allows the first description of a fossilised root stem cell niche
- The cellular organisation and therefore development of the meristem is unique
- The discovery reveals previously unknown diversity in plant meristem types

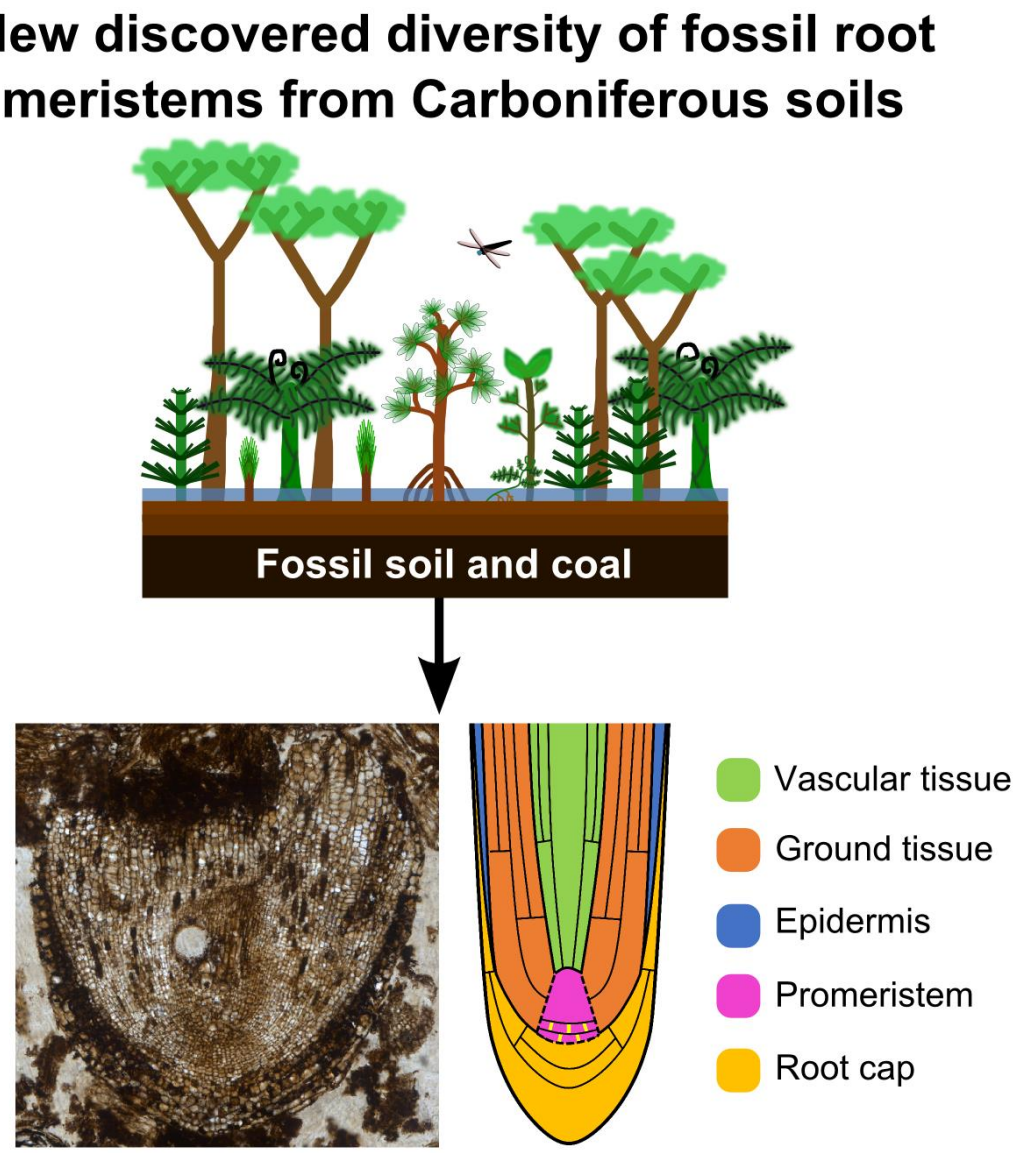

Figure 3.01 ***R. carbonica*** **graphical abstract.** Hetherington et al. report the discovery of the oldest fossilized remains of an actively growing root meristem from Carboniferous (>300 million year old) soil. The cellular organisation of stem cells and differentiating cells is unique. This discovery reveals new but now extinct meristem diversity in Carboniferous plants.

3.4 Results and Discussion

To characterise cellular development in the oldest root apices (Osborn, 1909; Stopes & Watson, 1909; Weiss, 1913; Halket, 1930, 1932) we inspected 139 thin sections made from Carboniferous coal balls from Britain (see Supplemental information). We identified two new apices (Fig. 3.02A, A', C). The presence of root caps covering each demonstrates that they are root apices. The first apex was the tip of a differentiated, non-growing root (Fig. 3.02A, A'). It was designated *Apex 76.1* and tentatively assigned to *Lyginopteris oldhamia* on the basis of cellular organisation (Fig. 3.02A, A' 1B, B' (Stopes & Watson, 1909); see Supplemental information). Finding *Apex 76.1* validated our search for root meristems in this coal ball material. The second apex (Fig. 3.02C) is larger, blunt and represents an entirely new root apex type and was named *Radix carbonica* (see Supplemental information for systematic palaeobotany and comparisons with other fossil apices).

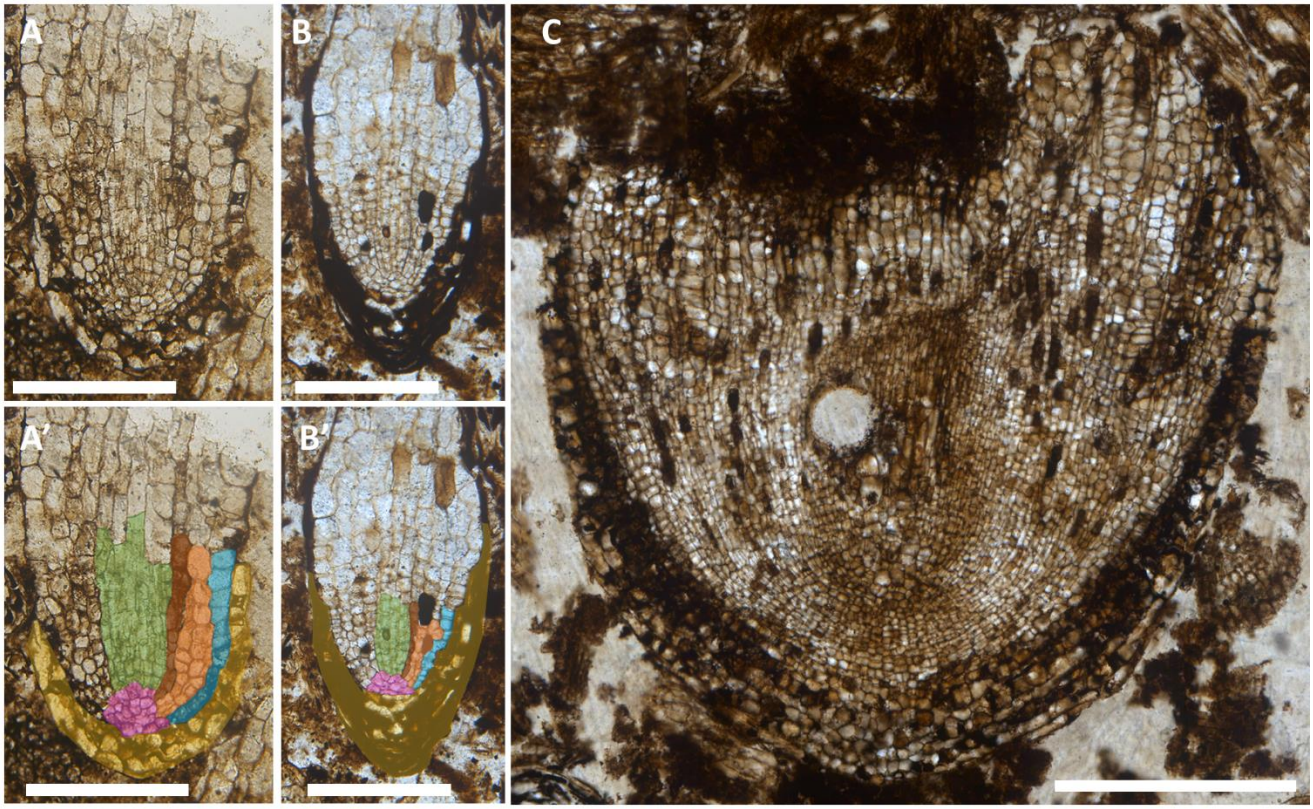

Figure 3.02 Two new fossil root apices from the Carboniferous period (A) *Apex 76.1* tentatively assigned to *Lyginopteris oldhamia*. (A') *Apex 76.1* with main tissue types colour coded; yellow – root cap, blue – epidermis, pink – differentiated cells at the position of the promeristem, orange – cortex, brown – endodermis, green – procambium. (B) *Lyginopteris oldhamia* root apex discovered by Stopes and Watson, 1909. (B') *L. oldhamia* overlaid with colours to represent the major tissues types as shown in (A'). (C) Holotype of the root apex of *Radix carbonica* (produced by the assembly of a series of continuous images of the root apex). Scale bars (A, B) 200 µm (C), 500µm. (A, A') Thin section 76; (C), thin section 81 Oxford University Herbaria (OXF). (B, B') Thin section R646 of the Manchester Museum (MANCH), The University of Manchester.

The cellular organisation of *Apex 76.1* and *R. carbonica* can be compared with root meristems of extant species because both thin sections are near median longitudinal in orientation. However, meristem organisation of extant plants can only be investigated in actively growing roots because meristem structure changes when root growth stops (Shishkova *et al.*, 2008). It is therefore essential to establish if the root apices were fossilised during active growths. In roots that have stopped growing, differentiated tissues, including thickened xylem cells, are found very close to the promeristem as in the fossil meristems of *Apex 76.1*, *Lyginopteris*, *Amyelon* and *Psaronius*, (Fig. 3.02A, A',B, B') (Osborn, 1909; Stopes & Watson, 1909; Weiss, 1913; Halket, 1930; Strullu-Derrien *et al.*,

2012), a feature not found in actively growing roots (Shishkova *et al.*, 2008). By contrast, there are no differentiated vascular cells; or distinguishable tissue types within the ground tissues found near the tip of *R. carbonica* (Fig. 3.02C). There is clear zonation of cell sizes in active root meristems of growing roots; meristematic cells are relatively small and vary in size by approximately two fold as dividing cells go through the cell cycle. Then cells expand as they differentiate. Consequently there is a gradient from the smaller cells of the promeristem to the larger cells in the differentiating tissues. In contrast there is no cell size gradient in inactive meristems where cell size abruptly increases from the small inactive initials which abut much larger differentiated cells close to the apex (Fig. 3.03A, B, D).The distribution of cell areas in the differentiating ground tissues of *R. carbonica* indicates that there is a gradual increase and roughly two fold difference in cell area (see heat map in Fig. 3.03C, D – there is a twofold difference in cell size between blue (<300 μm^2) and turquoise cells (300 – 600 μm^2) throughout the majority of the body of the root), typical of actively growing root apices. These data indicate that *R. carbonica* is the first and only example of a root fossilised during active growth which has preserved the cellular organisation of the meristem.

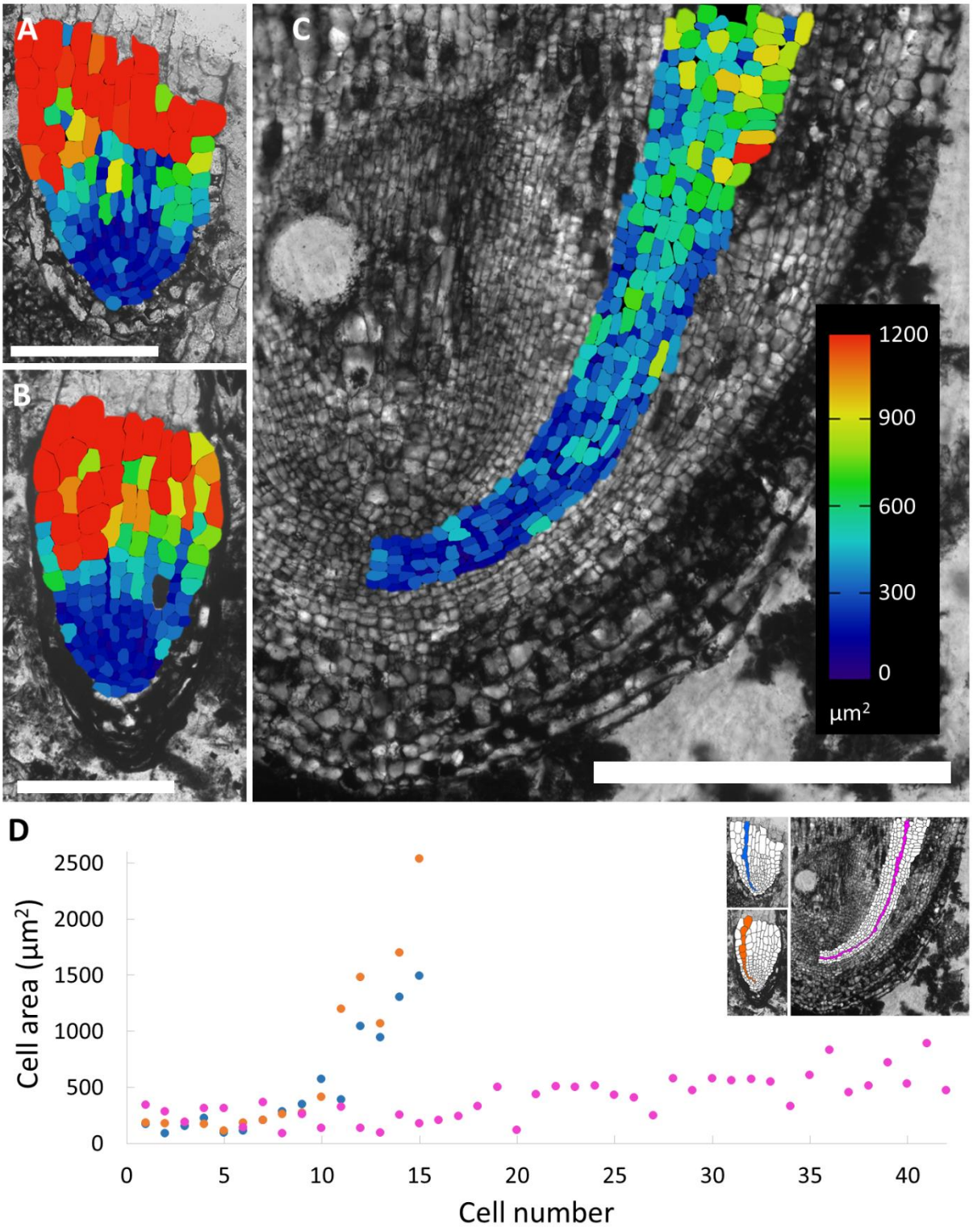

Figure 3.03 ***R. carbonica* is the first fossil of an active meristem in a growing root.** Cell surface area heat plots of (A) *Apex 76.1* (Fig. 3.02A) (B) *L. oldhamia* (Stopes & Watson, 1909) Fig. 3.02B) (C) *R. carbonica* (Fig. 3.02C). (D) Cell area increase along a single cell file in *Apex 76.1* (blue), *L. oldhamia* (Stopes and Watson, 1908 (orange) and *R. carbonica* (pink). Note the gradual increase in cell size within the ground tissue of *R. carbonica* compared to *Apex 76.1* and *L. oldhamia.* Scale bars (A, B) 200 µm (C) 500µm.

Comparison of the cellular organisation of the different regions of the root apex indicates that the cellular dynamics in *R. carbonica* conform to that observed in extant root meristems. The root apices of all extant roots are covered by a protective cap. Root caps are typically tapered – they are thinner in proximal positions than in distal positions – because older cell layers are sloughed off as the root grows through the soil. *R. carbonica* is covered by a protective root cap that tapers rapidly indicating that cells are sloughing, typical of roots of extant species (Clowes, 1961) (Fig. 3.04A yellow).The promeristem is the group of cells in a growing root which gives rise to all tissues (Clowes, 1961) and is identified in *R. carbonica* as the region where the files of cells of fundamental tissues converge (Fig. 3.04A pink).The *R. carbonica* promeristem is large, consisting of 138 cells arranged in 10-15 tiers when imaged in the longitudinal plane of section (Fig. 3.04A pink, 3B pink and lilac). It comprises two morphologically distinct pools of initials (Fig. 3.04B pink and lilac) (see Supplemental information) and initials give rise to many mature cell files, meaning that discrete initials for each cell layer do not exist. The *R. carbonica* promeristem is different from all vascular plant root meristems (see Supplemental information) because of its large size and the spatial organisation of cells that are arranged in more than 10 tiers of initials. The distal promeristem (Fig. 3.04B lilac) of *R. carbonica* takes the form of a regular block of cells – a similar organisation of the promeristem is found in almost all extant gymnosperms (Janczewski, 1874; Schüepp, 1926; Allen, 1947a; Guttenberg, 1961; Wilcox, 1962; Pillai, 1963, 1964, 1966, Voronin, 1969, 1964; Bogar & Smith, 1965; Milindasuta, 1975). However, the structure of *R. carbonica* differs from that of extant gymnosperm root meristems in two ways.

The first feature which distinguishes *R. carbonica* from extant gymnosperm root meristems is the discrete root cap that is not continuous with the distal promeristem in *R. carbonica* (Fig. 3.04B lilac). In extant gymnosperms it is not possible to distinguish a

boundary between the promeristem and the root cap (Janczewski, 1874; Schüepp, 1926; Allen, 1947a; Guttenberg, 1961; Wilcox, 1962; Pillai, 1963, 1964, 1966, Voronin, 1969, 1964; Bogar & Smith, 1965; Milindasuta, 1975). However, in *R. carbonica*, the promeristem is broad and not continuous with the root cap, which is discrete from other tissues. Furthermore, within this broad promeristem there are large numbers of anticlinal cell divisions (marked in yellow on Fig. 3.04C), which lead to the loss of the columnar organisation of cell files between the promeristem and the cap. While some gymnosperm promeristems are columellar (Allen, 1947a; Guttenberg, 1961; Wilcox, 1962; Pillai, 1963, 1964, 1966; Bogar & Smith, 1965; Milindasuta, 1975), and anticlinal division occur in the promeristem of others (Guttenberg, 1961; Voronin, 1964, 1969; Milindasuta, 1975), numerous anticlinal divisions within a columellar promeristem has not been described in any species. No similar organisation with broad promeristem and discrete root cap has been described in any root meristem to date (see Supplemental information).

The second feature that marks *R. carbonica* as distinct from the meristems of extant gymnosperms is the position of the Körper-Kappe boundary (Schüepp, 1917, 1926; Clowes, 1961; Evert, 2006) (see Supplemental information for extended description of the Körper-Kappe theory).The boundary between the Körper and Kappe complex is a highly conserved feature of all extant gymnosperms. In gymnosperms the Körper complex contains the vascular tissue and in some cases a small number of layers of the ground tissues (Schüepp, 1926; Pillai, 1963, 1964, 1966) (Fig. 3.05A, C – red). The Kappe complex on the other hand makes up the majority of the tissues (remainder of the ground tissues, epidermis and root cap) of the root meristem (Schüepp, 1926; Pillai, 1963, 1964, 1966) (Fig. 3.05A, C – blue). The Körper-Kappe boundary is therefore located very close to the junction between the provascular tissues and the ground tissue (Fig. 3.05A, C). However, the Körper-Kappe boundary is markedly different in *R. carbonica*. The *R.*

carbonica Körper complex constitutes the stele and almost all the ground tissue (Fig. 3.04C red; Fig. 3.05D – red). The Kappe complex (Fig. 3.04C blue shading; Fig. 3.05D – blue) comprises the root cap and the cell file abutting the root cap interpreted as the epidermis. Therefore the position of the Körper-Kappe boundary of *R. carbonica* is structurally different from all extant gymnosperm root meristems (Fig. 5).

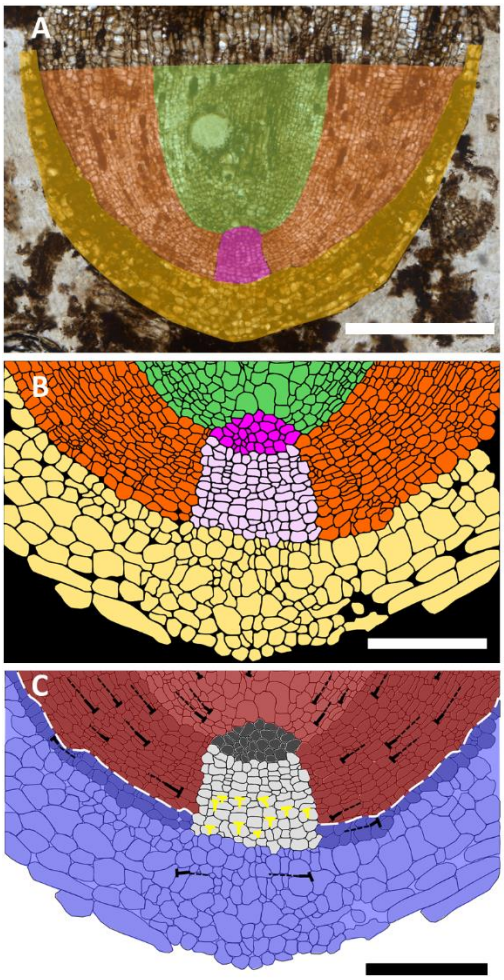

Figure 3.04 ***R. carbonica*** **has a unique cellular organisation** (A) *R. carbonica* (Fig. 3.02C) overlaid with colours representing the four major tissues found within the apex; yellow – root cap, pink – promeristem, orange – ground tissues and epidermis, green – procambium. (B) Line drawing of the apical portion of the *R. carbonica* holotype (Fig. 3.02C) colour coded to represent the major tissue types as in (A) except the promeristem is further divided to pink – proximal promeristem, lilac – distal columella-like promeristem. (C) Same line drawing as in (B) blue – Kappe complex, red – Körper complex, grey – promeristem. Black T's indicate T-divisions in both complexes with dashed lines showing the cell files which make the vertical stroke of the T. White dashed line marks the boundary between the Körper-Kappe complexes. Yellow T's mark positions of anticlinal cell divisions within the central columella-like region of the promeristem. Scale bars (A) 500 µm (B, C) 200µm.

R. carbonica is the only root meristem that has been preserved in which the patterns of cell division in the active apex can be elucidated. This allowed us for the first time to compare the organisation of cells in the promeristem of an extinct Carboniferous root with the organisation of cells in root meristems of extant plants. The organisation of stem cells and differentiating cells suggests that the same general cellular dynamics in the self-renewing populations and their derivatives occurred in *R. carbonica* as in extant root meristems. However, the discrete root cap, zone of anticlinal cell divisions in the group of columella-like promeristem, and the position of the Körper-Kappe boundary marks *R. carbonica* as structurally distinct from all other previously described meristems (Fig. 3.05).

Using the organisation of cells in the promeristem and the meristem as criteria, Schüepp (Schüepp, 1926) identified 9 classes of vascular plant root meristems. There were 5 classes of meristems in non-angiosperm tracheophytes (lycophytes, monilophytes and gymnosperms) combined. He identified 7 classes in angiosperms, of which 4 were angiosperm-specific. The evolution of novel meristem types (Schüepp, 1926; Groot *et al.*, 2004; Heimsch & Seago, 2008) in angiosperms has therefore been associated with their rise to dominance. The discovery of the organisation of stem cells and their derivatives in *R. carbonica* demonstrates that the diversity of developmentally-distinct root meristem types that existed before the origin of angiosperms (Fig. 3.05E) but now extinct was greater than previously described. It also shows that extant root meristem organisation represents a subset of the diversity that has existed since roots first evolved.

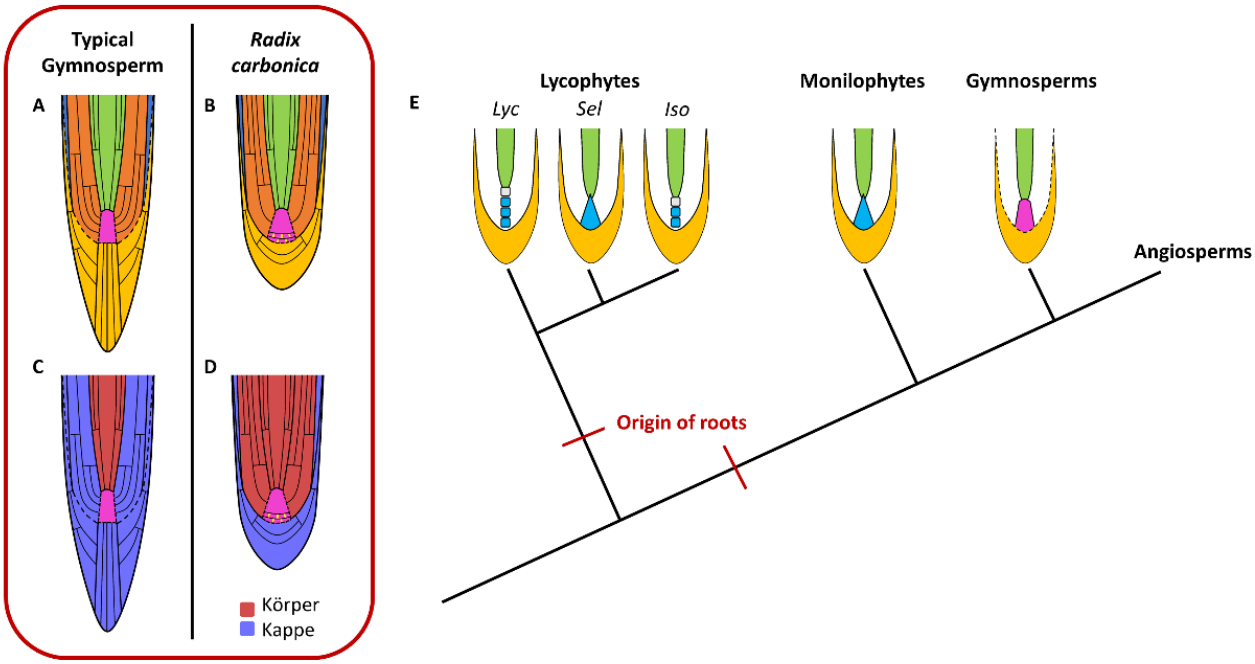

Figure 3.05 ***R. carbonica*** **is distinct from all extant root meristems** Schematic diagrams of the cellular organisation of a typical gymnosperm (A, C) and *Radix carbonica* meristem (B, D). (A, B) are colour coded for the major tissue types within the meristem; yellow – root cap, pink – promeristem (yellow lines in the *R. carbonica* promeristem indicate the positions of anticlinal cell divisions within the promeristem), orange – ground tissue; blue – epidermis, green – procambium. (C, D) same schematics as (A, B) but colour coded to mark the position of the Körper (red) – Kappe (blue) complexes. Note the difference in the Körper-Kappe boundary between *R. carbonica* (D) and the gymnosperm meristem (C). (E) a simplified vascular plant cladogram (Qiu *et al.*, 2006); showing the two hypothesised origins of roots (Raven & Edwards, 2001), and schematics of lycophyte, monilophyte and gymnosperm root meristems. *Lyc* stands for the Lycopodiales which typically have multicellular promeristems consisting of either 3 or 4 tiers of initials. *Sel* stands for the Selaginellales which typically have a single initial cell (apical cell). *Iso* stands for the Isoetales which typically have multicellular promeristems consisting of either 2 or 3 tiers of initials. Monilophyte root meristems typically have a single initial cell (apical cell). Gymnosperm root meristems have multicellular promeristems consisting of a zone of common initials for all tissues, or common initials for all non-vascular tissues and a separate set for all vascular tissues. (For detailed review of meristem types see Supplemental information).

3.5 Experimental Procedures

139 thin sections of Carboniferous coal balls from the Oxford University Herbaria (OXF) and the University of Oxford Natural History Museum were inspected for root meristems. The original *L. oldhamia* root apex described by Stopes and Watson (Stopes & Watson, 1909) and Weiss (Weiss, 1913) was also re-examined courtesy of the Manchester Museum (MANCH), The University of Manchester (Thin section R646). Meristems were imaged with an Olympus BX50 microscope and quantitatively characterised using Fiji (Schindelin *et al.*, 2012). To quantitatively characterise the cell shape, cell area and cell division pattern of *R. carbonica* a line drawing was made of the 988 cells which constitute the distal portion of the apex (Fig. 3.03C) and the 405 cells representing the development of the ground tissues (Fig. 3.04B, C). Line drawings were also made of the cells in the distal portion of *Apex 76.1* and *L. oldhamia* apices (Fig. 3.03A, B).

3.6 Acknowledgements

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3.7 Supplemental Information

3.7.1 Geological locality of *R. carbonica* and *Apex 76.1*

Apex 76.1 was identified on thin section 76 of the Oxford University Herbaria's (OXF) slide collection. The thin section was made by James Lomax petrologist (Howell, 2005), from a coal ball collected from Dulesgate – one of the key localities of the Lancashire and Yorkshire coal field dated to Westphalian A (Bashkirian) (319–317 million years old) (Scott & Rex, 1985; Scott *et al.*, 1996; Galtier, 1997; Richards, 2013). Thin section 81 on which *R. carbonica* was discovered is believed to come from a similar collection locality as *Apex 76.1*. In the collections of the Oxford University Herbaria (OXF) there are no thin sections which correspond or form a series with either thin section 76 or 81.

3.7.2 Assigning Apex 76.1 to the species *Lyginopteris oldhamia*

The cellular structure of the root cap, apical and sub-apical root portions are well preserved in *Apex 76.1* (Fig. 3.02A). The cellular organisation of *Apex 76.1* indicates that it was not actively growing when preserved; cell expansion has proceeded to the extreme apex (Fig. 3.03B). The maximum diameter of the root in the section is 313 μm. The section is oblique, but closer to the median plane in the distal region (nearest the root tip). The provascular cylinder (Fig. 3.02A' green) is narrow, 86 μm in diameter, and consists of seven cell layers. The ground tissues are split into two distinct tissue types, the endodermis (Fig. 3.02A' brown) and cortex (Fig. 3.02A' orange). Outside the ground tissues is a single epidermis layer (Fig. 3.02A' blue). The root cap (Fig. 3.02A' yellow) is inconspicuous and is four layers thick at the most distal root cap region. Cell walls in the central most region

of the root cap are not aligned in register typical of the columella of extant roots (Heimsch & Seago, 2008). The largest cells in the cap are the distal-most cells in the central cap region, and peripheral cells in the lateral-root-cap proximal portion, indicating a pattern of gradual maturation and sloughing typical of roots of extant species. All cell files converge on a group of cells that constitute the remains of the differentiated promeristem (Fig. 3.02A' pink). Based on its size and tissue organisation we tentatively assign *Apex 76.1* to *Lyginopteris oldhamia* based on similarities with the previously published description of *Lyginodendron oldhamium* (Stopes & Watson, 1909; Weiss, 1913) (Fig. 3.02B, B') later designated *Lyginopteris oldhamia* (see (Zimmerman, 1958; Taylor *et al.*, 2005)) which was collected at the same geological locality as *Apex 76.1*.

The diameter of *Apex 76.1* is larger (313 μm) than the *Lyginopteris oldhamia* apex described by Stopes and Watson (Stopes & Watson, 1909) which is 280 μm at its widest point (Fig. 3.02B, B'). The ground tissue comprises two distinct tissues in *Apex 76.1 and L. oldhami*a a single cell layer of endodermis (Fig. 3.02A', B' brown) and a multiple cell layered cortex (Fig. 3.02A', B' orange).There is a single layer of epidermis in both root apices (Fig. 3.02A', B' blue). The main difference between the two apices is that the *L. oldhamia* root cap (Fig. 3.02A', B' yellow) (Fig. 3.02B') is larger than *Apex 76.1* (Fig. 3.02A'). The length of the *L. oldhamia* root cap (Fig. 3.02B') is 460 μm from the most distal to the most proximal point, compared to 285 μm on *Apex 76.1* (Fig. 3.02A'). (Fig. 3.02B'), The distance from the most distal region of the differentiated *L. oldhamia* promeristem to the most distal portion of the cap is 124 μm long and contains approximately 8 cells along the root axis. In contrast the corresponding region on *Apex 76.1* (Fig. 3.02A') is roughly half the size (60 μm long and with approximately 4 cells). The overall similarity in the tissue organisation, root size and differentiated promeristem structure allowed us to tentatively assign *Apex 76.1* to *Lyginopteris oldhamia*.

3.7.3 Radix carbonica systematic Palaeobotany

Subdivision: Tracheophyta

Class, Order and Family: Incertae sedis

Genus: *Radix* Hetherington, Dubrovsky et Dolan gen. nov.

Species*: Radix carbonica* Hetherington, Dubrovsky et Dolan sp. nov.

Combined diagnosis: A large, blunt shaped root meristem in which the Körper complex constitutes the stele and almost all the ground tissue whereas the Kappe complex comprises the root cap and the cell file abutting the root cap interpreted as the epidermis. The root cap is distinct from the other tissues of the meristem. Cells of the root cap are typically larger and darker – due to the accumulation of opaque material within and between cells – than cells in the proximal region of the root. Most of the promeristem cells are arranged in a broad columella-shaped structure abutting the procambium, ground tissues proximally and root cap distally. The distal region of the promeristem is characterised by the presence of many anticlinal cell divisions.

Etymology: Generic name *Radix* is the Latin noun for root. Specific name *carbonica* is the Latin adjective for coal, because this fossil was found in a coal ball.

Type: *Radix carbonica sp. nov.*

Holotype: Thin section 81 Oxford University Herbaria (OXF) (Fig. 3.02C)

Repository: Oxford University Herbaria (OXF) (Oxford, United Kingdom).

Type locality and age: Thin section 81 lacks a collection locality but the coal ball from which the thin section was prepared is believed to have been collected from the Lancashire and Yorkshire coal field, dated to Westphalian A (319–317 million years old) (Scott &

Rex, 1985; Scott *et al.*, 1996; Galtier, 1997; Richards, 2013) (Bashkirian) in age along with the comparable thin sections housed in the Oxford University Herbaria (OXF).

Description: The cellular organisation of *R. carbonica* is well preserved; cell outlines are clearly demarcated. The presence of a root cap (Fig. 3.02C, 3.04A, B yellow) covering the apex indicates that this is a root apex. The number of cell layers in the root cap decreases proximally. Consequently the root cap tapers rapidly and is almost completely lost in the proximal region of the preserved apex indicating a pattern of gradual maturation and sloughing typical of roots of extant species. The root apex is 1.65 mm in length (from the distal-most point of the root cap to where it leaves the plain of section in the proximal-most region of the root). The apex is 1.55 mm in diameter at its widest point 1.01 mm from the tip of the root cap. It tapers slightly, forming a blunt apex, 1.19mm in diameter at the level of the meristematic initials (Fig. 3.04A pink). The apex is divided clearly into the three main tissue types characteristic of root meristems – the root cap (Fig. 3.04A, B yellow), epidermis combined with ground tissues (Fig. 3.04A, B orange) and procambium (Fig. 3.04A, B green). The promeristem is located at the point of convergence of theses tissues (Clowes, 1961) (Fig. 3.04A, pink) and is the group of cells from which all the fundamental tissues of the root develop (Clowes, 1961). The exceptional preservation allows identification of these tissues and the initials from which they developed (Fig. 3.04A, B pink and lilac).There are 138 initials when viewed in median longitudinal section in the *R. carbonica* promeristem comprising two morphologically distinct groups of initials. First, the proximal promeristem initials (Fig. 3.04B pink) are rounded with an average circularity of 0.83 (where 1 – is a perfect circle) (standard error (SE) 0.01, $n = 31$ cells). Second, the distal promeristem initials (Fig. 3.04B lilac), are more box shaped with an average circularity of 0.80 (SE 0.008, $n = 107$ cells) making these initials morphologically distinct from each other $P = 0.008$ (*t*-Test). Promeristem size and

organisation – 138 cells in longitudinal section arranged in two morphologically distinct pools of initials – marks *R. carbonica* as distinct from all other previously described fossil apices.

3.7.4 Cellular organisation of *R. carbonica* is different from all other root apices for the Carboniferous Period

R. carbonica differs from all other fossil root apices (Osborn, 1909; Stopes & Watson, 1909; Weiss, 1913; Halket, 1930, 1932; Dennis, 1969; Ehret & Phillips, 1977; Strullu-Derrien *et al.*, 2012) because it is the first and only example of a root apex fossilised during active growth. The cellular organisation in the actively growing *R. carbonica* apex differs from all other non-growing Carboniferous root apices in two further ways. First there are many more cells within the *R. carbonica* promeristem than in the differentiated promeristems of *Apex 76.1* (Fig. 3.02A, A'), *L. oldhamia* (Fig. 3.02B, B') (Stopes & Watson, 1909; Weiss, 1913; Halket, 1932), *Amyelon* (Osborn, 1909; Halket, 1930) and *Psaronius* (Ehret & Phillips, 1977). Second the *R. carbonica* apex is over three times larger in diameter than *Apex 76.1*(Fig. 3.02A, A'), *L. oldhamia* (Fig. 3.02A, B) (Stopes & Watson, 1909; Weiss, 1913; Halket, 1932) and *Amyelon* (Osborn, 1909; Halket, 1930). Comparisons cannot be made with the root meristem described by Dennis (Dennis, 1969) because the promeristem structure in that specimen is obscured. The large diameter of the *R. carbonica* apex and with the large number of cells which constitute the promeristem demonstrate that *R. carbonica* is distinct from all other previously described fossil root apices.

3.7.5 Comparison of *R. carbonica* with the root meristems of vascular plants

Roots are classified into distinct classes (Reinke, 1872; Janczewski, 1874; De Bary, 1884; Schüepp, 1926; Esau, 1953; Clowes, 1961; Newman, 1965; Groot *et al.*, 2004; Heimsch & Seago, 2008) on the basis of the organisation of cells within the meristem. The cellular

organisation of *R. carbonica* was compared with all previously described meristem types to determine if it could be classified into any of the existing root meristem classes.

3.7.5.1 R. carbonica is different from all extant lycophyte root meristems

The lycophytes are the oldest group of extant vascular plants (Kenrick & Crane, 1997). Lycophytes comprises three clades (Kenrick & Crane, 1997; Rydin & Wikström, 2002; Qiu *et al.*, 2006) – the Selaginellales, Lycopodiales and Isoetales – each with a distinct root meristem organisation. There is a single tetrahedral apical cell in the majority of *Selaginella* species which have been described (Fig. 3.05E) (Nägeli & Leitgeb, 1868; Guttenberg, 1966; Grenville & Peterson, 1981; Imaichi & Kato, 1989, 1991; Lu & Jernstedt, 1996; Imaichi, 2008; Otreba & Gola, 2011) although some species have more than one initial (Bruchmann, 1909; Guttenberg, 1966). *R. carbonica* with its multicellular pomeristem consisting of 138 cells is distinct from all *Selaginella* species with a single tetrahedral apical cell. Additionally *R. carbonica* is distinct from *Selaginella* species with multicellular pomeristems (Bruchmann, 1909; Guttenberg, 1966). In *Selaginella* species with multiple initials the initials are arranged in three tiers (Bruchmann, 1909; Guttenberg, 1966), whereas the initials of *R. carbonica* are organised in over 10 tiers a number far greater than any *Selaginella* species described to date.

Multicellular promeristems consisting of either three (Strasburger, 1872; Saxelby, 1908; Guttenberg, 1964, 1966) or four (Bruchmann, 1874; Stokey, 1907; Guttenberg, 1964, 1966; Tsukaya, 2014) tiers of initials develop in Lycopodiales root apices (the most ancient extant clade of the lycophytes (Kenrick & Crane, 1997; Qiu *et al.*, 2006) (Fig. 3.05E). Schüepp (Schüepp, 1926) classified the Lycopodiales root meristems into his IIID group. The procambium and ground tissue comprise the Körper complex, and the root cap comprises the Kappe complex. The epidermis develops independently of the Körper and Kappe; and is located between the two complexes. The cellular organisation of *R.*

carbonica is different from the Lycopodiales meristem organisation in at least two ways. The first and most striking difference between *R. carbonica* and extant Lycopodiales is the size and organisation of the promeristem. The promeristem of *R. carbonica* is broad and contains over 10 tiers of cells which far exceeds the 3 or 4 tiers described in extant Lycopodiales. Second, the epidermis of *R. carbonica* is not distinct from all other layers but instead is interpreted to develop as the innermost layer of the Kappe complex. There are similarities between *R. carbonica* and the root meristem of *Lycopodium clavatum* (Imaichi, 2008) which develops a domain with initials that are not clearly separated into distinct tiers. However, unlike *L. clavatum* (Imaichi, 2008) where there is a central group of many initials, the *R. carbonica* promeristem is organised in a regular block of cells in a columnar arrangement.

The promeristem of the Isoetales forms either two (Farmer, 1890; Campbell, 1891; Bhambie, 1963; Paolillo, 1963; Guttenberg, 1966) or three tiers (Bruchmann, 1874; Scott & Hill, 1900; Guttenberg, 1966; Yi & Kato, 2001) of initials (Fig. 3.05E).The *R. carbonica* promeristem is much larger than Isoetales; there are over 10 tiers of cells in the *R. carbonica* promeristem and only three in typical Isoetales root meristems. In summary the size and organisation of the *R. carbonica* promeristem distinguishes it from all extant lycophytes.

3.7.5.2 The organisation of the *R. carbonica* promeristem is different from all extant monilophyte root meristems

The monilophytes are amonophyletic group that includes the ferns and horsetails (Pryer *et al.*, 2001, 2004; Qiu *et al.*, 2006; Rothfels *et al.*, 2015). There is a single apical initial in most monilophyte root meristems (Bower, 1889; Foster & Gifford, 1959; Guttenberg, 1966; Ogura, 1972) (Fig. 3.05E). However there are more than one initials in the Marattidae and the Osmundaceae where they range between 1 and 4 (Bower, 1889;

Campbell, 1891; West, 1917; Foster & Gifford, 1959; Guttenberg, 1966; Bhambie & Rao, 1972; Freeberg & Gifford, 1984). The broad multicellular promeristem of *R. carbonica* consisting of 138 cells is therefore strikingly larger than the promeristems of all extant monilophytes with either a single or a small number of initials cells. In summary the size and organisation of the *R. carbonica* promeristem distinguishes it from all extant monilophytes.

3.7.5.3 R. carbonica most closely resembles extant gymnosperm meristems

The root meristems of gymnosperms are distinctive from the meristems of other vascular plants because of the presence of a broad promeristem with common initials for all or the majority of the mature tissues of the root (Reinke, 1872; Strasburger, 1872; Janczewski, 1874; De Bary, 1884; Schüepp, 1926). In gymnosperms all mature tissues converge on a broad promeristem which takes the form of an upturned cup shape (the base of the cup is where the procambium and root cap columella converge and the sides of the cup are where the ground tissue, epidermis and lateral root cap converge (Fig. 3.05)). The initial cells in the central region of the promeristem are arranged as a columella where the columnar organisation results from the alignment of longitudinal cell walls in files, which Allen (Allen, 1947a,b) refers to as the 'column mother initial zone' (Allen, 1947a,b). Individual sets of initials for specific tissue types are not distinct. There are three types of initial cell organisation in gymnosperm promeristems. 1. There is a common set of initials for all fundamental tissues or all fundamental tissues except the vasculature tissues (Cycadales (Pillai, 1963, 1966, Voronin, 1964, 1969; Milindasuta, 1975; Webb, 1983); Ginkgoales (Ball, 1956a,b; Guttenberg, 1961; Pillai, 1963) and some members of the Pinopsida (Schopf, 1943; Allen, 1947a,b; Wilcox, 1954)). 2. There is one set of common initials for the root cap columella and the procambium and another set for all of the ground tissue, epidermis and lateral root cap (Spurr, 1949; Wilcox, 1962; Pillai, 1964, 1966; Bogar &

Smith, 1965) which is referred to as the 'conifer type' (Pillai, 1964, 1966) (Pinopsida and Cupressophyta). 3. There is one set of common initials for ground tissue, epidermis and lateral root cap and another set of initials for root cap columella and procambium (Gnetophyta) (Deshpande & Bhatnagar, 1961; Guttenberg, 1961; Pillai, 1966; Peterson & Vermeer, 1980).

The organisation of cells in *R. carbonica* is more similar to the cellular organisation of meristems in extant gymnosperms than any other group of tracheophytes. The *R. carbonica* promeristem like the promeristems of all gymnosperms is broad and shaped like an upturned cup and cells in the central region are organised as a columella. *R. carbonica* most closely resembles the promeristem organisation characteristic of the Cycadales, Ginkgoales and some members of the Pinopsida because the promeristem of both comprises a set of common initials for all fundamental tissues except the vascular tissues. However, it is distinct from all of these extant gymnosperms in three main ways. First the position of the Körper-Kappe boundary. Second, the discrete nature of the *R. carbonica* root cap. Third, the presence of anticlinal cell divisions in a regular broad promeristem – as discussed in detail in the main text.

3.7.5.4 R. carbonica is not an angiosperm root meristem

R. carbonica cannot be an angiosperm root. First, it is approximately 320 million years old and angiosperms did not appear in the fossil record until almost 200 million years later (Clarke *et al.*, 2011). Second, the cellular organisation in the *R. carbonica* promeristem is entirely different from any of the recognised 15 classes of angiosperm meristem (Heimsch & Seago, 2008).

In summary the root meristem of *R. carbonica* is most similar to extant gymnosperm meristems because all fundamental tissues converge on a broad promeristem

with a regular columella-like organisation. However, the three major differences between *R. carbonica* and typical gymnosperm meristems are the position of the Körper-Kappe boundary, the discrete nature of the *R. carbonica* root cap and the presence of anticlinal cell divisions in a regular broad promeristem – as discussed in the main text. These three character states combined mark *R. carbonica* as distinct from all root meristems previously described.

3.7.6 Description of the Körper-Kappe theory

Schüepp (Schüepp, 1917, 1926) identified that root meristems could be split into two discrete zones defined by the distribution of two distinct cell division types termed Körper (inner body) and Kappe (outer cap) T-divisions. When a root meristem in viewed in median longitudinal section files of cells can be followed from the initials to the mature regions of the root. Occasionally a single cell file splits in two and this break leads to the formation of characteristic T shape (where the horizontal stroke of the T represents a transverse cell division and the vertical stroke of the T represents the longitudinal division and the split of one cell file into two). T-divisions can be found throughout the root meristem however the orientation of the T shape varies. Within the Körper (body) complex the vertical stroke of the vertically inverted T points away from the meristematic initials towards the base of the root – resulting from the transverse cell division occurring before the longitudinal division (Schüepp, 1917, 1926; Clowes, 1961; Romberger *et al.*, 1993). Inverted T-divisions where the vertical stroke of the T points away from the meristematic initials facilitate increase in cell layer number within the root body and are therefore termed Körper T-divisions (Fig. 3.05). However, within the Kappe (cap) the vertical stroke of the T points towards the meristematic initials resulting from the longitudinal division occurring before the transverse division (Schüepp, 1917, 1926; Wagner, 1939; Clowes, 1961; Romberger *et al.*, 1993; Evert, 2006). T divisions where the vertical stroke of the T

points away from the meristematic initials facilitate increase in cell number within the root cap and are therefore termed Kappe T-divisions (Fig. 3.05). The distribution of Körper and Kappe T-divisions therefore defines the Körper-Kappe boundary, and critically the position of this boundary varies between species and provides a way to distinguish between different classes of root meristems (Schüepp, 1917, 1926; Clowes, 1961; Romberger *et al.*, 1993; Evert, 2006).

3.7.7 Extended Figure 3.05 legend

Fig. 3.05 displays a summary of meristem types in the extant vascular plant lineages. The Selaginellales are shown to have a single tetrahedral apical cell, as is found in the majority of *Selaginella* species (Fig. 3.05E) (Nägeli & Leitgeb, 1868; Guttenberg, 1966; Grenville & Peterson, 1981; Imaichi & Kato, 1989, 1991; Lu & Jernstedt, 1996; Imaichi, 2008; Otreba & Gola, 2011). The root apices of the Lycopodiales are shown with initials arranged in either three (Strasburger, 1872; Saxelby, 1908; Guttenberg, 1964, 1966) or four (Bruchmann, 1874; Stokey, 1907; Guttenberg, 1964, 1966; Tsukaya, 2014) tiers, representing the promeristem structure in the majority of the Lycopodiales described to date. The promeristem of the Isoetales is depicted with initials arranged in either two (Farmer, 1890; Campbell, 1891; Bhambie, 1963; Paolillo, 1963; Guttenberg, 1966) or three tiers (Bruchmann, 1874; Scott & Hill, 1900; Guttenberg, 1966; Yi & Kato, 2001) as described in all Isoetales examined to date (Fig. 3.05E).

The root meristems of monilophytes are depicted in Fig. 3.05E with a single apical initial. We hypothesize that there was a single initial in the root meristem of the last common ancestor of the monilophytes (Fig. 3.05E). The most recent monilophyte phylogenies indicate that there is a single initial in the root meristem of the basal monilophytes taxa (Rothfels *et al.*, 2015); Equisetales (Johnson, 1933; Foster & Gifford, 1959; Guttenberg, 1966; Gifford JR & Kurth, 1982) and the Ophioglossales (Bower, 1889;

Farmer & Freeman, 1899; Campbell, 1921, 1922; Guttenberg, 1966). Osmundales and Marattiales develop between 1 and 4 initials and there are single initials in the six more derived classes (Hymenophyllales, Gleicheniales, Schizaeles, Saliniales, Cyatheales and Polypodiales). Given that the most basal monilophyte lineages develop root meristems with single initials, and clades with a single apical (initial) cell are more common than those with multiple initials, it is most parsimonious to conclude that a single apical (initial) cell was the ancestral root meristem state in the monilophytes and that multiple initials subsequently evolved in the ancestors of the Osmundales and Marattiales. Therefore the root meristems of monilophytes are depicted with a single apical initial in Fig. 3.05E.

The gymnosperm root meristem in Fig. 3.05E comprises a central zone of common initials for all tissues, or common initials for all non-vascular tissues and a separate set for all vascular tissues. The meristem is shown with common initials for all tissues or common initials for all non-vascular tissues and a separate set for all vascular tissues because this is the most parsimonious interpretation for the ancestral root meristem type in gymnosperms. A root meristem with common initials for all tissues or all non-vascular tissues is found in the Cycadales (Pillai, 1963, 1966, Voronin, 1964, 1969; Milindasuta, 1975; Webb, 1983); Ginkgoales (Ball, 1956a,b; Guttenberg, 1961; Pillai, 1963) and some members of the Pinopsida (Schopf, 1943; Allen, 1947a,b; Wilcox, 1954) which are the most ancestral lineages of the gymnosperms (Chaw *et al.*, 2000; Qiu *et al.*, 2006; Xi *et al.*, 2013; Lu *et al.*, 2014; Ruhfel *et al.*, 2014).

Chapter 4: Gene expression data support the lycopsid root rather than the modified shoot hypothesis for the evolution of Isoetalean rootlets

Hetherington AJ, Kelly S, Dolan L. Gene expression data support the lycopsid root rather than the modified shoot hypothesis for the evolution of Isoetalean rootlets. **(Unpublished)**

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A.J.H. and L.D. designed research; A.J.H. performed research with assistance from S.K.; A.J.H. wrote the paper with assistance from L.D.

4.2 Summary

- The longstanding modified shoot hypothesis predicts that the rootlets of the Isoetales are modified microphylls (leaves). An alternative hypothesis is that rootlets of the Isoetales are homologous to other lycopsid roots. However, neither of these hypotheses have been tested using gene expression data.
- In this study we carried out a comparative transcriptomic comparison between the rootlets and microphylls of *Isoetes echinospora* and the roots and leafy shoots of the related lycopsid *Selaginella moellendorffii*.
- We identified a high similarity between the rootlets of *I. echinospora* and the roots of *S. moellendorffii* by comparing differentially expressed genes, putative rootlet regulators and putative rootlet developmental regulators.
- The high similarity between the gene expression profiles of the rootlets of *I. echinospora* and the roots of *S. moellendorffii* is evidence consistent with the lycopsid root hypothesis. We conclude that *Isoetes* rootlets are roots, homologous to the roots of other lycopsids (rather than modified microphylls).

4.3 Introduction

The first giant (> 50 m) trees to grow on Earth (arborescent members of the Isoetales (*sensu* (DiMichele & Bateman, 1996)) were tethered to the ground by remarkable rooting structures termed stigmarian systems (Stewart & Rothwell, 1993). What distinguishes these rooting systems are the numerous parallels in both anatomy and development between the belowground stigmarian system and the aboveground leafy shoot system (Stewart & Rothwell, 1993). Stigmarian systems of both extinct and extant members of the Isoetales develop underground shoot-like structures termed rhizomorphs which develop leaf-like lateral appendages, termed rootlets, in a regular pattern or rhizotaxy (Williamson,

1887; Stewart, 1947; Karrfalt, 1980; Paolillo, 1982; Rothwell & Erwin, 1985; Stewart & Rothwell, 1993). It was the great similarity between the shooting and rooting systems of the Isoetales that led to the evolutionary hypothesis that stigmarian systems represent a direct modification of a leafy shoot system (Schimper, 1872). The theory describing this direct modification is termed the modified shoot hypothesis (MSH), and has a heritage dating back over a century, and it has represented the consensus in the literature for the past 30 years (Schimper, 1872; Frankenberg & Eggert, 1969; Rothwell & Erwin, 1985; Phillips & DiMichele, 1992; Stewart & Rothwell, 1993; Rothwell *et al.*, 2008, 2014; Taylor *et al.*, 2009; Sanders *et al.*, 2011).

Although the MSH represents one theory to account for stigmarian systems (and has support from multiple lines of evidence, including anatomy, embryology and development (described in detail by Rothwell & Erwin, 1985)), it is not the only theory. An alternative hypothesis is that the rootlets of stigmarian systems are in fact roots, homologous to the roots of other lycopsids, rather than modified microphylls. These rootlets are attached to the shoot-like rhizomorph, which is interpreted as a unique root-bearing structure with an unclear evolutionary origin (Gensel & Berry, 2001). Although the evolutionary origin of the rhizomorph is unknown the rhizomorph has similarities, but not necessarily homologies, with other root-bearing structures found in the lycopsids (reviewed by Hetherington & Dolan, accepted). The theory describing the homologous nature of lycopsid roots, including the rootlets of the Isoetales, can be termed the lycopsid root hypothesis (LRH) and although it also has a heritage dating back over a century it has been largely overshadowed by the MSH during the last 30 years (Rothwell & Erwin, 1985).

Both hypotheses agree that rhizomorphs are remarkably shoot-like, but disagree strikingly in their interpretation of the evolutionary origin of rootlets. The MSH predicts

that rootlets are direct modifications of photosynthetic, determinate, ligulate, stomata bearing, microphylls with bilateral symmetry. It postulates that this ancestral microphyll was modified by the loss of all of these characters and the convergent evolution of indeterminacy, profuse apical dichotomous branching (dependent on auxin as in other lycopsid roots (Sanders, 2007; Sanders & Langdale, 2013)), a root meristem developing a root cap, root hairs developing in type two root hair epidermal patterning and radial symmetry. The MSH finds support for this interpretation in the presence of microphyll-like characters in the rootlets of the Isoetales, most notably the collateral position of the phloem, the regular arrangement or rhizotaxy of rootlets (although it should be noted that rhizotaxy results from the developmental patterning from the rhizomorph meristem and is therefore not solely a feature of the rootlets), and the abscission of rootlets from the rhizomorph (Karrfalt, 1980; Rothwell & Erwin, 1985). By contrast, the LRH predicts that rootlets of the Isoetales are homologous to other lycopsid roots. The LRH is supported by the shared anatomical and developmental features between lycopsid roots and the rootlets of the Isoetales including; indeterminacy, profuse apical dichotomous branching, a root meristem developing a root cap, root hairs developing in type two root hair patterning and radial symmetry (reviewed by Hetherington & Dolan, accepted). The LRH predicts that although rootlets evolved from an ancestral root they convergently evolved microphyll-like characters including the collateral position of the phloem, the regular arrangement or rhizotaxy of the rootlets, and rootlet abscission. Based solely on character evolution the LRH is most parsimonious as it involves only three character changes (the evolution of the collateral position of the phloem, regular arrangement or rhizotaxy of rootlets and rootlet abscission) compared with the MSH which predicts over 8 character transitions (loss of photosynthetic ability, loss of stomata, loss of a ligule, change from determinate to indeterminate growth; change from bilateral to radial symmetry, gain of apical

dichotomous branching dependent on auxin; the gain of a root meristem developing a root cap, gain of root hairs developing in type 2 epidermal patterning). However, both hypotheses involve significant modification from an ancestral root or microphyll, followed by convergent evolution of root or microphyll characters leading to the anatomy and development of rootlets present in extant *Isoetes* species.

Convergent evolution on this scale is not uncommon in land plants, both roots and leaves as well as many other traits have had multiple independent origins in the vascular plant lineage (Kenrick & Crane, 1997; Boyce, 2005). However, the direct modification of a leaf to a root, including the convergent evolution of root hairs and root meristem developing a root cap has never been described (Goebel, 1905) (the closest analogy are the modified leaves of species of *Utricularia* (Goebel, 1905; Adlassnig *et al.*, 2005)). By contrast, roots have been described from a range of species which have converged on leaf-like characters such as; rhizotaxy, collateral phloem and active abscission. Regular root patterning, albeit not spiral rhizotaxy found in the extinct Isoetales (Charlton & Watson, 1982), has been described in a number of species including *Ceratopteris species* (Mallory *et al.*, 1970; Hou *et al.*, 2004) *Arachis hypogaea*, *Victoria trickeri*, *Eichhornia crassipe* (Mallory *et al.*, 1970), and *Pontederia cordata* (Charlton, 1975). The development of a collateral phloem has also been described in both the aerial roots of *Rhizophora mangle* (Gill & Tomlinson, 1971) and the primary root of *Opuntia basilaris* (Freeman, 1969). Moreover, a collateral phloem is also characteristic of other lycopsid roots (Stokey, 1907; Wigglesworth, 1907). Finally, active root abscission, although thought of as primarily a leaf character has been documented in a range of species including *Oxalis Esculenta* (Duncan, 1927); *Abies balsamea*, *Tsuga canadensis*, *Pinus strobus* (Tippett, 1982), and *Azolla species* (Konar & Kapoor, 1972; Uheda *et al.*, 1995; Gurung *et al.*, 2012; Fukuda *et al.*, 2013; Yamada *et al.*, 2015). Taken together these data from the diversity of rooting

structures present in extant plants demonstrate that, although roots of extant plant species have converged on leaf-like characteristics, leaves have never been modified to roots with root caps and root hairs. Although this does not rule out the character evolution predicted by the MSH it highlights that the character evolution needed for the LRH has occurred multiple times in vascular plants.

Both the modified shoot and the LRH require the rootlets of extant Isoetales to have diverged substantially from an ancestral root or microphyll. The LRH is a more parsimonious interpretation as it requires less character evolution and all anatomical character transitions required have evolved convergently in the roots of other extant species. However, it is clear that more lines of evidence are necessary to evaluate the predictions of both hypotheses. An additional and previously unexplored line of evidence is to examine the genetic similarities between rootlets and microphylls of the Isoetales and compare them with the genetic similarities between roots and microphylls in other lycopsids. The LRH predicts a high degree of similarity between all lycopsid roots and a marked difference between the rootlets and microphylls of the Isoetales. By contrast, if the MSH is correct we may predict that rootlets and microphylls of the Isoetales should show a high degree of genetic similarity (supporting their common origin from an ancestral leaf). Moreover, if the MSH is correct we might predict that there would be a high degree of difference between the rootlets of the Isoetales and other lycopsids roots. The current study reports the investigation of gene expression profiles of *Isoetes echinospora* and the related lycopsid species *Selaginella moellendorffii* in order to test the predictions of both the modified shoot and lycopsid root hypotheses.

4.4 Materials and Methods

4.4.1 Plant collection and growth

Mature *I. echinospora* plants were collected in September 2013 and 2014 from North West Sutherland (Scotland, UK) with the permission of the John Muir Trust and the Scourie Estate. *I. echinospora* plants were identified on the basis of their echinate megaspore ornamentation (Taylor, 1993). Mature *I. echinospora* plants were grown submerged in aquaria in Levington M2 compost topped with coarse gravel in a glasshouse at Oxford University. Plants were grown at 18°C under a 16 h light : 8 h dark photoperiod, submerged in tap water.

4.4.2 Growth of *I. echinospora* in axenic culture

In order to solely extract RNA from *I. echinospora* tissues and to minimise contamination, RNA was extracted from plants grown in axenic culture. A procedure was developed to surface sterilise *I. echinospora* spores and germinate a population of axenically grown plants, based on previously developed procedures (Kott & Britton, 1982; Oh *et al.*, 2013). Sporophylls were removed from the mature plant population growing in aquaria in September (2013 and 2014) when sporangia were mature (Kott & Britton, 1982). Using forceps (under a Leica M165 FC stereo microscope) mega- and micro-sporangia were extracted from sporophylls. Intact sporangia were washed in 1% (v/v) sodium dichloroisocyanurate (NaDCC) for 5 min. After which sporangia were broken and loose spores were washed in 0.1% NaDCC for a further 5 min. Following the washes with NaDCC loose spores were rinsed three times in ddH_2O, each wash for 5 min (microspores were centrifuged for 5 min at 5000 rpm between washes). After surface sterilisation mega- and micro-spores were mixed together in ddH_2O within a Petri dish. Petri dishes were sealed with parafilm, and kept in darkness at 4^0C for 2 wk. After 2 wk, Petri dishes were moved to a 16 h light : 8 h dark photoperiod at 18^0C. Approximately 30% of surface

sterilised megasporangia contained megaspores which germinated. Within the 30% of megasporangia which contained developing megaspores *c*. 25% of the total megaspore population germinated. It was possible to identify germinating megaspores due to the cracking of the megaspore wall and the development of archegonia on the megagametophyte. Developing sporophytes were first identified by the presence of the first microphyll. Sporophytes were left to continue to grow in ddH_2O water until the two microphyll two rootlet stage where they were moved to magenta boxes containing; ½ Gamborg's medium (Gamborg *et al.*, 1968), supplemented with 1% phytogel (Sigma). Plants were embedded in Gamborg media and submerged in liquid Bold's Basal Medium (Sigma, UK).

4.4.3 RNA extraction and sequencing

Total RNA was extracted from root, corm and microphyll tissues from *c*. 100 *I. echinospora* plants. Total RNA from microphylls (two independent replicates), corm (two independent replicates) and rootlets (one replicate) was extracted with the RNeasy plant mini kit (Qiagen). On-column DNase I treatment was performed with RNase-free DNase I (Qiagen), according to the manufacturer's instructions. cDNA was synthesised with ProtoScript II reverse transcriptase (New England Biolabs) according to the manufacturer's instructions, using oligo(dT) primer. Total cDNA samples were quantified with a Nanodrop ND-1000 spectrophotometer. RNA purity and quality were checked with an Agilent 2100 Bioanalyzer. cDNA was sequenced by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, University of Oxford. Sequencing resulted in 195,072,304 paired end reads separated into five samples: 2 microphyll samples (35,718,157; 35,555,048 paired end reads), 2 corm samples (38,728,989; 44,379,751 paired end reads) and one rootlet sample (40,690,359 paired end reads).

4.4.4 *De novo* transcriptome assembly

Raw reads were quality trimmed using Trimmomatic-0.32 (Bolger *et al.*, 2014), to remove remaining Illumina adaptors and low quality tails. Ribosomal RNA was filtered out using Sortmerna-1.9 (Kopylova *et al.*, 2012) and error corrected using BayesHammer (SPAdes-16 3.5.0) (Nikolenko *et al.*, 2013) (with setting --only-error-correction) and Allpaths-LG-4832 (Butler *et al.*, 2008) (with setting PAIRED_SEP=-20 and ploidy = 2). Reads were normalised using Khmer-0.7.1 with a khmer size of 21. Before assembly, paired end reads were stitched together using Allpaths-LG-4832 (Butler *et al.*, 2008). A *de novo* transcriptome assembly was made with the cleaned, stitched reads using SGA (Simpson & Durbin, 2012), SSPACE-v3 (Boetzer *et al.*, 2011), and CAP3 (Huang & Madan, 1999). Finally assembled scaffolds were corrected using Pilon-1.6 (Walker *et al.*, 2014). Transcriptome assembly statistics were calculated using TransRate (Smith-Unna *et al.*, 2016).

4.4.5 Expression and differential expression (DE) analysis

The expression level for each transcript was calculated using RSEM (Li & Dewey, 2011). Transcripts were deemed expressed in each organ if average expression was ≥ 0.5 Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Identification of differentially expressed (DE) transcripts was carried out with Deseq2 (Love *et al.*, 2014) in R (R-studio). Expected read counts from RSEM were used to create normalised expression levels in Deseq2 using default parameters (Love *et al.*, 2014). Pairwise comparisons between the microphyll and rootlet samples were made to identify DE transcripts, using Deseq2 (Love *et al.*, 2014). DE transcripts were identified as transcripts which had a Benjamini-Hochberg corrected $P \leq 0.05$ (Benjamini & Hochberg, 1995).

4.4.6 Protein predictions and assignment of gene orthology

Proteins were predicted from the *de novo* transcriptome assembly using GeneMarkS-T (Tang et al 2015), Prodigal (Hyatt et al., 2010) and Transdecoder (part of the Trinity assembly program Grabherr et al 2011). Two methods were used to determine gene orthology, between *I. echinospora* and other species. The first method used a reciprocal best blast analysis (developed by Aubry *et al.*, (2014) and modified to run on the *I. echinospora* predicted proteins with an E-value of 1E-10). Blast searches were made respectively between *I. echinospora* proteins and the predicted proteome of *S. moellendorffii* (Banks *et al.*, 2011). The reciprocal best blast analysis results in the prediction of orthologous genes between *I. echinospora* and *S. moellendorffii* based on their blast scores.

The second method aimed to determine gene orthology from 20 species (including *I. echinospora* from the current study) representing the breadth of the Archaeplastida lineage (including red algae, chlorophytes, and streptophytes) (Collén *et al.*, 2013), using Orthofinder (Emms & Kelly, 2015). The taxa and genome / transcriptome versions used in the analysis are listed in Supplementary Information Table S1. In addition to the protein sequences from 15 species with fully sequenced genomes (Table S1), protein sequences predicted from *de novo* transcriptome assemblies of five additional species were included to increase the representation of monilophytes (*Equisetum giganteum* (Vanneste *et al.*, 2015) and *Lygodium japonicum* (Aya *et al.*, 2015)) and lycophytes (*Phylloglossum drummondii* and *Isoetes drummondii* (data courtesy of Josh Mylne (University of Western Australia))) and *I. echinospora* from the current study. Orthofinder (Emms & Kelly, 2015) was run on the predicted proteins from these genomes and transcriptomes, resulting in the clustering of orthologous proteins into orthogroups (OGs). A phylogenetic analysis was carried out for the protein sequences in each OG (using *trees_for_orthogroups.py*; (Emms

& Kelly, 2015)). Alignments for the protein sequences for each OG were carried out using MAFFT (Katoh & Standley, 2013) (orthogroups with < 500 sequences were aligned using the linsi option; orthogroups with > 500 sequences were aligned using default parameters). Based on these alignments gene trees were generated using FastTree2 (Price *et al.*, 2010), with branch support based on a Shimodaira-Hasegawa test.

4.4.7 Comparison of gene expression with *S. moellendorffii*

The results of the current study based on *I. echinospora* were compared with previously published gene expression data for *S. moellendorffii*, from Huang & Schiefelbein, (2015), and Frank *et al.*, (2015). Average gene expression in the roots of *S. moellendorffii* (FPKM) was generated by calculating the average expression of *S. moellendorffii* roots across all root samples described in Huang & Schiefelbein, (2015). Gene expression in the leafy shoots of *S. moellendorffii* was based on an average expression of the leafy shoot sample from Frank *et al.*, (2015). Expression in leafy shoots from Frank *et al.*, (2015) was reported in Reads Per Million (RPM). *S. moellendorffii* proteins were characterised as expressed in either the leafy shoot or root if the RPM or FPKM, respectively, were ≥ 0.5.

Although the expression levels for both are reported with different units of expression the units are related and can therefore be compared. The formula for RPM (Eqn 1) and FPKM (Eqn 2) are identical except for the addition of the variable L (the length of the transcript in kilo bases pairs (Kb)) (Eqn 2, L). The average value for L in the *S. moellendorffii* genome is 1.28 kb (Banks *et al.*, 2011), so the expression level reported by Frank et al., (2015) was normalised and presented in nRPM (Eqn 3). In order to directly compare DE transcripts between *I. echinospora* and *S. moellendorffii*, transcripts were interpreted as DE if a 3-fold expression difference was observed between root and leaf samples.

$$RPM = \frac{R}{T/10^6}$$

Eqn 1 Formula for Reads Per Million (RPM). *R*, the number of reads that map to a transcript. *T*, total number of reads sequenced.

$$FPKM = \frac{R}{L\left(T/10^6\right)}$$

Eqn 2 Formula for Fragments Per Kilobase of Transcript Per Million Mapped Reads (FPKM). *R*, the number of reads that map to a transcript. *L*, length of the transcript in kilobases. *T*, total number of reads sequenced.

$$nRPM = \frac{R}{1.28\left(T/10^6\right)}$$

Eqn 3 Formula for normalised Reads Per Million (nRPM). *R*, the number of reads that map to a transcript. *T*, total number of reads sequenced.

4.5 Results

4.5.1 Assembly of an *Isoetes echinospora* sporophyte transcriptome

To examine gene expression in the organs of the Isoetales, the sporophyte transcriptome of *I. echinospora* was generated. RNA was extracted from the three major organs which constitute the sporophyte body of *Isoetes* species; microphyll, corm, and rootlet. RNA was sequenced, the raw reads were quality checked and then assembled to form contiguous lengths of sequence referred to as transcripts. 114,038 transcripts with a mean sequence length of 936 base pairs (bp) constituted the *de novo* transcriptome assembly of *I. echinospora*. The transcriptome comprised 35,616 sequences over 1 kilo base pairs (Kb),

and an N50 of 1311 bp. Of the 13,583,544 paired-end reads used to generate the assembly, 95.6% aligned to the final assembly, and 73.8% of the read pairs aligned concordantly one time. Furthermore, proteins were predicted for *c*. 95% (107,931) of the 114,038 transcripts. These metrics indicate that the transcriptome assembly was of high quality and incorporated the majority of the sequence reads.

4.5.2 *I. echinospora* rootlets and microphylls display a similar degree of gene expression overlap as the roots and leafy shoot of *S. moellendorffii*

The MSH predicts that rootlets of *I. echinospora* are modified microphylls and are, therefore, fundamentally different from the roots of the closely related lycopsid *S. moellendorffii*. If true, it may be expected that rootlets and microphylls of *I. echinospora* would have a greatly overlapping gene expression profile (as both organs evolved from a common microphyll). Furthermore, it may be expected that the similarity between the gene expression profiles of *I. echinospora* rootlets and microphylls would be greater than that seen between roots and microphylls of *S. moellendorffii* (where roots and microphylls have had distinct evolutionary histories). To test these hypotheses, we utilised our *I. echinospora* sporophyte transcriptome. Mapping the error corrected reads for the three organ types (microphyll, corm, and rootlet) to the sporophyte transcriptome we calculated the number of transcripts expressed in each organ. Transcripts were interpreted as expressed if the average expression level was ≥ 0.5 Fragments Per Kilobase of transcript per Million mapped reads (FPKM). With organ expression levels for each transcript it was possible to compare expression levels in both microphylls and rootlets. In total, 95,134 transcripts were expressed in microphylls and 88,718 transcripts in rootlets. This indicates that 89% (101,857 transcripts) of the total *I. echinospora* sporophyte transcriptome were expressed in either microphylls or rootlets. Of these 101,857 transcripts, 80.5% (81,995

transcripts) were expressed in both rootlets and microphylls (Fig. 4.01). An overlapping gene expression of *c*. 80% between *I. echinospora* rootlets and microphylls demonstrates the similarity in gene expression profiles in both organs.

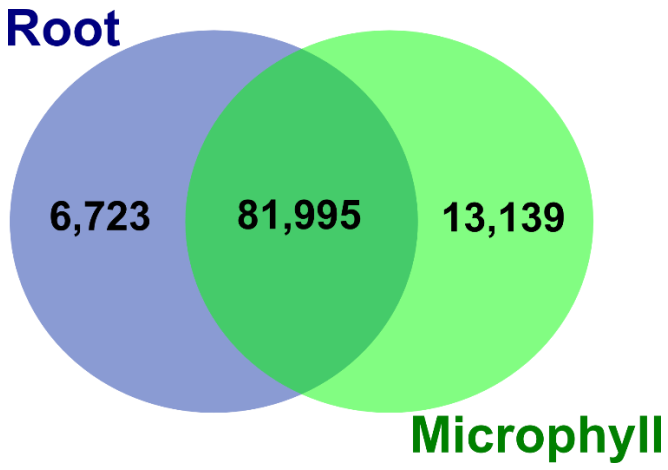

Figure 4.01 80% of transcripts expressed in either rootlets or microphylls of *Isoetes echinospora* are expressed in both organs. Venn diagram displaying the numbers of transcripts expressed uniquely in rootlets (blue), microphylls (light green) or with overlapping expression in both organs.

The similar gene expression profiles of *I. echinospora* microphylls and rootlets may be taken as support for the predictions of the MSH, that *I. echinospora* rootlets and microphylls are homologous. However, comparison of similar organs in the related lycopsid *S. moellendorffii* – where it is clear that microphylls and rootlets are not homologous – indicates that a high degree of overlapping gene expression is common between organs of other lycopsids. The study by Frank *et al.*, (2015) identified 13,502 *S. moellendorffii* genes expressed in leafy shoots. Of these genes expressed in leafy shoots, 88.1% were also reported as expressed in the roots of *S. moellendorffii* (Huang & Schiefelbein, 2015) (Fig. 4.02). This indicates that 77.1% of the total number of genes

expressed in either *S. moellendorffii* leafy shoots or roots were expressed in both organs (Fig. 4.02). The percentage of genes expressed in both leafy shoots and roots of *S. moellendorffii* (77.1%) is therefore comparable to the percentage overlap of microphylls and rootlets of *I. echinospora* (80.5%).

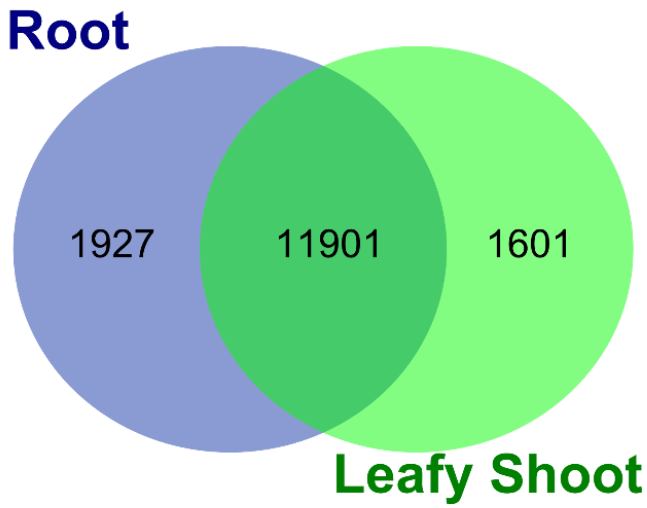

Figure 4.02 77% of genes expressed in either roots or leafy shoots of *Selaginella moellendorffii* are expressed in both organs. Venn diagram displaying the numbers of transcripts expressed uniquely in root (blue), leafy shoots (light green) or with overlapping expression in both organs.

4.5.3 60% of the *S. moellendorffii* orthologs of *I. echinospora* putative rootlet regulators (PRR's) are differentially or uniquely expressed in *S. moellendorffii* roots

If roots of *S. moellendorffii* and rootlets of *I. echinospora* are homologous (as predicted by the LRH) we expect putative rootlet regulators (PRR's) of *I. echinospora* to be also expressed in the roots of *S. moellendorffii*. In order to test this hypothesis, we first identified PRR's in *I. echinospora*. Of the 81,995 *I. echinospora* transcripts with expression in both rootlets and microphylls, 23.7% (19,406 transcripts) were differentially expressed (DE) between the two organs. Transcripts are interpreted as DE if there is a significant difference in expression between the two organs (supported by a Benjamini-

Hochberg corrected $P \leq 0.05$ (Benjamini & Hochberg, 1995)). 11,278 of the total 19,406 DE transcripts were identified as DE in the rootlets of *I. echinospora* and these transcripts were assigned as PRR's.

To identify the orthologs of each of these *I. echinospora* PRR's in *S. moellendorfii* a reciprocal best blast analysis was carried out (Aubry *et al.*, 2014). The 11,278 *I. echinospora* PRR's corresponded to 3,363 *S. moellendorffii* orthologous genes identified by the reciprocal best blast analysis. Of the 3,363 genes, 1,561 were either differentially or uniquely expressed in leafy shoots or roots of *S. moellendorffii*. 60.7% of these genes were differentially or uniquely expressed in roots of *S. moellendorffii*. This higher percentage of root genes than leafy shoot genes indicates that *I. echinospora* rootlets are more similar to the roots than the leafy shoots of *S. moellendorffii*.

4.5.4 15 of the 24 PRR orthogroups were present in *S. moellendorffii*

Although a reciprocal best blast analyses provides one method for assigning orthology it has its limitations (Ward & Moreno-Hagelsieb, 2014), especially when assigning orthology between predicted proteomes which vary greatly in size (the predicted *I. echinospora* proteome is *c*. 5 times larger than that *S. moellendorffii* (Banks *et al.*, 2011)). We therefore undertook a further analysis to define the precise orthology relationships between *I. echinospora* PRR's and *S. moellendorffii* using the programme Orthofinder (Emms & Kelly, 2015). Orthofinder has advantages over reciprocal best blast approaches as it allows orthology to be examined across a range of species, blast scores are corrected for sequence length bias, and it is possible to generate gene trees of each orthologous group to provide confident orthology assignment. Gene orthology of the entire *I. echinospora* predicted proteome was assigned using Orthofinder (Emms & Kelly, 2015)

with a set of 20 species spanning the Archaeplastida lineage (which includes red algae, chlorophytes, and streptophytes) (Collén *et al.*, 2013) (Table S1).

We took the top 25 differentially expressed rootlet transcripts in *I. echinospora* as the top PRR candidates, and undertook a manual examination of 24 orthogroups (OGs) which included these transcripts (Table 1). There was a single *I. echinospora* protein, and no *S. moellendorffii* genes, or genes from any other species, in 5 OGs (OG0095825, OG0084889, OG0082280, OG0077270, and OG0065114; Table 1). A further manual examination of the nucleotide and protein sequences predicted from these transcripts revealed that none was similar to other proteins or nucleotides on the NCBI database. This indicates that these transcripts, and their predicted proteins, may either represent chimeras produced during the *de novo* transcriptome assembly, or are unique *I. echinospora* sequences dissimilar to previously described sequences. There were *I. echinospora* genes and genes from other species, but no *S. moellendorffii* genes in four other OGs (OG0013412, OG0010801, OG0003352, and OG0002682; Table 1). Taken together these data indicate that 15 of the 24 PRR orthogroups were present in *S. moellendorffii*.

Table 1 The top 25 putative rootlet regulator (PRR) candidates ordered by Orthogroup number

Orthogroup	*I. echinospora* transcript	Figure	Table	Notes
OG0095825	Transcript_55812	/		Single *I. echinospora* protein
OG0084889	Transcript_111139	/		Single *I. echinospora* protein
OG0082280	Transcript_96910	/		Single *I. echinospora* protein
OG0077270	Transcript_39992	/		Single *I. echinospora* protein
OG0065114	Transcript_35272	/		Single *I. echinospora* protein
OG0013412	Transcript_33199	/		No *S. moellendorffii* proteins
OG0010801	Transcript_110189	/		No *S. moellendorffii* proteins
OG0004398	Transcript_74941	Fig. ES1	Table ES1	
OG0003352	Transcript_21046	/		No *S. moellendorffii* proteins
OG0002682	Transcript_111336	/		No *S. moellendorffii* proteins
OG0001880	Transcript_112637	Fig. ES2	Table ES2	
OG0000996	Transcript_59924	Fig. ES3	Table ES3	
OG0000848	Transcript_65821	Fig. ES4	Table ES4	
OG0000358	Transcript_72072	Fig. ES5	Table ES5	
OG0000249	Transcript_85041	Fig. ES6	Table ES6	
OG0000186	Transcript_75044	Fig. ES7	Table ES7	
OG0000163	Transcript_97028	Fig. ES8	Table ES8	
OG0000121	Transcript_89393	Fig. ES9	Table ES9	
OG0000077	Transcript_88653	Fig. ES10	Table ES10	
OG0000052	Transcript_31902	Fig. ES11	Table ES11	
OG0000042	Transcript_82861	Fig. ES12	Table ES12	
OG0000028	Transcript_97095	Fig. ES13	Table ES13	
OG0000028	Transcript_91303	Fig. ES13	Table ES13	
OG0000022	Transcript_32031	Fig. ES14	Table ES14	
OG0000016	Transcript_10348	Fig. ES15	Table ES15	

4.5.5 14 of 15 orthogroups (OGs) which included an *I. echinospora* PRR also included a differentially or uniquely expressed root gene in *S. moellendorffii*

To test if the *I. echinospora* PRR's were also expressed in the roots of *S. moellendorffii* we compared gene expression profiles between the *I. echinospora* PRR's and their *S. moellendorffii* orthologs. In total 15 OGs included both an *I. echinospora* PRR (a transcript which is differentially expressed in rootlets relative to microphylls) and a *S. moellendorffii* gene. 14 of the 15 OGs including an *I. echinospora* PRR also included a differentially or uniquely expressed root gene in *S. moellendorffii* (Table 2). Furthermore, of these 14 OGs, 67% (10 out of 15) were characterised by dominant root expression in both *I. echinospora* and *S. moellendorffii* (Table 2). Root expression is described as dominant when the total number of differential and uniquely expressed root genes was greater than the total of the differential and uniquely expressed microphyll genes. These results demonstrate that the majority of OGs including an *I. echinospora* PRR also included a *S. moellendorffii* DE or uniquely expressed root gene. Additionally, the majority of the OGs also included dominant root expression in both *I. echinospora* and *S. moellendorffii*. Orthogroups including *I. echinospora* PRRs are therefore more likely to include root differentially and uniquely expressed genes in *S. moellendorffii* than leafy shoot expressed genes.

Table 2 The number of differentially expressed (DE) and uniquely expressed genes in roots and microphylls in each of the top 15 putative rootlet regulator (PRR) orthogroups (OGs) in *Isoetes echinospora* and *Selaginella moellendorffii*

	Fig.	Total Iso	Total Sel	Isoetes		Selaginella		Isoetes		Selaginella	
				DE Leaf	DE Root	DE Leaf	DE Root	Unique Leaf	Unique Root	Unique Leaf	Unique Root
OG0004398	S01	4	3	0	2	0	1	0	2	0	1
OG0001880	S02	5	1	0	2	1	0	0	0	0	0
OG0000996	S03	10	4	0	1	0	1	0	1	0	1
OG0000848	S04	9	3	0	4	0	1	0	0	0	1
OG0000358	S05	4	4	0	3	0	0	0	1	0	2
OG0000249	S06	17	8	0	2	0	2	1	4	1	0
OG0000186	S07	24	9	0	5	2	1	1	2	2	0
OG0000163	S08	26	11	0	8	2	3	1	0	0	0
OG0000121	S09	11	6	0	3	0	1	0	2	0	1
OG0000077	S10	59	14	0	7	1	4	1	4	0	1
OG0000052	S11	37	39	0	10	10	8	0	1	3	4
OG0000042	S12	51	19	3	15	0	9	4	8	0	6
OG0000028	S13	108	27	5	41	1	3	7	20	0	5
OG0000022	S14	75	24	2	22	3	2	6	10	4	0
OG0000016	S15	87	50	4	10	10	0	11	6	10	1

Table showing the genes expressed in the 15 OGs characterised by both an *I. echinospora* PRRs and a *S. moellendorffii* gene. From left to right the table reports the total number of both *I. echinospora* (Total Iso) and *S. moellendorffii* (total Sel) genes in each orthogroup. The number of differentially expressed (DE) genes present in leaf tissues (DE Leaf) or root / rootlet tissues (DE Root) for both *I. echinospora* and *S. moellendorffii*. The number of uniquely expressed genes present in leaf tissues (Unique Leaf) or root / rootlet tissues (Unique Root) for both *I. echinospora* and *S. moellendorffii*. Both DE and uniquely expressed genes are colour coded based on their dominant expression profile (orange, roots; green, microphylls; white, none).

4.5.6 73% off the closest *S. moellendorffii* orthologs to *I. echinospora* putative rootlet regulators (PRRs) are differentially or uniquely expressed in roots of *S. moellendorffii*

The majority of differentially or uniquely expressed genes in the same orthogroups (OGs) as *I. echinospora* putative rootlet regulators (PRRs) were also expressed in roots in *S. moellendorffii*. This finding supports the similarity between the roots of *S. moellendorffii* and rootlets of *I. echinospora*. However, many of the OGs included multiple *I. echinospora* and *S. moellendorffii* genes (Table 2), therefore comparing orthology of the entire orthogroup may not provide a direct comparison of the closest *S. moellendorffii*

genes to *I. echinospora* PRRs. To test if the closest orthologs of *I. echinospora* PRRs were also expressed in roots of *S. moellendorffii* we further divided orthogroups down into the smallest monophyletic group containing both an *I. echinospora* PRR and a *S. moellendorffii* gene. Of the 15 monophyletic groups including an *I. echinospora* PRR, 73% (11 of the 15) also contained a DE or uniquely expressed root gene in *S. moellendorffii* (Table 3). Furthermore, 60% (9 of these 15) of the monophyletic groups were characterised by dominant root expression in both *I. echinospora* and *S. moellendorffii* (Table 3). The examination of the closest *S. moellendorffii* orthologs of *I. echinospora* PRRs therefore indicate a high similarity between root and rootlet expression in *S. moellendorffii* and *I. echinospora.*

Table 3 The number of differentially expressed (DE) and uniquely expressed genes in roots and microphylls in each of the top 15 monophyletic groups containing both an *Isoetes echinospora* putative rootlet regulator (PRR) and *Selaginella moellendorffii* gene

	Total Iso	Total Sel	***Isoetes*** DE Leaf	***Isoetes*** DE Root	***Selaginella*** DE Leaf	***Selaginella*** DE Root	***Isoetes*** Unique Leaf	***Isoetes*** Unique Root	***Selaginella*** Unique Leaf	***Selaginella*** Unique Root
OG0004398	4	3	0	2	0	1	0	1	0	1
OG0001880	5	1	0	2	1	0	0	0	0	0
OG0000996	6	4	0	1	0	1	0	1	0	1
OG0000848	9	3	0	4	0	1	0	0	0	1
OG0000358	2	1	0	1	0	0	0	1	0	1
OG0000249	10	5	0	2	0	1	1	4	1	0
OG0000186	6	1	0	3	0	0	0	0	0	0
OG0000163	20	8	0	3	2	3	1	0	0	0
OG0000121	4	2	0	1	0	0	0	2	0	1
OG0000077	8	2	0	4	1	0	0	2	0	0
OG0000052	18	25	0	5	5	7	0	1	2	4
OG0000042	25	3	2	8	0	1	0	4	0	2
OG0000028	103	4	5	40	0	1	5	20	0	0
OG0000022	27	1	0	13	0	0	4	5	4	5
OG0000016	8	5	0	6	0	0	0	0	0	0

Table showing the genes expressed in the 15 OGs monophyletic groups containing both an *Isoetes echinospora* putative rootlet regulator (PRR) and *Selaginella moellendorffii* gene. From left to right the table reports the total number of both *I. echinospora* (Total Iso) and *S. moellendorffii* (total Sel) genes in each monophyletic group. The number of differentially expressed (DE) genes present in leaf tissues (DE Leaf) or root / rootlet tissues (DE Root) for both *I. echinospora* and *S. moellendorffii.* The number of uniquely

expressed genes present in leaf tissues (Unique Leaf) or root / rootlet tissues (Unique Root) for both *I. echinospora* and *S. moellendorffii.* Both DE and uniquely expressed genes are colour coded based on their dominant expression profile (orange, roots; green, microphylls; white, none).

4.5.7 Identification of 13 putative rootlet developmental regulators based on their similarities to known root system developmental regulators in other land plants

We can make predictions about potential rootlet developmental regulators based on the similarities of *I. echinospora* transcripts with known rooting system regulators from other land plants. The MSH predicts that an ancestral microphyll was modified by the loss of microphyll characters and the acquisition of; root hairs developing in type two root hair patterning, an indeterminate root meristem developing a distal root cap, with profuse apical branching (dependent on auxin (Sander, 2007)), radial symmetry and potentially also the development of an endodermis (it is unclear if the ancestral microphyll had an endodermis as the microphylls of *Selaginella* species do not develop an endodermis (Gibson, 1897; Lersten, 1997) whereas *Isoetes* species do (Romeo *et al.*, 2000)). We therefore identified developmental regulators known to control the development of each of these root characters (or analogous structures (Scotland, 2010)) in other land plants (Table 4).

Table 4 List of 13 putative rootlet developmental regulators based on their functional characterisation in other land plants

Function	Protein type	Arabidopsis gene name	Orthogroup	Fig	Table	Ref
Root Hair Development	bHLH TF	LRL	OG0000015	ES16	Table ES16	(Tam *et al.*, 2015)
					Table ES17	(Menand *et al.*, 2007; Proust et al., 2016)
	bHLH TF	RSL	OG0000015	ES17		
Root Hair Patterning	HD TF	GL2	OG0000165	ES18	Table ES18	(Petricka *et al.*, 2012)
	bHLH TF	GL3	OG0000015	ES19	Table ES19	(Petricka *et al.*, 2012)
	MYB	WER	OG0000012	ES20	Table ES20	(Petricka *et al.*, 2012)
Root Cap Development	NAC	FEZ	OG0000021	ES21	Table ES21	(Willemsen *et al.*, 2008)
	NAC	SMB	OG0000021	ES22	Table ES22	(Willemsen *et al.*, 2008)
Indeterminacy	HD	WOX	OG0001820	ES23	Table ES23	(Zhou *et al.*, 2015)
Branching / Auxin	Auxin influx transporter	AUX	OG0000327	ES24	Table ES24	(Marchant *et al.*, 2002)
	LOB Domain	LOB	OG0000108	ES25	Table ES25	(Coudert *et al.*, 2013)
	PIN	PIN	OG0000485	ES26	Table ES26	(Blilou *et al.*, 2005)
Organ Symmetry	HD	PHB	OG0000580	ES27	Table ES27	(Ilegems *et al.*, 2010)
Endodermis Development	GRAS	SHR	OG0006905	ES28	Table ES28	(Petricka *et al.*, 2012)

Of the 13 orthogroups (Tables 4, 5), 11 included *I. echinospora* DE or unique rootlet expressed genes (LRL, RSL, GL2, WER, SMB, WOX, AUX, LOB, PIN, PHB, SHR), of these, 7 (LRL, RSL, WER, SMB, WOX, LOB, PIN) orthogroups also included differentially or uniquely expressed root genes in *S. moellendorffii*. Of the 11 OGs containing *I. echinospora* differentially or uniquely expressed genes, 8 are characterised by dominant rootlet expression (the total number of differential and uniquely expressed root genes was greater than the total of the differential and unique leaf expressed genes). 5 of the 8 OGs with dominant rootlet expression in *I. echinospora* were also characterised by dominant rootlet expression in *S. moellendorffii* (Table 5). The gene expression of 13

putative rootlet developmental regulators therefore further confirms the similarity between *I. echinospora* rootlets and the roots of *S. moellendorffii*.

These data – overall expression, comparison of DE genes based on orthology assigned by a reciprocal best blast analysis, 25 putative rootlet regulators and 15 putative rootlet developmental regulators with orthology assigned using orthofinder – indicate that gene expression is similar in *I. echinospora* rootlets and *S. moellendorffii* roots. The degree of similarity is found to be higher between *I. echinospora* rootlets and *S. moellendorffii* roots than leafy shoots.

Table 5 The number of differentially expressed (DE) and uniquely expressed genes in roots and microphylls in each of the 13 PRDRs based on their functional characterisation in rooting systems of other land plants

Arabidopsis gene name	Total Iso	Total Sel	**Isoetes**		**Selaginella**		**Isoetes**		**Selaginella**	
			DE Leaf	DE Root	DE Leaf	DE Root	Unique Leaf	Unique Root	Unique Leaf	Unique Root
LRL	3	5	0	1	0	2	0	1	0	2
RSL	7	5	0	2	1	0	0	2	0	3
GL2	27	1	2	0	0	0	10	1	0	0
GL3	6	1	0	0	0	0	0	0	0	0
WER	1	1	0	0	0	1	0	1	0	0
FEZ	2	4	0	0	0	0	0	0	0	0
SMB	5	4	0	1	0	0	1	2	0	3
WOX	11	5	0	0	0	0	2	1	0	2
AUX	61	58	4	2	10	0	3	0	7	0
LOB	8	12	0	2	0	1	0	2	0	3
PIN	9	6	1	3	1	2	0	2	1	0
PHB	9	3	0	2	2	0	0	1	0	0
SHR	1	3	0	0	0	0	0	1	0	0

Table showing the genes expressed in each of the 13 PRDRs. From left to right the table reports the total number of both *I. echinospora* (Total Iso) and *S. moellendorffii* (total Sel) genes in each monophyletic group. The number of differentially expressed (DE) genes present in leaf tissues (DE Leaf) or root / rootlet tissues (DE Root) for both *I. echinospora* and *S. moellendorffii*. The number of uniquely expressed genes present in leaf tissues (Unique Leaf) or root / rootlet tissues (Unique Root) for both *I. echinospora* and *S. moellendorffii*. Both DE and uniquely expressed genes are colour coded based on their dominant expression profile (orange, roots; green, microphylls; white, none).

4.6 Discussion

The MSH has been the dominant theory in the literature for the evolution of the roots of the Isoetales for the past 30 years (Rothwell & Erwin, 1985; Phillips & DiMichele, 1992; Stewart & Rothwell, 1993; Rothwell *et al.*, 2008, 2014; Taylor *et al.*, 2009; Sanders *et al.*, 2011). It is supported by multiple lines of evidence (Rothwell & Erwin, 1985), but is most contentious in its interpretation of Isoetales rootlets. Although rootlets share great anatomical and developmental similarities with the roots of other lycopsids (reviewed by Hetherington & Dolan, accepted) they are interpreted as modified microphylls. An alternative hypothesis, the LRH, proposes instead that rootlets of the Isoetales are homologous to the roots of other lycopsids. This interpretation is most parsimonious in terms of the number of anatomical character changes and also the types of character evolution (all character evolution necessary for the LRH has occurred multiple times in the roots of other extant plant lineages). Furthermore, this study reports the finding that the rootlets of *I. echinospora* share a high degree of genetic similarity with the roots of *S. moellendorffii*. This offers an additional line of similarity between the rootlets of *I. echinospora* and the roots of *S. moellendorffii*, consistent with the predictions of the LRH.

Regardless of the origin and homology of the rhizomorph of the Isoetales (from which rootlets develop) the results reported in this study find no evidence to interpret rootlets as modified microphylls. In much the same way that leaves and shoots that develop from roots in other extant vascular plants (e.g. in *Platycerium bifurcatum* (Richards *et al.*, 1983)) or in the *Podostemaceae* (Imaichi *et al.*, 1999; Koi & Kato, 2003; Katayama *et al.*, 2010) are not interpreted as 'modified lateral roots' the study finds no evidence to interpret rootlets of Isoetales as 'modified microphylls' due to their attachment to a shoot-like rhizomorph. Instead the high degree of similarity based on anatomy, development and now gene expression data highlights that rootlets of the Isoetales share a

great similarity with other lycopsid roots. Without further lines of evidence supporting the leaf-like characteristics of the rootlets of the Isoetales it is, therefore, most parsimonious to interpret the rootlets as roots, homologous to the roots of other lycopsids.

4.7 Acknowledgements

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4.8 Supplementary Information

Table S1. The taxa and genome / transcriptome versions used in the orthofinder analysis

Species	Genome / Transcriptome version
Arabidopsis thaliana	TAIR10
Chlamydomonas reinhardtii	v5.5
Chondrus crispus	ASM35022v2.31
Cyanidioschyzon merolae	ASM9120v1
Equisetum giganteum	http://bioinformatics.psb.ugent.be/supplementary_data/kenes/Equisetum/
Isoetes drummondii	Josh Mylne (University of Western Australia)
Isoetes echinospora	This study
Klebsormidium flaccidum	V1
Lygodium japonicum	http://bioinf.mind.meiji.ac.jp/kanikusa/index.php
Marchantia polymorpha	V1 (NCBI LVLJ00000000)
Oryza sativa	v7.0
Ostreococcus tauri	v2.0
Phylloglossum drummondii	Josh Mylne (University of Western Australia)
Physcomitrella patens	v3.0
Picea abies	v1.0
Porphyridium purpureum	http://cyanophora.rutgers.edu/porphyridium/ (28th June 2016)
Selaginella moellendorffii	v1.0
Volvox carteri	v2.0

4.9 Electronic Supplementary Information

Electronic supplementary information included with the thesis includes: Table ES1-28, and Figures ES1-28.

Chapter 5: General discussion

The goal of the work described in this thesis was to investigate the evolution and morphology of lycopsid root systems. The thesis was divided into four main aims, based on a review of the literature described in Chapter 1. Each of these four aims and the way that they were achieved are now outlined:

1. Examine root evolution solely in the lycopsids

In order to establish a clear framework for lycophyte root evolution the work described in this thesis investigated root evolution in the lycopsids rather than in total group lycophytes. Lycopsids, unlike earlier diverging lycophytes, are better preserved in the fossil record and are represented by more robust phylogenies. These features are both essential for reconstructing character evolution of roots. Furthermore, investigating root evolution solely in the lycopsids has the advantage that fossils can be interpreted in light of the anatomy and development of roots in extant lycopsids.

2. Identify major trends in lycopsid root evolution from extant species, and test if the roots of extinct lycopsids also display the same patterns

The research in this thesis took a top-down approach to studying lycopsid root evolution (starting by reviewing the literature of rooting structures in extant lycopsids – Chapter 1). This revealed that lycopsid rooting structures displayed two contrasting patterns: conservatism and disparity. The identification of these two trends provided a framework for reinvestigating the fossil evidence for lycopsid rooting structures. The study of roots, the conserved part of the system, has received less attention than the disparate parts in the evolutionary literature. Research in this thesis, concentrated on describing the evolution of lycopsid roots. A better understanding of which is essential for constructing a unifying theory for both the conserved and disparate parts of lycopsid rooting structures.

3. Identify and describe key rooting characters in the fossil record, such as root hairs and root meristems

With the identification of the two major trends of lycopsid rooting structure evolution, conservatism and disparity, the research in the thesis aimed to better understand the structures of the extinct lycopsid roots. The research aimed to discover and characterize two of the defining features of lycopsid roots, namely root hairs and root meristems, in extinct lycopsids. The aim of the work in Chapters 2 and 3 was to identify both of these illusive structures to better characterize the morphology of extinct lycopsid roots. The research described in Chapter 2 reports the discovery of root hairs on the rootlets of the arborescent lycopsids and that stigmarian rootlets had the same branching architecture as the rootlets of extant *Isoetes* species. The results presented in Chapter 2 ended a long-standing mystery about the apparent lack of root hairs on stigmarian rootlets and highlighted the remarkable conservatism found in the rootlets of the Isoetales. These findings were of particular importance as they challenged the hypothesis that stigmarian rootlets were modified microphylls. Instead, the results presented in Chapter 2 provide support for the hypothesis that Isoetalean rootlets are homologous to the roots of all other lycopsids.

Chapter 3 reports the discovery of the first fossilised remains of an actively growing root meristem in the description of *Radix carbonica*. It was not possible to determine if *R. carbonica* was a lycopsid. Indeed, the evidence from its anatomy allied the meristem more closely to extant gymnosperm than lycopsid roots. However, the discovery marks a significant increase in our knowledge of root evolution. The discovery provided the first description of the fossilised remains of a root meristem, containing both fossilised stem and differentiating cells. A comparison of *R. carbonica* with the root meristems of extant plants indicates that the cellular organization, and therefore development, of *R. carbonica*

was unique. This demonstrates that extant root meristem organization and development represents only a subset of the diversity that has existed since roots first evolved. The research in Chapter 3 illustrates how examining fossils can shed light on the evolution of plant development and should encourage more studies at the interface between palaeontology and developmental biology. The results in Chapters 2 and 3 also showed that Carboniferous coal balls contain a wealth of undescribed, exceptionally well preserved root material that has the potential to hugely increase our understanding of root evolution. It is likely that further examinations of coal balls will lead to the discovery of the illusive lycopsid root meristem and further descriptions of lycopsid root hairs.

4. Define a molecular mechanism controlling root development in the genus *Isoetes*

The final aim of the work in this thesis was to define a molecular mechanism controlling root development in the genus *Isoetes*. Then to integrate this molecular data into existing hypotheses concerning lycopsid root evolution. This was accomplished in Chapter 4 by the generation and investigation of rootlet expressed transcripts in the sporophyte transcriptome of *Isoetes echinospora*. The study by Huang & Scheifelbein, (2015) (published during the course of the thesis) produced a dataset of gene expression in the roots of the lycopsid *Selaginella moellendorffii* and this dataset provided a resource to compare gene expression between *I. echinospora* rootlets and *S. moellendorffii* roots. The results reported in Chapter 4 indicate that *I. echinospora* rootlets and *S. moellendorffii* roots have similar gene expression profiles. The similarity in gene expression profiles was taken as a further line of evidence supporting the conservation of lycopsid roots. The results in Chapter 4 demonstrate how transcriptomic data from extant lycopsids can shed new light on hypotheses developed primarily from extinct lycopsids.

As discussed above, the work presented in this thesis had the research goal of increasing our understanding of lycopsid root morphology and evolution. This was achieved by tackling four main aims. By investigating root evolution solely in lycopsids (Aim 1), and taking a top down approach to reviewing the anatomy of lycopsids (Aim 2) two major trends in lycopsid root evolution, conservatism and disparity, were identified. Using this framework the research presented in this thesis aimed to characterise both the anatomy (Aim 3) and genetic basis (Aim 4) of the highly conserved roots of extinct and extant lycopsids. The results presented in this thesis have also helped shape theory more generally about the evolution of the lycopsid rooting structure. This has been achieved through the reinvestigation of the modified shoot hypothesis (MSH), and the reinterpretation of lycopsid root fossils. Next, I will outline the key conclusions from the work and discuss the broader implications of the work described in the thesis

5.1 Isoetalean rootlets are root homologous to all lycopsid roots

A review of the literature (Chapter 1) indicates that the roots of all extant lycopsids have a highly conserved structure. Roots of extant lycopsids have similar morphology and development forming isotomously branched systems covered in root hairs developing from a root meristem covered by a protective cap. From examining extant lycopsid roots it may therefore be expected that all lycopsid roots are homologous. However, the dominant theory in the literature is that lycopsid roots are not homologous. Instead the modified shoot hypothesis (MSH) predicts that that the rootlets of both extant and extinct Isoetales are modified microphylls and are therefore different from all other lycopsid roots. This prediction sits jarringly at odds with the highly conserved structure of extant lycopsid roots

described in Chapter 1. The work presented in Chapters 2 and 4 find no support for the predictions of the MSH but instead highlight the similarity of all lycopsid roots.

Roots of extant lycopsids are unified by the presence of root hairs developing from their epidermal surface. One line of evidence in support of the MSH was the previous interpretation that rootlets of extinct Isoetales (stigmarian rootlets) lacked root hairs. My discovery of root hairs on stigmarian rootlets (Chapter 2), instead provided a further line of evidence consistent with the hypothesis that all lycopsid roots are homologous. By contrast, if rootlets were modified microphylls it might have been expected that rootlets would develop leaf epidermal characters such as stomata. In fact, the founder of the MSH, Schimper (1872), described extinct stigmarian rootlets as lacking stomata, implying that stomata might develop on rootlets. The finding of root hairs on stigmarian rootlets provided an additional line of evidence demonstrating the shared anatomy of both living and extinct Isoetalean rootlets and the roots of all lycopsids. Furthermore, the results presented in Chapter 4 found additional evidence supporting the hypothesis that all lycopsid root are homologous. Chapter 4 reports a high degree of similarity between the gene expression profiles of rootlets of *Isoetes echinospora* and the roots of the related lycopsid *Selaginella moellendorffii*. The evidence from gene expression data provided additional support for the hypothesis that rootlets and roots are homologous. These findings led to a re-examination of the literature supporting the MSH (that rootlets are modified microphylls). The review of the literature in Chapter 4 concluded that it is most parsimonious, based on both the number (only 3 character transitions compared to 8) and types of character transitions required, to interpret Isoetalean rootlets as roots, homologous to the roots of other lycopsids (LRH). The similarities of rootlets with the roots of other lycopsids led to the dominant interpretation in the early 20th century (Scott, 1900; Bower, 1908; Weiss, 1933), that stigmarian rootlets were similar to roots of all other lycopsids.

The research described in this thesis therefore moves the interpretation back to the view held in early 20th century.

To summarize, the discovery of root hairs on the stigmarian rootlets (Chapter 2), and the genetic similarity between the rootlets of *I. echinospora* and roots of *S. moellendorffii* (Chapter 4), alongside a reinvestigation of the literature supporting the MSH in Chapter 4, call into question the interpretation that Isoetalean rootlets as modified microphylls. Instead these three lines of evidence are taken as support for the homology of all lycopsids (which is termed in this thesis the lycopsid root hypothesis (LRH)).

5.2 Extinct Lycopsid rooting structure evolution: conservatism and disparity

An additional significant finding presented in this thesis is the identification of two main evolutionary patterns of extant lycopsid rooting structures: conservatism and disparity. Combining the new evidence presented in this thesis with a review of the literature indicates that extinct lycopsids also show these two patterns of rooting structure evolution.

5.2.1 Conservatism: root structure is highly conserved in extinct lycopsids

I discovered root hairs and the highly conserved branching architecture of the roots of the Isoetales (Chapter 2). In this respect Isoetalean rootlets are morphologically similar to the roots of other lycopsids. Isoetalean roots therefore show a remarkable degree of conservatism and we therefore investigated if this was a similar pattern for all lycopsid roots. Roots branch isotomously in the majority of extinct lycopsids, and were structurally similar to the roots of extant lycopsids. The earliest diverging, now extinct group of lycopsids – Drepanophycales (Kenrick & Crane, 1997) (Fig. 1.02) – includes a number of well-described fossil genera such as *Drepanophycus*, *Asteroxylon* and the recently described Cottonwood Canyon lycophyte from the Devonian period with preserved

isotomous branching axes (Fig. 5.01a–d) (Kidston & Lang, 1920b; Schweitzer, 1980; Schweitzer & Giesen, 1980; Rayner, 1984; Li & Edwards, 1995; Gensel *et al.*, 2001; Matsunaga & Tomescu, 2016). Where fossil preservation permits it is clear that these axes lacked a cuticle and were therefore likely to be functional roots (Kidston & Lang, 1920b, 1921; Bhutta, 1969; Matsunaga & Tomescu, 2016). Therefore, the earliest diverging groups of extinct lycopsids, like their extant relatives, developed terminal appendages, that formed strictly isotomously branched systems specialized for water uptake.

This isotomously branched root architecture was conserved in more derived groups of lycopsids despite the dramatic radiation in morphological diversity that occurred during the Devonian period (Stewart & Rothwell, 1993; Kenrick & Crane, 1997). Isotomously branched roots are found in Middle and Late Devonian lycopsids such as *Chamaedendron multisporangiatum* and *Longostachys latisporophyllus* (Fig. 5.01e,f) (Cai & Chen, 1996; Schweitzer & Li, 1996). Furthermore dichotomously branched roots (often termed rootlets) are present in the anatomically diverse fossil members of the Isoetales (*sensu* (DiMichele & Bateman, 1996)) (Fig. 1.02). The earliest diverging members of the Isoetales (DiMichele & Bateman, 1996; Xue, 2011); (Fig. 1.02) such as *Oxroadia gracilis* and *O. conferta* (Long, 1986; Bateman, 1992), *Paurodendron fraiponti* (Phillips & Leisman, 1966) (Fig. 5.01g) and *Longostachys latisporophyllus* (Cai & Chen, 1996) (Fig. 5.01f) developed isotomously branched roots. Isotomously branched roots (Williamson, 1887) were also conserved in the giant relatives of these extinct plants, the giant 50-m lycopsid trees which were dominant components of the Carboniferous wetland coal forests. I discovered that the roots of the giant tree lycopsids developed root hairs (Fig. 5.01h) and the same root architecture as extant Isoetes (Chapter 2) (Hetherington *et al.*, 2016a). This root architecture is conserved in all geological periods – from Devonian through to today (Kidston & Lang, 1920b, 1921; Foster & Gifford, 1959; Phillips &

Leisman, 1966; Bhutta, 1969; Stewart & Rothwell, 1993; Hetherington *et al.*, 2016a; Matsunaga & Tomescu, 2016). Furthermore, these roots develop root hairs (Hetherington *et al.*, 2016a; McLoughlin & Bomfleur, 2016). Taken together, the data from both extinct and extant lycopsids suggest that a conserved developmental programme operated, and continues to operate, during the development of the roots of all lycopsids. This conservatism is remarkable because the aboveground shoot and reproductive structures of the lycopsids diversified extensively over this same timescale (Stewart & Rothwell, 1993).

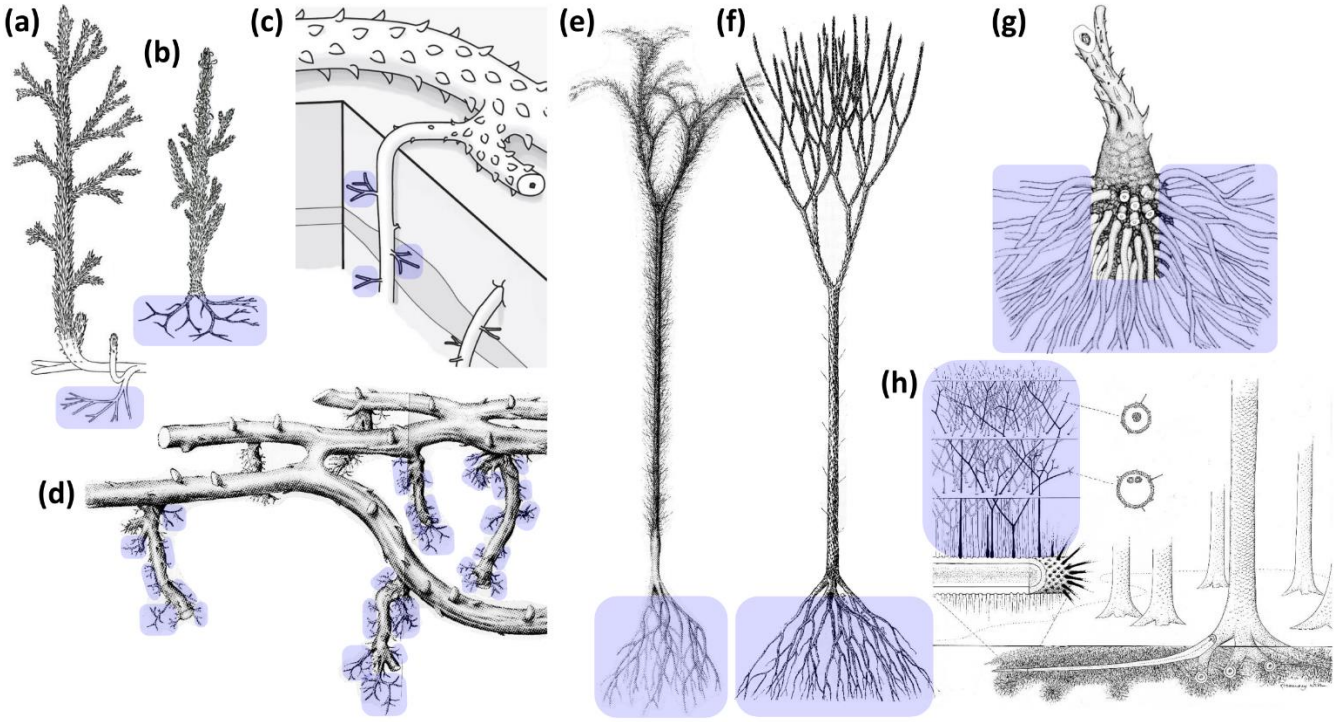

Figure 5.01 Conservatism of roots in extinct lycopsids. Conserved isotomously branched roots (highlighted with blue shading) develop from anatomically disparate root-bearing organs in Palaeozoic lycopsids. Isotomously branching roots develop in *Asteroxylon mackiei* (a, b), reconstruction based on Kidston & Lang, 1921 (a) and Bhutta 1969 (b); Cottonwood Canyon lycophyte (c), reconstruction based on Matsunaga & Tomescu, 2016; *Depanophycus devonicus* (d), reconstruction based on Schweitzer & Giesen, 1980; *Chamaedendron multisporangiatum* (e), reconstruction based on Schweitzer & Li, 1996; *Longostachys latisporophyllus* (f); reconstruction based on (Cai & Chen, 1996); *Paurodendron fraiponti* (g), reconstruction based on (Phillips & Leisman, 1966); tree lycopsids (h), reconstruction based on Hetherington *et al.*, 2016a. In all cases isotomously branched roots develop from disparate structures of the extinct lycopsids.

5.2.2 Disparity: disparity of root producing structures in extinct lycopsids

The roots of the Drepanophycales (earliest diverging fossil group of the lycopsids (Kenrick & Crane, 1997) (Fig. 1.02) develop from a range of disparate structures, as described in the living lycopsids above. Roots developed directly from leafy (Rayner, 1984; Li & Edwards, 1995; Gensel *et al.*, 2001) or leafless (Rayner, 1984) shoot systems in *Drepanophycus spinaeformis* and *Drepanophycus qujingensis*. However, roots were also attached to positively gravitropic shoot-like axes which grow from leafy shoot axes into the sediment in *Drepanophycus spinaeformis*; *Drepanophycus devonicus* and the Cottonwood Canyon lycophyte (Schweitzer, 1980; Schweitzer & Giesen, 1980; Matsunaga & Tomescu, 2016) (Fig. 5.01c,d). *Asteroxylon mackiei* roots (referred to by Kidston & Lang, 1920, 1921 and Bhutta, 1969 as rhizomes) develop from the region termed the transition zone which connects the leafy shoot with the roots and shows a gradual transition in anatomy between the two (Kidston & Lang, 1920b, 1921; Bhutta, 1969) (Fig. 5.01a,b). These observations indicate that roots were attached to diverse shoot and shoot derived structures in the earliest diverging groups of lycopsids. The only unifying feature among these structures is that they all have shoot-like characteristics. Some may be leafy shoots (Rayner, 1984; Li & Edwards, 1995; Li *et al.*, 2000; Gensel *et al.*, 2001) and others appear to be transitional structures between shoots and roots – lacking microphylls but with cuticle, stomata and scale leaves (Kidston & Lang, 1920b, 1921; Bhutta, 1969; Schweitzer, 1980; Schweitzer & Giesen, 1980; Matsunaga & Tomescu, 2016) (Fig. 5.01a–d). From these diverse structure in the Drepanophycales root-bearing organs further increased in diversity. This can be seen most clearly in the Isoetales where the rhizomorph displays a variety of forms from the small rhizomorph of *Oxroadia* (Long, 1986; Bateman, 1992) and *Paurodendron* (Phillips & Leisman, 1966; Rothwell & Erwin, 1985) (Fig. 5.01g); cormose rhizomorphs of groups such as the *Chaloneriaceae* (Pigg & Rothwell, 1983a,b); *Pleuromeiaceae* (Grauvogel-

Stamm, 1993) and *Isoetaceae* (Paolillo, 1963; Retallack, 1997) to the giant rhizomorph axes known as stigmarian axes (Stewart & Rothwell, 1993) which extended up to 15 m and occasionally further from the trunks of large trees (Fig. 5.01h). In summary, a diversity of organs and meristems gave rise to roots in fossil taxa as in living lycopsids.

The rooting structures of both extant (reviewed in Chapter 1) and extinct (reviewed above) lycopsids display striking conservatism and disparity. Recent research, including the results presented in this thesis, indicates that strictly dichotomising roots are ancient and have been conserved in lycopsids for over 400 million years. By contrast, the organs that gave rise to roots have diversified greatly over this same time period. The theory that describes the conserved and therefore homologous nature of all lycopsid roots (including Isoetalean rootlets) is termed in this thesis the lycopsid root hypothesis (LRH). The LRH interprets all lycopsid roots as homologous, rejecting the interpretation that the rootlets of the Isoetales are modified microphylls. Although further anatomical work on extinct Lycopsis roots is required (including the description of a fossil lycopsid root meristem) to verify the LRH, the theory predicts that all lycopsid roots will be anatomically highly conserved. Furthermore, given this highly conserved anatomy, it is likely that the mechanism controlling development is also conserved and this can be tested further using comparative developmental genetic approaches with extant taxa (as described in Chapter 4). Examination of rooting structures of both extant and extinct lycopsids also recognised a set of highly disparate structures to which roots attach. The origin and evolution of these disparate root-bearing structures is still largely unclear and it will require a combination of developmental genetics (Kawai *et al.*, 2010), paleo-developmental biology (Rothwell *et al.*, 2014; Hetherington *et al.*, 2016b; Tomescu, 2016) and the analysis of character-trait evolution of extinct taxa to better characterize these structures in the future. In summary, the rooting structures of both extinct and extant lycopsids are divided into two contrasting

parts: highly conserved roots and disparate root-bearing structures. Identifying these two separate lines provides a framework to develop further questions about lycopsid root evolution. The LRH predicts that all highly conserved roots will develop from root meristems (a prediction which can be tested with fossils in the future (as demonstrated in Chapter 2)), and that conserved Lycopsid roots should have a similar underlying genetic toolkit (which can also be tested in a similar fashion as demonstrated in Chapter 4). In contrast to the highly conserved morphology and evolution of roots, this investigation identifies a range of disparate structures to which roots attach. The origin and evolution of these disparate structures is unclear, however a goal of future work will be to try and better characterize their morphology and evolution. A better understanding of both the conserved and disparate parts of lycopsid rooting structures will allow a more complete theory to be formed that can account for the two contrasting patterns of rooting structure evolution: conservatism and disparity.

5.3 Conclusion

The evolution of roots is widely recognized as a key event that fundamentally changed the Earth system. Roots helped in the development of deep soils, transformed hydrological systems, and increased the weathering of silicate rocks—the dominant sink for atmospheric CO_2 in the long-term carbon cycle (Algeo & Scheckler, 1998; Berner, 1998; Berner & Kothavala, 2001; Bergman *et al.*, 2004; Gibling & Davies, 2012; Jasechko *et al.*, 2013; Gibling *et al.*, 2013). The acquisition of roots provided plants with anchorage and the capacity for high rates of nutrient and water uptake essential for the evolution of trees and the first extensive forest ecosystems (Raven & Edwards, 2001). The lycophytes were the first group of plants to evolve roots and the study of their morphology and evolution

has been a major goal for morphologists and evolutionary scientists working on both extant and extinct lycopsids. In the research described in this thesis the evidence for rooting structures in extant lycopsids was reviewed (Chapters 1). Two contrasting patterns of rooting structure evolution were revealed: conservatism and disparity. The conserved part of the rooting system has been given less attention in the evolutionary literature and further characterizing these structures was a main aim of the work in this thesis. The discovery of the first root hairs on stigmarian rootlets (Chapter 2), and the comparison of the gene expression similarities between extant lycopsids (Chapter 4) both indicated the similarity of Isoetalean rootlets with the roots of other lycopsids. These two lines of evidence challenged the MSH. The results presented in this thesis propose instead that all lycopsid roots are homologous (described by the LRH) and have a highly conserved structure. Finally, alongside the discoveries made of Isoetalean rootlets was the discovery of *Radix carbonica* (Chapter 3) the oldest fossilised remains of an actively growing root meristem. Although it was not possible to establish if the fossil named *R. carbonica* was a lycopsid the finding demonstrates the possibility that the rock record contains examples of fossilised root meristems. The results described here demonstrate how interdisciplinary studies combining evidence of both anatomy and development of extant and extinct lycopsid roots will be essential to continue to increase our understanding of both the conserved and disparate parts of lycopsid root structures.

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Appendices

Appendix 1 Catarino B, Hetherington AJ, Emms DM, Kelly S, Dolan L. 2016. The stepwise increase in the number of transcription factor families in the Precambrian predated the diversification of plants on land. Molecular Biology and Evolution. doi:10.1093/molbev/msw155

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The Stepwise Increase in the Number of Transcription Factor Families in the Precambrian Predated the Diversification of Plants On Land

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Abstract

The colonization of the land by streptophytes and their subsequent radiation is a major event in Earth history. We report a stepwise increase in the number of transcription factor (TF) families and subfamilies in Archaeplastida before the colonization of the land. The subsequent increase in TF number on land was through duplication within existing TF families and subfamilies. Almost all subfamilies of the Homeodomain (HD) and basic Helix-Loop-Helix (bHLH) had evolved before the radiation of extant land plant lineages from a common ancestor. We demonstrate that the evolution of these TF families independently followed similar trends in both plants and metazoans; almost all extant HD and bHLH subfamilies were present in the first land plants and in the last common ancestor of bilaterians. These findings reveal that the majority of innovation in plant and metazoan TF families occurred in the Precambrian before the Phanerozoic radiation of land plants and metazoans.

Key words: **transcription factors, plant evolution, Homeodomain, bHLH.**

Introduction

Complex multicellular plants and metazoans diversified at the taxonomic, morphological, and genetic levels during the Phanerozoic eon (541 million years ago to present) (Kenrick and Crane 1997a, b; Carroll 2001; Powell and Kowalewski 2002; Erwin et al. 2011; Droser and Gehling 2015). Transcription factors (TFs) control a plethora of developmental mechanisms in eukaryotic organisms that appear during the course of evolutionary radiations and transitions (Menand et al. 2007; de Mendoza et al. 2013; Holland 2013). The role of TF diversification around one of these major transitions—the colonization of the land by streptophyte plants—is not understood (Mukherjee et al. 2009; Pires and Dolan 2010). The move to land by plants was paralleled by a major body plan transition; the evolution of complex 3D tissues and key morphological characters crucial for life on land such as the epidermis, rooting and shooting systems, stomata, and water conducting tissues that are associated with the function of characteristic TFs (Kenrick and Crane 1997a, b; Graham et al. 2000; Menand et al. 2007; Pillitteri et al. 2007; MacAlister and Bergmann 2011; De Rybel et al. 2013; Pires et al. 2013; Xu et al. 2014; Tam et al. 2015; Sakakibara 2016). The recent availability of the genome sequence of an aquatic streptophyte alga from a clade that is sister to the land plants (Wickett et al. 2014), *Klebsormidium flaccidum* (Hori et al. 2014), and the liverwort, one of the earliest divergent lineages of land plants (Kenrick and Crane 1997a, b; Wickett et al. 2014), *Marchantia polymorpha* (NCBI GenBank accession LVLJ00000000.1) allowed us to track the change in the number and size of TF families and subfamilies during streptophyte evolution. Here, we demonstrate that the origin of the majority of TF families and subfamilies predate the radiation of extant land plant lineages.

Results and Discussion

47 of the 48 Land Plant TF Families Evolved before the Colonisation of the Land

We identified 48 (see Supplementary Material online) TF families in the genomes of 15 species of Archaeplastida (the lineage that includes red algae, chlorophytes, and streptophytes) (Adl et al. 2005; Archibald 2009). These taxa comprise the red algae *Cyanidioschyzon merolae*, *Porphyridium purpureum*, *Chondrus crispus*; the chlorophytes *Ostreococcus tauri*, *Micromonas pusilla*, *Chlorella variabilis*, *Coccomyxa subellipsoidea*, *Volvox carteri*, and *Chlamydomonas reinhardtii*; the streptophyte alga *K. flaccidum* and the streptophyte land plants *M. polymorpha*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Oryza sativa*, and *Arabidopsis thaliana* (see supplementary table 1, Supplementary Material online). TF genes were defined using established rules for the identification and categorisation of TFs on the basis of their domain architecture (Finn et al. 2014; Jin et al. 2014, Supplementary Material online). The number of TF families that are present at each internal node in the phylogeny was inferred using parsimony. This revealed that the number of TF families increased progressively from node 1 to node 6 (fig. 1). There were 17 TF families in the common ancestor of all species in this analysis (node 1, fig. 1) and 28 in the common ancestor of chlorophytes and streptophytes (node 2, fig. 1). 39 TF families were present in the aquatic

Letter

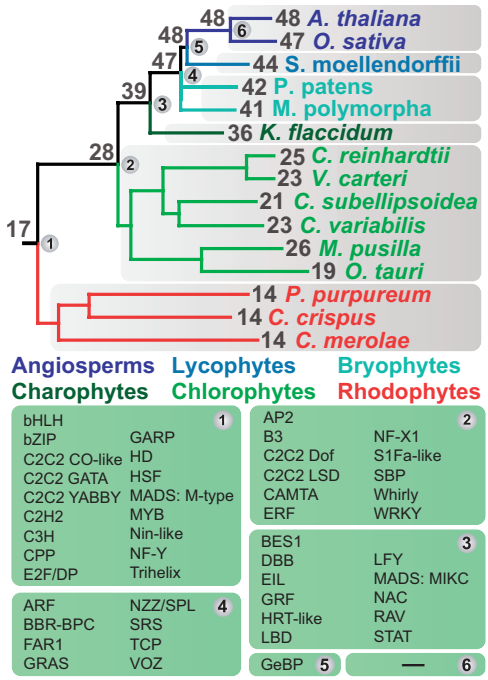

Fig. 1. Distribution of TF families in plants. Cladogram of Archaeplastida phylogeny (based on Finet et al. 2010; Cox et al. 2014; Wickett et al. 2014) with the origin of the TF families (within green boxes) and total number of TF families (grey) present at each ancestral node (circled numbers) and in extant species (coloured according to clade).

common ancestor of *K. flaccidum* and land plants (node 3, fig. 1) and 47 TF families had evolved in the first land plants (node 4, fig. 1). The stepwise origin of TF families in the aquatic ancestors of land plants—from 17 TFs in the last common ancestor of all species at node 1 to 47 in the first land plants—contrasts strikingly with the subsequent evolution of TF families on land. During the radiation of plants in the terrestrial realm TF number increased from 47 to 48; only one TF family, GeBP (node 5, fig. 1), evolved after the divergence of bryophytes and vascular plants (fig. 1). This indicates that there was a stepwise increase in the number of TF families in Archaeplastida before the radiation of land plants and since that time there has been relatively little change in TF family number (fig. 1).

Land plants radiated both taxonomically and morphologically after the colonization of the land (Kenrick and Crane 1997a, b; Bateman et al. 1998; Nickrent et al. 2000; Qiu et al. 2006; Finet et al. 2010; Wickett et al. 2014). However, this analysis revealed that this radiation was not accompanied by an increase in the number of novel TF families (fig. 1). Instead, the number of TFs within each family increased as land plants evolved after the colonisation of the land (Mukherjee et al. 2009; Pires and Dolan 2010; de Mendoza et al. 2013). It is possible that TF evolution was accompanied by the origin of new subfamilies during land plant evolution. To test the hypothesis that the origin of novel TF subfamilies paralleled the radiation of plants on land a gene tree analysis was conducted on two of the major pan-eukaryotic TF families (included in the 48 TF families examined above), Homeodomain (HD) and basic Helix-Loop-Helix (bHLH) (Nam and Nei 2005; Simionato et al. 2007; Degnan et al. 2009; Mukherjee et al. 2009; Pires and Dolan 2010; Holland 2013; Gyoja 2014; Sakakibara 2016).

13 of the 14 Subfamilies of HD Transcription Factors Are Present in Streptophyte Algae

Phylogenetic analysis of Archaeplastida HD proteins (fig. 2A, supplementary fig. S1, Supplementary Material online) resolved the 14 monophyletic subfamilies previously reported by Mukherjee et al. (2009). 11 of the 14 subfamilies are supported by Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-like aLRT) values over 0.85 (supplementary fig. S1, Supplementary Material online). There are 26 HD TFs in 13 subfamilies in the genome of the streptophyte algae *K. flaccidum* (fig. 2A, supplementary table 2, Supplementary Material online); NDX is the only land plant subfamily that is not present in the *K. flaccidum* genome. This indicates that at least 13 HD subfamilies had originated in an aquatic streptophyte ancestor before colonisation of the land. Phylogenetic analysis of the 19 *M. polymorpha* HD TFs indicates that all 14 HD subfamilies previously described in land plants are present in this early diverging land plant. Furthermore, there is a single gene in the majority of *K. flaccidum* and *M. polymorpha* subfamilies, whereas there are multiple genes in the same subfamilies of angiosperms (supplementary table 2, Supplementary Material online). The majority (13/14) of TF subfamilies was also present before the colonization of the land, as described above in the analysis of all TF families. Thus the colonization and subsequent diversification of plants on land was not accompanied by the substantial evolution of new HD subfamilies.

26 of the 30 Land Plant Basic Helix-Loop-Helix Subfamilies Were Present in the First Land Plants

The analysis of the gene tree of Archaeplastida bHLH genes indicated that the major subfamilies had evolved in the common ancestor of land plants as observed with HD subfamilies. This analysis revealed that there is a single bHLH protein in the red alga, *C. merolae* and 3 bHLH proteins encoded in the genomes of the chlorophytes *C. reinhardtii and V. carteri* (supplementary table 3, Supplementary Material online). However, none of the 30 streptophyte bHLH subfamilies were found in either red algae or chlorophytes (fig. 2B). There are 10 *K. flaccidum* bHLH TFs in 6 subfamilies all of which were previously described in land plants—IVb, IVc, Vb, VII(a + b), XI, and XIII. The genome of the liverwort *M. polymorpha* encodes at least 49 bHLH proteins which comprise 25 of the 30 plant bHLH subfamilies (fig. 2B, supplementary table 3, Supplementary Material online). The *M. polymorpha* sequences, together with the sequences of *P. patens*, *S. moellendorffii*, *O. Sativa*, and *A. thaliana*, defined four new monophyletic bHLH subfamilies: IVd(2), VIIIc(3), XVI, and XVII. 23 of the 30 bHLH subfamilies are monophyletic clades supported by SH-like aLRT values greater than 0.85 (supplementary fig. S2, Supplementary Material online). The numbers of bHLH subfamilies in the earliest divergent land plants (*M. polymorpha* and *P. patens*) allow us to infer that 26 bHLH subfamilies were present in the first land plants.

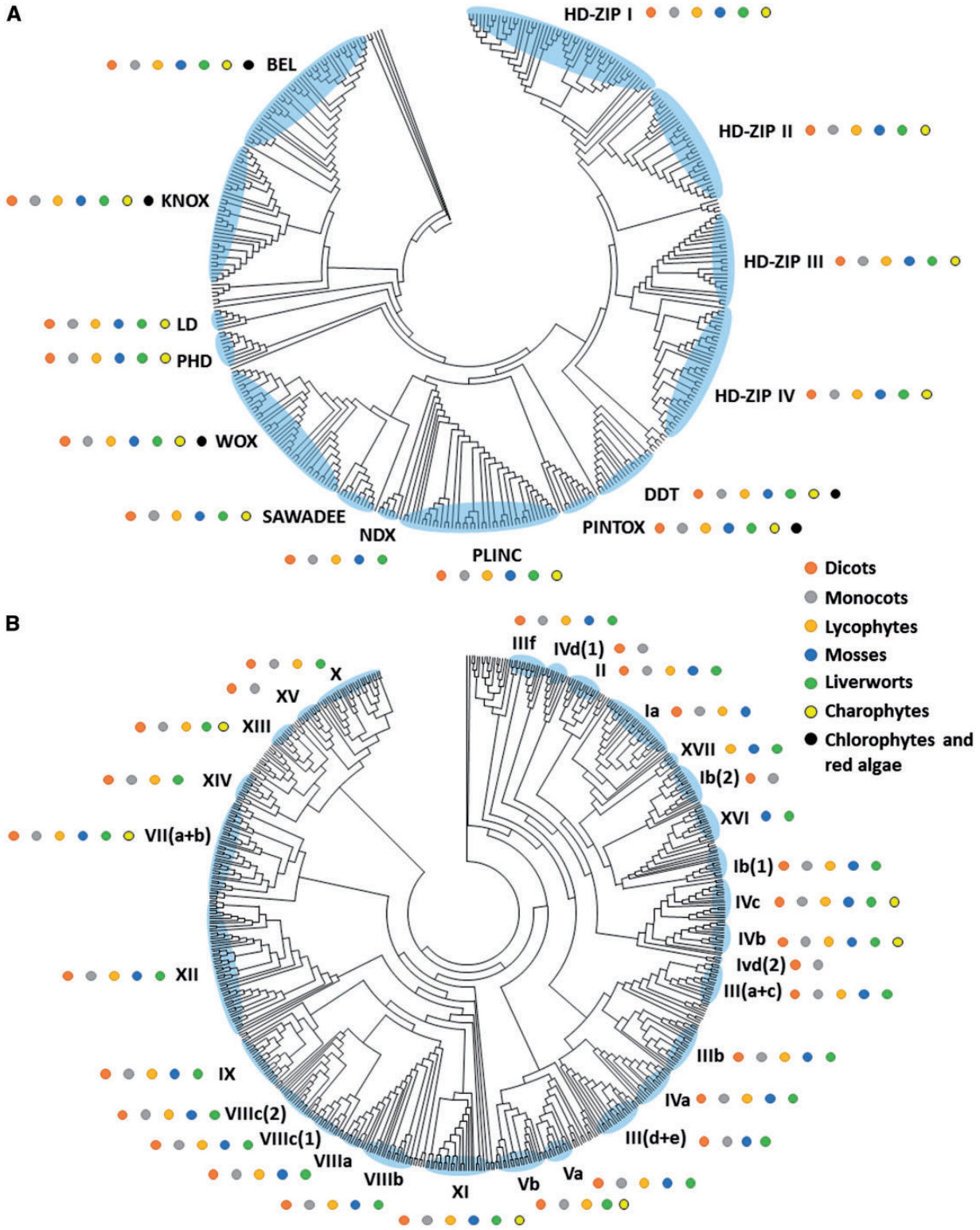

Fig. 2. Phylogenetic analysis of HD and bHLH Archaeplastida TFs. Circular cladograms representing the Maximum Likelihood (ML) analysis of Archaeplastida HD (*A*) and bHLH (*B*) TF proteins. The trees are unrooted ML generated with the software PhyML 3.0, (Guindon et al. 2010) using the JTT model of amino acid substitution, an estimated gamma distribution parameter and an SH-like aLRT test. Subfamiles are grouped with blue ellipses. Coloured dots represent protein presence in each subfamily. *A. thaliana* (orange); *O. sativa* (grey); *S. moellendorffii* (gold); *P. patens* (blue); *M. polymorpha* (green); *K. flaccidum* (yellow) and Chlorophytes- *C. reinhardtii*; *O. tauri*; *V. carteri* (black). See supplementary figs. S1 and S2, Supplementary Material online for fully annotated trees.

These data indicate that some new bHLH subfamilies evolved while other subfamilies were lost during the course of land plant evolution. Subfamilies VIIIc(3) and XVI are present only in bryophytes (liverworts and moss) suggesting that subfamilies VIIIc(3) and XVI were either lost before the origin of vascular plants or that they evolved independently in the case that bryophyte constitute a monophyletic clade (Cox et al. 2014; Wickett et al. 2014). There are only nonseed plant proteins in subfamily XVII. The most parsimonious interpretation of this result is that subfamily XVII was lost in the lineage giving rise to the seed plants after the divergence of the lycophytes from the seed plants (fig. 2*B*, supplementary table 3, Supplementary Material online). Moreover, subfamilies Ib(2), IVd(1), IVd(2), and XV are present in angiosperms only (fig. 2*B*, supplementary table 3, Supplementary Material online), suggesting that these four subfamilies evolved in the lineage leading to the angiosperms after the divergence of lycophytes and seed plants. These data suggest that in contrast to HD subfamilies evolution, there were losses and origins of new bHLH subfamilies during the evolution of plants on land. The presence of 6 bHLH subfamilies in *K. flaccidum* compared with 26 bHLH subfamilies in bryophytes (*M. polymorpha* and *P. patens*) suggests that the majority of bHLH subfamilies originated around the time that plants colonised the land, or at very least after the time when *K. flaccidum* and early diverging land plants last shared a common ancestor.

Precambrian Evolution of the Majority of the HD and bHLH Subfamilies in Plants and Metazoans

To independently assess if morphological diversification was predated by the evolution of the majority of TF subfamilies in another eukaryotic radiation we compared the evolution of both HD and bHLH families in metazoans (Larroux et al. 2008; Degnan et al. 2009; Ryan et al. 2010). Strikingly, in metazoans, like plants, there was an early origin followed by a stepwise increase of the majority of HD and bHLH subfamilies before the radiation of bilaterians (fig. 3). This observation indicates that the evolution of the majority of HD and bHLH subfamilies in the Precambrian predated the major morphological diversification in both plants (radiation of land plants) and metazoans (radiation of bilaterians) in the Phanerozoic (Kenrick and Crane 1997a, b; Clarke et al. 2011; Erwin et al. 2011; Parfrey et al. 2011).

We demonstrate that there was a stepwise increase in the number of TF families during the course of Archaeplastida evolution before the colonization of the land by plants. The majority of plant TF families had already evolved by the time the first plants colonized the land. This stepwise increase in the number of families before the radiation of land plants is paralleled by a similar stepwise increase in the number of subfamilies in two of the major pan-eukaryotic TF families (HD and bHLH). Taken together, these data demonstrate that there was a gradual increase in the number of TF families and subfamilies before the radiation of plants on land. This was followed by a relatively small increase in the number of TF families and subfamilies during and after the land plant radiation. Therefore, the morphological radiation of land plants was accompanied by an increase in number of TF proteins within these highly conserved TF subfamilies. Similarly metazoan TFs originated in the Precambrian before the bilaterian radiation. This suggests that TF diversification in the Precambrian preceded two major radiations in organismal diversity in distant branches of the tree of life.

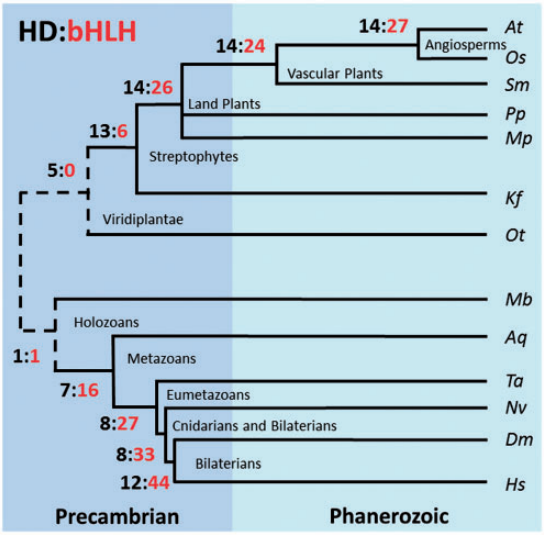

Fig. 3. A Precambrian origin the majority of HD and bHLH TF subfamilies. The number of HD (black) and bHLH (red) subfamilies present at each node of a simplified time calibrated phylogeny of animals and Archaeplastida. Subfamily number for Archaeplastida based on the current study and for animals based on Larroux et al. (2008), Degnan et al. (2009), Ryan et al. (2010). Phylogenetic tree for Archaeplastida based on Cox et al. (2014) and Wickett et al. (2014), time calibrations for land plants based on Clarke et al. (2011), time calibration for the origin of streptophytes based on Parfrey et al. (2011). Phylogeny and time calibration of animals based on Erwin et al. (2011). Dashed lines lack time calibrations. Abbreviated taxa names are *At, A. thaliana*; *Os, O. sativa*; *Sm, S. moellendorffii*; *Pp, P. patens*; *Mp, M. polymorpha*; *Kf, K. flaccidum*; *Ot, O. tauri*; *Aq, Amphimedon queenslandica*; *Nv, Nematostella vectensis*; *Sp, Strongylocentrotus purpuratus*; *Hs, Homo sapiens*; *Dm, Drosophila melanogaster*; *Lg, Lottia gigantea*.

Materials and Methods are described in Supplementary Material online.

Supplementary Material

Supplementary material, tables S1–S3 and figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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Appendix 2 Hetherington AJ, Berry CM, Dolan L. 2016a. Networks of highly branched stigmarian rootlets developed on the first giant trees. Proceedings of the National Academy of Sciences 113: 6695–6700.

Networks of highly branched stigmarian rootlets developed on the first giant trees

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Lycophyte trees, up to 50 m in height, were the tallest in the Carboniferous coal swamp forests. The similarity in their shoot and root morphology led to the hypothesis that their rooting (stigmarian) systems were modified leafy shoot systems, distinct from the roots of all other plants. Each consists of a branching main axis covered on all sides by lateral structures in a phyllotactic arrangement; unbranched microphylls developed from shoot axes, and largely unbranched stigmarian rootlets developed from rhizomorphs axes. Here, we reexamined the morphology of extinct stigmarian systems preserved as compression fossils and in coal balls from the Carboniferous period. Contrary to the long-standing view of stigmarian systems, where shoot-like rhizomorph axes developed largely unbranched, root-hairless rootlets, here we report that stigmarian rootlets were highly branched, developed at a density of ∼25,600 terminal rootlets per meter of rhizomorph, and were covered in root hairs. Furthermore, we show that this architecture is conserved among their only extant relatives, herbaceous plants in the *Isoetes* genus. Therefore, despite the difference in stature and the time that has elapsed, we conclude that both extant and extinct rhizomorphic lycopsids have the same rootlet system architecture.

evolution | paleobotany | Carboniferous forests | stigmarian root systems | *Isoetes*

The spread of the first wetland forests with tall trees during the Carboniferous period (359–300 million years ago) had a dramatic impact on the carbon cycle by burying large amounts of organic carbon in the form of peat in coal swamps (1, 2). Lycophyte trees up to 50 m in height (3, 4) were dominant components of coal swamp forests (5, 6). They were key components of coal-forming environments throughout the Carboniferous period but dominated in the lower–middle Pennsylvanian (Namurian–Wetsphalian) where they typically contribute between 60% and 95% of the biomass in buried peat (7–13). The preserved remains of lycophyte trees form some of the most extensive fossil plant deposits of any geological period. This is in part because of their size and ecological dominance but also the result of the high probability of preservation in the waterlogged conditions in which these trees grew (4). Detailed descriptions of the morphology of these plants on a range of scales—from entire in situ tree lycophyte forests (14, 15) to cellular descriptions of developing spores (16)—have made these trees some of the best understood fossil plants of the Carboniferous coal swamps.

The rooting system of the arborescent lycopsids—stigmarian systems—consist of large shoot-like axes (rhizomorphs) that develop lateral organs called rootlets (4, 17–20). Rootlets, which have been described as largely unbranched and root hairless (4, 5, 17–24), are arranged in a characteristic pattern or rhizotaxy on the rhizomorph (25). It is the arrangement of these largely unbranched leaf-like rootlets on a shoot-like axis that first led to the theory that stigmarian systems were modified leafy shoots (26–28). The modified shoot hypothesis got more support toward the end of the 20th century. The discovery of fossilized embryos showing that the shoot and root axes were derived from a branching event during embryogenesis (16, 29), the documentation that rootlet abscission resembled foliar abscission (17, 18), observations on well-preserved fossil rhizomorph apices (30–32), and their interpretation within a phylogenetic context (31, 33) led to a complete revival of Schimper's (27) modified shoot hypothesis.

The only living relatives of these Carboniferous giant trees are small herbaceous plants in the genus *Isoetes* (24, 33–35). The rooting system of *Isoetes* also consists of a rhizomorph meristem that develops rootlets in a regular rhizotaxy (31, 36, 37). Aside from the reduction and modification of the rhizomorph, the rooting systems of *Isoetes* and the tree lycopsids are morphologically similar (4, 19, 21, 31, 38–40). However, current models suggest that rootlet architecture is different in extant *Isoetes* and extinct stigmarian rootlets. *Isoetes* rootlets form dense, highly branched networks of rootlets covered in root hairs (41, 42), whereas stigmarian rootlets are thought to be largely unbranched and root hairless (4, 5, 17–24). This difference is even more puzzling because the cellular anatomy of stigmarian rootlets and *Isoetes* rootlets is almost identical (21, 38).

Given that the architecture of the stigmarian rootlet systems differs markedly from rooting systems of their extant relatives, we hypothesized that the rootlet architecture of the stigmarian rooting system may have previously been misinterpreted. Here, we report the discovery of the complex structure of stigmarian rootlet systems from quantitative analysis of rootlet branching and multiple lines of geological evidence. The proposed model reveals that the highly branched rootlet architecture has been conserved over the past 300 million years and is found in the closest living relatives of arborescent lycophytes.

Results

To compare rootlet architecture of the stigmarian systems with their extant relatives, we first defined quantitatively rootlet branching in *Isoetes echinospora* Durieu and *Isoetes histrix* Bory

Significance

Coal swamps were the carbon burial factories of the Carboniferous period, forming huge coal deposits and driving climate cooling. The Carboniferous forests were also home to the first giant (>50 m) trees to grow on the planet. These trees were anchored by a unique structure termed a stigmarian system, which is hypothesized to represent a leafy shoot modified to function as a root. Here, we report the discovery of the complex, highly branched rootlet structure of these trees. Our findings demonstrate that rootlet architecture is conserved from the giant extinct trees of the Carboniferous to the small extant herbs of today's flora.

Author contributions: A.J.H. and L.D. designed research; A.J.H. performed research; A.J.H. analyzed data; A.J.H. and L.D. wrote the paper; and C.M.B. provided the first coal ball peels that were found to contain root hairs on stigmarian rootlets and provided advice on fossil material throughout the project.

The authors declare no conflict of interest.

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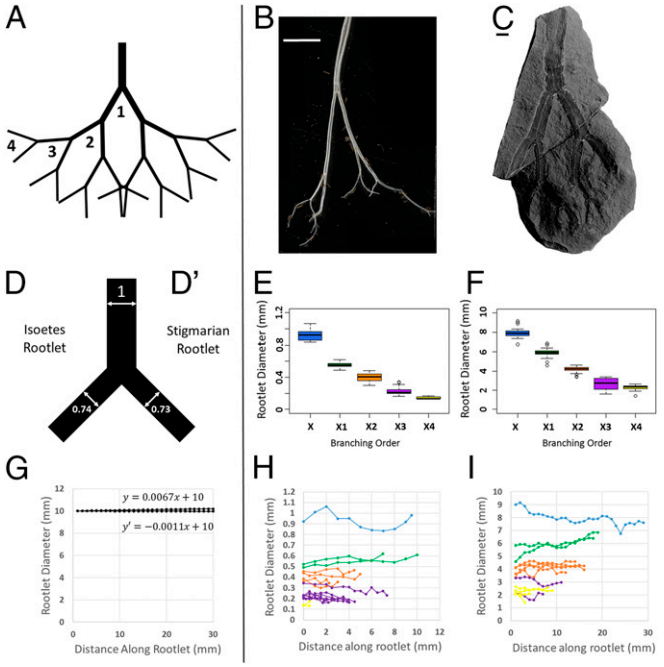

Fig. 1. Stigmarian and *Isoetes* rootlets do not taper but branch dichotomously, decreasing in diameter in a stepwise manner through multiple orders of branching. (*A*) Cartoon of branched rootlet showing four orders of dichotomous branching with branching orders labeled. (*B*) *Isoetes* and (*C*) stigmarian rootlets branch in a strictly dichotomous manner through multiple orders of branching. (Scale bar: 5 mm.) After a branching event, daughter rootlets have an average diameter 74% of the parent rootlet in *Isoetes* (*D*) and 73% of the parent rootlet in stigmaria (*D'*). *Isoetes* (*E*) and stigmarian (*F*) rootlet diameter decreases in a stepwise manner over four orders of branching (X–X4). (*G*) Average gradients of the diameter of *Isoetes* (*y'*) and stigmarian (*y*) rootlet segments indicate that rootlets do not taper between branch points. *Isoetes* (*H*) and stigmarian (*I*) rootlets do not taper but decrease in diameter in a stepwise manner through multiple orders of branching: first-order branch (blue), second-order branch (green), third-order branch (orange), fourth-order branch (purple), and fifth-order branch (yellow). Stigmarian rootlet (*C*) reproduced by permission of the British Geological Survey, Asset number: 687585 (CP15/032). Photograph taken and fossil determined by Robert Kidston in 1912. Collection locality, Ilkeston (United Kingdom).

(Fig. S1). *Isoetes* rootlets branch dichotomously along their length, and rootlet diameter decreases by ~25% at each dichotomy (Fig. 1 *A*, *B*, *D*, *E*, and *H*); the average diameter of the rootlet that develops from the rhizomorph is 0.73 mm (SD, ±0.21 mm; SE, ±0.02 mm), and the average rootlet diameter of the fifth-order branch is 0.21 mm (SD, ±0.04 mm; SE, ±0.002 mm) after four rounds of dichotomous branching (X4 in Fig. 1*E*). Rootlet diameter does not decrease between branch points, i.e., the branches do not taper (Fig. 1 *G* and *H*). These data indicate that *Isoetes* rootlets are highly branched—there are up to five orders of branching on each rootlet—and decrease in diameter by ~25% at each dichotomy but do not taper.

To test the hypothesis that stigmarian rootlets formed branched networks like *Isoetes*, we characterized the branching morphology of rootlets preserved as compression fossils in Carboniferous sediments (*Supporting Information* and Fig. S2). We found rootlets with up to four orders of branching (Fig. 1*C*). Furthermore, rootlet diameter decreased by ~25% with each order of branching (Fig. 1 *D*, *F*, and *I*) with no evidence of tapering (Fig. 1 *G* and *I*). Together, these data from rootlets preserved as compression fossils demonstrate that stigmarian rootlets were branched and rootlet diameter decreased by ~25% at each branch point and did not taper. This indicates that the pattern of rootlet branching is similar in extinct stigmarian and extant *Isoetes* rootlets.

To test independently the hypothesis that stigmarian rootlets were highly branched, we modeled the predicted frequency of rootlet diameters sampled from thin sections of coal balls (Fig. S3). Coal balls are permineralized peat from the coal swamps in which the anatomical and cellular detail of growing stigmarian rootlets are preserved in situ (4, 43–45). If rootlets branched dichotomously (Fig. 2*A*), as we observed in the compression fossils described above (Fig. 1*C*), we hypothesized that there would be more thin rootlets than thick rootlets in coal ball-preserved stigmarian systems (Fig. 2*C*, red). This is because of the geometric increase in the number of progressively smaller terminal rootlets in the dichotomously branching stigmarian rootlet system. However, if rootlets were relatively unbranched, as the long-standing model suggests, and therefore decreased in diameter by tapering (Fig. 2*B*), we would expect to observe equal numbers of small- and large-diameter rootlets in a sample of roots preserved in coal balls (Fig. 2*C*, blue). Our model therefore allows us to determine whether stigmarian rootlets preserved in coal balls were unbranched or branched.

We measured the diameter of 785 stigmarian rootlets preserved in 94 coal ball thin sections (*Supporting Information*)

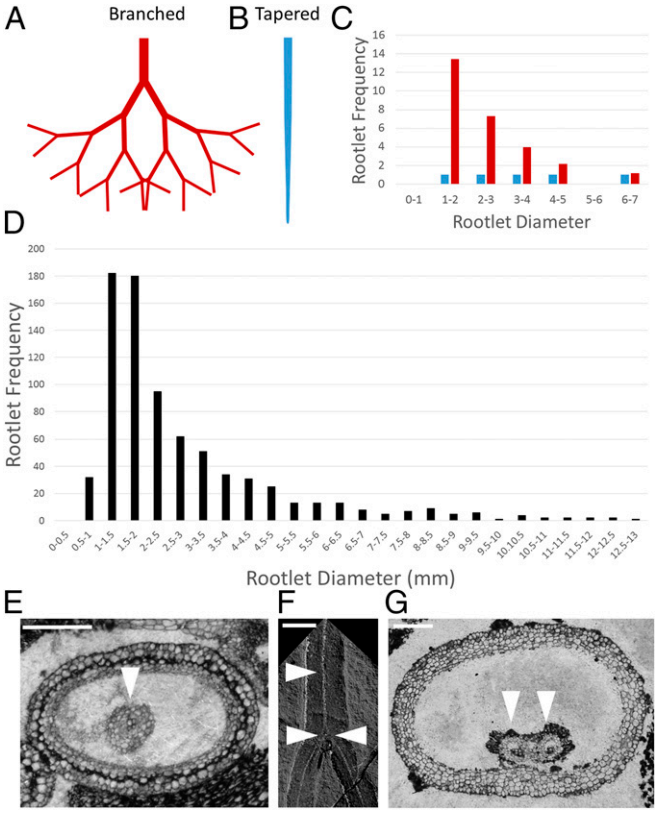

Fig. 2. Measurement of 785 stigmarian rootlets preserved in coal balls indicates that branching was common. Modeling the predicted frequency of rootlet diameters in coal balls based on a branched (*A*) and tapered (*B*) rootlets reveals that the two rootlet types have characteristically different frequency distributions (*C*; red, branched model; blue, tapered model). (*D*) Frequency histogram of the diameters of 785 measured stigmarian rootlets preserved in coal balls—note the similarity between the predicted branched-rootlet model (red) and the measured results (*D*). (*E*) Stigmarian rootlets have a single vascular strand (vascular strand indicated by white arrowheads in *E*–*G*) except where it bifurcates just before a branching point (*F*). Twin vascular strands (*G*) were found in 51 rootlets, indicating that rootlets in the coal balls branched frequently. (Scale bars: *E* and *G*, 0.5 mm; *F*, 5 mm.) Thin sections slide 54 (*E*) and slide 25 (*G*) by permission of Oxford University Herbaria; photograph taken by A.J.H. (*F*) Magnified image of Fig. 1*C*; reproduced by permission of the British Geological Survey, Asset number: 687585 (CP15/032). Photograph taken and fossil determined by Robert Kidston in 1912. Collection locality, Ilkeston (United Kingdom).

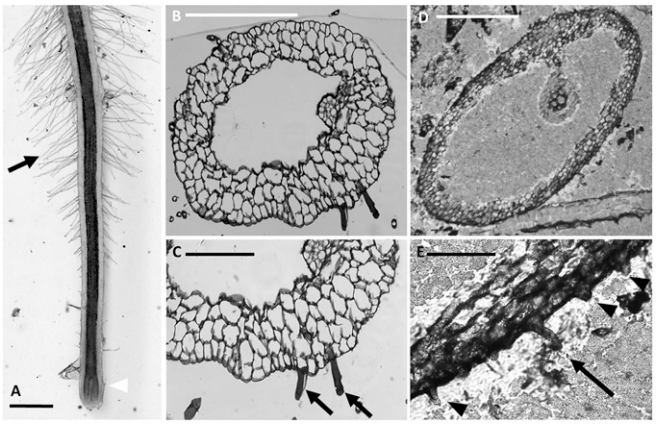

Fig. 3. Stigmarian rootlets possessed root hairs. (*A*) *Isoetes* rootlets develop root hairs (black arrows in *A*–*E*) (note the *Isoetes* rootlet is in the process of branching at the apex—white arrowhead). (*B*–*E*) Transverse sections of *Isoetes* (*B* and enlarged region of *B* shown in *C*) and stigmarian (*D*, enlarged region of *D* shown in *E*) rootlets showing root hair outgrowths from the epidermis. Black arrowheads indicate the base of root hairs that have been broken off. (Scale bars: *A* and *D*, 500 μm; *B*, 200 μm; *C* and *E*, 100 μm.) (*D* and *E*) Stigmarian rootlet reproduced by kind permission of Amgueddfa Cymru – National Museum Wales [NMW 2016.9G.1]; photograph taken by A.J.H.

(Fig. S4). Rootlet diameter was then calculated, and the frequency of rootlets in each 0.5-mm-diameter class was calculated (Fig. 2*D*). These data demonstrate that there are many more small-diameter rootlets than large-diameter rootlets, which supports the dichotomous branching rootlet model. To ensure that the distribution of rootlet diameter is not due to variation in local growth conditions we plotted separately the data from 16 individual thin sections with more than 15 stigmarian rootlets and thin sections collected from a variety of collection sites in Central Britain [Yorkshire and Lancashire coal fields (46)] (Figs. S5 and S6). In all cases, the same frequency distribution was observed—there were more (Figs. S5 and S6) small-diameter rootlets than large-diameter rootlets. This demonstrates that the relationship between rootlet diameter and frequency does not vary from place to place or in different samples. This is consistent with the model in which the stigmarian rootlets are highly branched, and this branching pattern did not vary from site to site or from sample to sample.

To determine whether young, developing rootlets near the rhizomorphs apex could have contributed to the large numbers of thin rootlets in our sample, we measured the diameters of the earliest stages of rootlet development on two preserved apices (Fig. S7 *A* and *C*). Mean rootlet scar diameter within the first 10 cm from the rhizomorph apex was 3.48 mm (SD, ±0.95). The measurements of the rootlet scars on these apices are similar to those observed on other stigmarian apices (30, 32). The diameter of these young rootlets were more than twice the diameter of the most abundant, small-diameter rootlets that we observed in coal balls (Fig. 2*D*). This demonstrates that the youngest developing rootlets near the apex of the rhizomorph could not account for the large numbers of thin rootlets observed in coal balls. Instead, it supports the hypothesis that the thin rootlets were formed through progressive rounds of dichotomous branching of large rootlets.

To verify independently that stigmarian rootlets branched, we searched the coal ball thin sections for rootlets in which there were two vascular strands. Stigmarian rootlets have a single vascular strand (4, 17, 21, 22) (Fig. 3*E*, white arrowhead); however, above a point of branching, the vascular strand locally bifurcates (Fig. 2*F*, white arrowheads). Finding rootlets in coal balls containing two vascular strands therefore indicates that rootlets in coal balls branched (Fig. 2*G*, white arrowheads). Of the 785 samples observed, twin vascular strands were found in 51 rootlets (Fig. 2*G*, white arrowheads), demonstrating that these sections were made just above a branch point (Fig. S8). Of these, the circumference of 42 rootlets could be measured. Twin vascular strands were observed in rootlets with diameters ranging from 1.1 to 12.8 mm, indicating that rootlets of all size classes branched (Fig. S8). The frequency distribution of the diameters from the 42 rootlets further supported the branched-rootlet model (Fig. S8). The observed peak frequency diameter of branching rootlets was 2–2.5 mm (Fig. S8), indicating that the smallest and most frequent rootlets undergoing dichotomous branching were in the 2- to 2.5-mm-diameter range. We previously showed that daughter rootlet diameter is ∼73% the diameter of the rootlet from which they formed. According to this measure, the peak diameter that we would expect to see produced from the branching of 2- to 2.5-mm-diameter rootlets would be in the 1.5- to 1.8-mm-diameter range. The peak frequency rootlet diameter found in all rootlets examined being between 1 and 2 mm (Fig. 2*D*). Taken together, diameter frequency distributions of rootlets preserved in permineralized coal balls demonstrate that stigmarian rootlets were highly branched.

Root hairs have not previously been found on stigmarian rootlets and their absence led to the suggestion that root hairs did not develop in these plants (5, 20, 22, 23). However, because root hairs develop on *Isoetes* rootlets (41, 42, 47) (Fig. 3 *A*–*C*, black arrows), we hypothesized that root hairs would have formed on stigmarian rootlets. A total of 21 root hairs were discovered on nine stigmarian rootlets on seven individual thin sections made from different coal balls (Fig. 3 *D* and *E*; *Supporting Information* and Fig. S9). Mean stigmarian root hair diameter was 14.3 μm (SD, ±2.6 μm; SE, ±0.56 μm), and the root hair highlighted with an arrow in Fig. 3*E* was 13.9 μm in diameter. Root hair diameter of the two *Isoetes* root hairs shown in Fig. 3*C* were 9.2 and 10.7 μm. These data indicate that root hairs developed on stigmarian rootlets and that they were morphologically similar to the root hairs that develop on extant *Isoetes* species.

The lycopsid trees of the British Carboniferous wetland forests comprised both sigillarian and nonsigillarian species (7, 33, 46). To determine whether the rootlet branching pattern was the same in each, we scored the presence of rootlets with twin vascular strands and determined the distribution of rootlet diameters in both sigillarian and nonsigillarian rootlets. There is a "connective" of cortical tissue between the vascular trace and the outer cortex in sigillarian rootlets (4, 17, 18, 21, 48) (Fig. S10*A*). By contrast, there is no connective in the central cavity of the nonsigillarian rootlet and the central vascular trace is free within the rootlet cavity (4, 17, 21) (Fig. S10*B*). First, we identified twin vascular stands in both sigillarian and nonsigillarian rootlets, which indicates both sigillarian and nonsigillarian rootlets in the coal balls sampled were branching (*Supporting Information*). Second, the frequency distribution of rootlet diameters is similar for sigillarian and nonsigillarian rootlets (Fig. S10*C*) (*Supporting Information*). These data indicate that both sigillarian and nonsigillarian rootlets branched three to four times (*Supporting Information*). Furthermore, root hairs are present on both sigillarian and nonsigillarian rootlet types (*Supporting Information*). We conclude that both sigillarian and nonsigillarian rootlets formed similar bifurcating rootlets systems to those found in *Isoetes* today.

Using the quantitative data from this analysis, we constructed a model for the stigmarian system (Fig. 4). Because rootlets developed at densities of ∼1,600 rootlets per m of rhizomorph (25) (this study) and we assumed that each rootlet branched at least four times (this study), we calculated a density of 25,600 terminal rootlets per m of rhizomorph with a surface area 5.5 times larger than unbranched rootlet systems (assuming that living root hairs are present only on the terminal two orders of branching) (*Methods*). This model shows a stigmarian system

with a densely packed cylinder of interwoven rootlets around the rhizomorph axes (Fig. 4).

Discussion

We demonstrate that the rootlets of stigmarian systems were highly branched—branching dichotomously up to five times—and were covered in root hairs. We verified the highly branched architecture through quantitative analysis of the numbers and diameters of stigmarian rootlets preserved in coal balls. Analysis of the size distribution of stigmarian rootlets in coal balls provided us with the unique opportunity to investigate the entire population of stigmarian rootlets growing in situ regardless of either the diameter of the rootlet or the proximity of the rootlet to the rhizomorph axis. This analysis was possible because stigmarian rootlets are ubiquitous in coal balls (49, 50), and can be readily identified because of their unique cellular anatomy composed of three zones of cortex, the middle of which rapidly disintegrates, leading to the formation of a large air space containing the inner cortex and central vascular strand (17, 21). This anatomical detail allows stigmarian rootlets to be easily distinguished from the rooting structures of other plants that grew in the coal swamps (Fig. S4). Furthermore, the exquisite cellular preservation of these in situ fossils allowed the visualization of root hairs developing from the rootlet epidermal surface for the first time (to our knowledge). Such an extensive branched system would have formed a subterranean network with a large surface area available for nutrient uptake and tethering these giant trees in place.

We suggest that the previous model for stigmarian rooting systems was incomplete because it was based on compression fossils in which the full extent of the rootlet network was obscured. Furthermore, isolated stigmarian rootlets preserved in compression fossils have few features distinguishing them at this coarse level of preservation, making them difficult to identify (51). Therefore, isolated branched rootlets have not contributed to the construction of the long-standing model of stigmarian rootlet architecture. This means that previous interpretations of stigmarian systems were biased; reconstructions were based on the proximal portions of the rootlets where they attach to the rhizomorphs and could be identified unequivocally. However, because rootlets can extend for over 90 cm from the rhizomorph surface (52–55), this bias means that the morphology of the distal branched regions of the rootlets remained undescribed. Through quantitative analysis of rootlet architecture in both compression and in situ-preserved permineralized fossils, we have been able to demonstrate that stigmarian rootlets were highly branched.

Highly branched rootlets would have contributed to the anchoring of these giant trees. Branched root structures are between twice and seven times more resistant to pull-out compared with unbranched structures (56–58) and the discovery of root hairs would not only have increased the surface area but would have further contributed to anchorage (59). The tree lycophytes would have formed large root plates as individual rhizomorph axes could extend for over 12 m (19) from the trunks of large trees. Given that tree lycopsids have additionally been reported to grow at high densities [up to 1,769 stems per ha (15)] in coal swamp forests (14, 60), root plates would have also interlocked with neighboring stigmarian systems. Highly branched rootlets would have further consolidated these extensive root plates (Fig. 4). It is the ability of root plates to resist movement when the aerial parts of the tree are subjected to lateral force that provides structural support to tall trees (61). We predict that highly branched stigmarian rootlets would have contributed to the anchorage of these giant trees.

The first giant wetland trees to grow on Earth with their unique stigmarian rooting systems have attracted the attention of scientists for well over 150 y (4, 17–19, 21, 31, 52, 62). Recent studies have built on this foundation of knowledge and have shed fresh light on physiological mechanism controlling their development, structure, and interaction with other organisms (63–66). The discovery

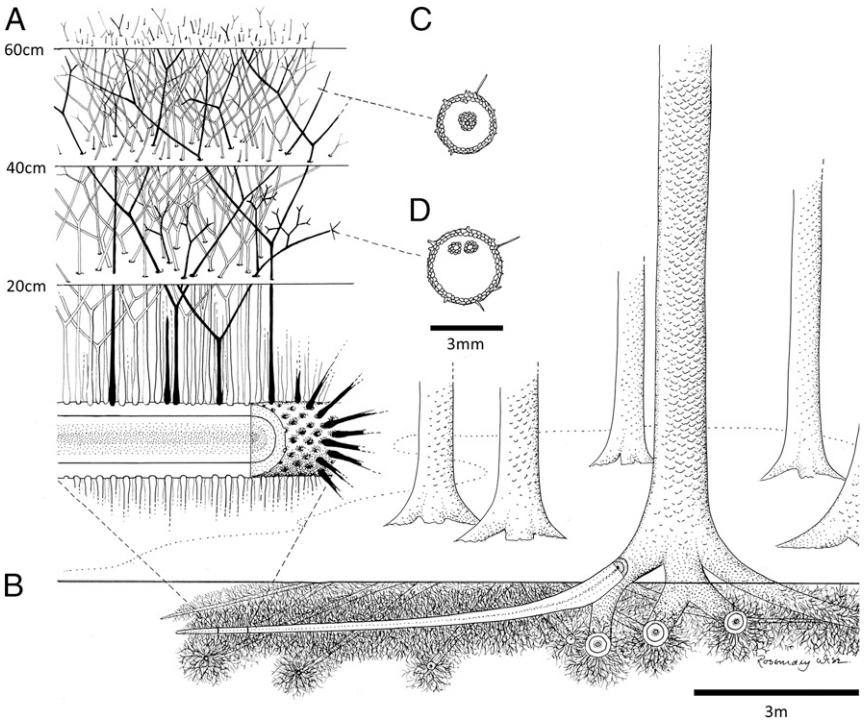

Fig. 4. Reconstruction of stigmarian root systems with highly branched systems of rootlets. (*A*) Reconstruction of a population of branching rootlets that could grow to over 90 cm in length. (*B*) Reconstruction of the root plate that comprised rhizomorph axes covered with rootlets producing ∼25,600 terminal rootlets per m. (*C* and *D*) Rootlets were covered in root hairs. (*C*) The single vascular strand characteristic of stigmarian rootlets bifurcated at each branch point (*D*), and the diameter of the two daughter branches produced was ∼25% that of the parent rootlet.

that stigmarian rootlets were highly branched, developed root hairs and share the same branching architecture as extant *Isoetes* rootlets reveals a remarkable conservatism in rootlet architecture between the first giant trees and their only living herbaceous relatives.

Methods

***Isoetes* Collection and Plant Growth.** *Isoetes histrix* was collected in March 2014 on the Lizard Peninsula (Cornwall, UK) with the permission of the National Trust and Natural England. *Isoetes echinospora* was collected in September 2013 and 2014 from North West Sutherland (Scotland, UK) with the permission of the John Muir Trust and the Scourie Estate. *Isoetes histrix* plants were grown in Levington M2 compost. *Isoetes echinospora* were grown submerged in aquaria in Levington M2 compost topped with coarse gravel. Both were grown at 20 °C with a 16-h photoperiod.

Quantifying *Isoetes* and Stigmarian Rootlet Architecture. *Isoetes* rootlets were imaged with a Leica M165 FC (Fig. 1*B* and Fig. S1 *A–I*). *Isoetes* rootlet diameter was measured using Fiji (67) (Fig. 1*H* and Fig. S1 *A′–I′*). SD and SE were calculated using Microsoft Excel 2013. Graphs were plotted using Microsoft Excel 2013. Box plots were made in RStudio (2013) (68). To establish whether rootlets taper, the diameter of 167 rootlet segments (only including segments covered by five or more diameter measurements) were plotted against distance along their respective rootlets. A linear trend line was then applied to each data series allowing the gradient of each trend line to be calculated (Microsoft Excel 2013). An average gradient of $y' = -0.0011x$ (Fig. 1*G*) was calculated from the 167 rootlet segments. Decrease in rootlet diameter at each branching point was calculated by comparing the average diameter of 227 daughter rootlets with the average diameter of their parent rootlet. Daughter rootlets had an average diameter of 74% of their parent rootlet. Stigmarian rootlet architecture was quantified using the same method described above for *Isoetes* rootlets (Fig. 1*C* and Fig. S2 *A–D*). The same method was used to investigate whether stigmarian rootlets tapered; this time, the average rootlet gradient was calculated from 40 rootlet segments giving an average rootlet gradient of $y = 0.0067x$ (Fig. 1*G*). Average decrease at each branching point was calculated in a similar fashion to *Isoetes* using the measurements of 36 daughter rootlet diameters compared with their parent rootlets. Daughter rootlets had an average diameter of 73% of their parent rootlet.

Modeling the Predicted Frequency of Rootlet Diameters in Coal Balls. The model is based on the principle that the length of a rootlet segment is equal to the frequency of finding that segment in a random rootlet sample from coal balls.
The branched rootlet (Fig. 2A and Fig. S3A). The branched rootlet model undergoes four rounds of dichotomous branching, resulting in 16 terminal rootlets. At each dichotomy, the diameter of the daughter rootlet is 0.73 that of the parent rootlet (based on the measurements made in this study). After a bifurcation point, the daughter rootlet segment is 0.92 the length of the previous segment. This value is based on measurements of 96 *Isoetes* daughter rootlet segments compared with their parent rootlet segments (only using rootlet segments that started and terminated with a branching point to avoid the bias of using rootlets that had not finished growing or had been broken off).
The tapered rootlet (Fig. 2B and Fig. S3B). The tapered rootlet is made up of five segments of equal length. Each segment is 0.73 the diameter of the previous segment, such that the size decrease (tapering) between the branched rootlet and the tapered rootlet is the same.
The model (Fig. S3C). To compare between the two types of rootlets an initial starting diameter (*D*) and a combined length of the five segments (*L*) was assumed for both rootlets. From this, it is possible to calculate the length (frequency) of finding a particular diameter (*D*) of rootlet segment. To determine a realistic value for diameter (*D*), a starting diameter of 6 mm (4, 12, 17, 20) was used (Fig. 2*C*). After four orders of decreasing in diameter by 0.74, this results in a terminal rootlet diameter of 1.7 mm, a value similar to the terminal rootlets of the compression fossil (Fig. 1*C*) and approaching the smallest sizes of isolated stigmarian rootlets previously reported from coal balls (48) interpreted as coming from distal portions of stigmarian systems (18).

Measuring the Diameter of Stigmarian Rootlets from Coal Balls. Thin sections prepared from Carboniferous coal balls held in the Oxford University Herbaria (97 slides) and Oxford University Museum of Natural History (42 slides) were inspected, and stigmarian rootlets were identified. All of the available slides were used rather than only those that were made to display stigmarian systems, to take an unbiased approach. Images were captured of 785 rootlets from 94 thin sections with a Leica M165 FC stereo microscope. The circumference of 785 rootlets was measured using Fiji (67). The rootlets were grouped into 0.5-mm size bins and plotted on a histogram (Fig. 3*C*) (Microsoft Excel 2013).

Measurement of Rootlet Scar Diameter on Rhizomorph Apices. Two rhizomorph apices were photographed by A.J.H. in the collections of The University of Manchester, Manchester Museum. The diameter of 36 rootlet scars were measured from the well-preserved apex (Collection No. LL. 15952.470; Fig. S7*A*), and 12 rootlet scars we measured form the poorly preserved apex (Collection No. LL. 15952.471; Fig. S7*C*) using Fiji (67) (Fig. S8 *A* and *B*). Average rootlet scar diameter was plotted for each 2-cm interval from the apex (Fig. S7 *A′* and *B′*) (Microsoft Excel 2013).

***Isoetes* and Stigmarian Root Hairs.** The distal portion of a single *Isoetes echinospora* rootlet was imaged using a Leica M165 FC stereo microscope (Fig. 3*A*). Additional rootlets were embedded in paraffin, sectioned, and stained with toluidine blue. Slides were imaged with an Olympus BX50 compound microscope using bright field (Fig. 3 *B* and *C*). Stigmarian root hairs were imaged with an Olympus BX50, and root hair diameter was measured with Fiji (67).

Estimating Surface Area Increase. To estimate the increase in surface area due to branching, we again used the simplified rootlet models (branched and unbranched; Fig. 3 *A* and *B*, and Fig. S3 *A–C*). Rootlets were assumed to be cylindrical and the surface area of each segment of rootlet was calculated with $SA = \pi dh$. The presence of branching results in the branched-rootlet model having a surface area 3.9 times larger than the tapered model. Next, we included an estimate of the increased surface area provided by root hairs. Dittmer (69) estimated that the surface area of the rye (*Secale cerale* L.) root systems was 6,875.4 ft^{-2}, with root axes contributing 2,554.09 ft^{-2} and the root hairs contributing 4,321.31 ft^{-2}. We therefore assumed that an axis with root hairs has a surface area 1.7 times that of the same axis lacking root hairs. The Dittmer (69) estimate is applicable for the stigmarian system as 50% of the epidermal cells in *Isoetes* form root hairs (70) as they do in rye (71). As a conservative estimate, we did not take into account root hairs on the top three branching orders (where they may have been sloughed off in the soil) but estimated that root hairs would contribute an additional 1.7 times the surface area over the final two orders of branching. This resulted in our branched-root model having a surface area 5.5 times that of the tapered model (4, 17, 20–22).

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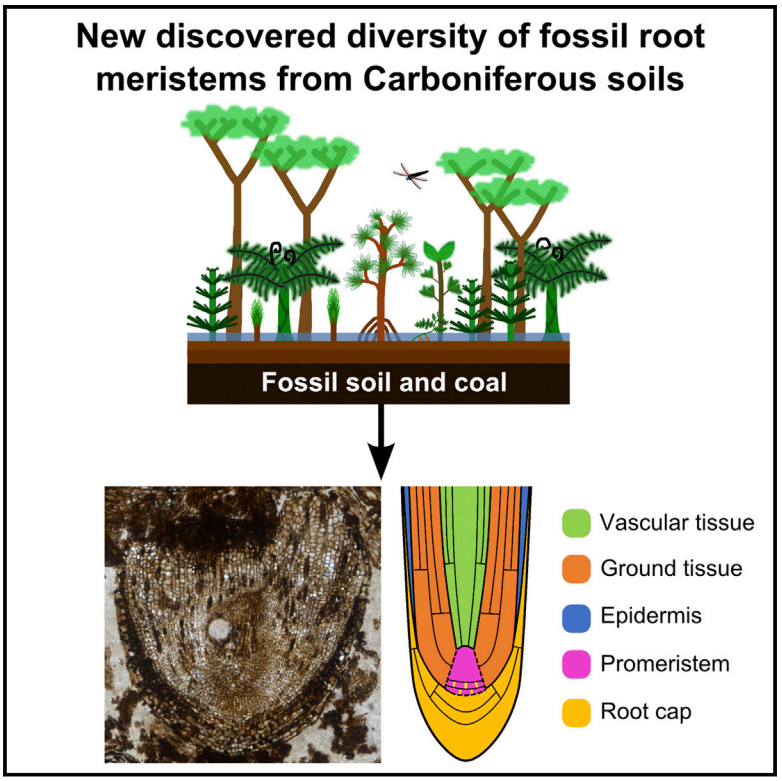
Appendix 3 Hetherington AJ, Dubrovsky JG, Dolan L. 2016b. Unique cellular organization in the oldest root meristem. Current Biology 26: 1629–1633.

Report

Current Biology

Unique Cellular Organization in the Oldest Root Meristem

Graphical Abstract



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In Brief

Hetherington et al. report the discovery of the oldest fossilized remains of an actively growing root meristem from Carboniferous (>300-million-year-old) soil. The cellular organization of stem cells and differentiating cells is unique. This discovery reveals new but now extinct meristem diversity in Carboniferous plants.

Highlights

- The oldest fossilized root meristem is described from >300-million-year-old soil
- The discovery allows the first description of a fossilized root stem cell niche
- The cellular organization and therefore development of the meristem is unique
- The discovery reveals previously unknown diversity in plant meristem types

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CellPress

Current Biology
Report

Unique Cellular Organization in the Oldest Root Meristem

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SUMMARY

Roots and shoots of plant bodies develop from meristems—cell populations that self-renew and produce cells that undergo differentiation—located at the apices of axes [1].The oldest preserved root apices in which cellular anatomy can be imaged are found in nodules of permineralized fossil soils called coal balls [2], which formed in the Carboniferous coal swamp forests over 300 million years ago [3–9]. However, no fossil root apices described to date were actively growing at the time of preservation [3–10]. Because the cellular organization of meristems changes when root growth stops, it has been impossible to compare cellular dynamics as stem cells transition to differentiated cells in extinct and extant taxa [11]. We predicted that meristems of actively growing roots would be preserved in coal balls. Here we report the discovery of the first fossilized remains of an actively growing root meristem from permineralized Carboniferous soil with detail of the stem cells and differentiating cells preserved. The cellular organization of the meristem is unique. The position of the Körper-Kappe boundary, discrete root cap, and presence of many anticlinal cell divisions within a broad promeristem distinguish it from all other known root meristems. This discovery is important because it demonstrates that the same general cellular dynamics are conserved between the oldest extinct and extant root meristems. However, its unique cellular organization demonstrates that extant root meristem organization and development represents only a subset of the diversity that has existed since roots first evolved.

RESULTS AND DISCUSSION

To characterize cellular development in the oldest root apices [3–7], we inspected 139 thin sections made from Carboniferous coal balls from Britain (see Supplemental Information). We identified two new apices (Figures 1A and 1C). The presence of root caps covering each demonstrated that they were root apices. The first apex was the tip of a differentiated, non-growing root (Figure 1A). It was designated *Apex 76.1* and tentatively assigned to *Lyginopteris oldhamia* on the basis of cellular organization (Figures 1A and 1B [3]; see Supplemental Information). Finding *Apex 76.1* validated our search for root meristems in this coal ball material. The second apex (Figure 1C) was larger, blunt, and represents an entirely new root apex type; it was named *Radix carbonica* (see Supplemental Information for systematic paleobotany and comparisons with other fossil apices).

The cellular organization of *Apex 76.1* and *R. carbonica* can be compared with root meristems of extant species, because both thin sections are near median longitudinal in orientation. However, meristem organization of extant plants can be investigated only in actively growing roots, because meristem structure changes when root growth stops [11]. It is therefore essential to establish if the root apices were fossilized during active growth. In roots that have stopped growing, differentiated tissues, including thickened xylem cells, are found very close to the promeristem as in the fossil meristems of *Apex 76.1*, *Lyginopteris*, *Amyelon*, and *Psaronius*, (Figures 1A and 1B) [3, 4, 6, 7, 9], a feature not found in actively growing roots [11]. By contrast, there were no distinguishable tissue types within the ground tissues or differentiated vascular cells found near the tip of *R. carbonica* (Figure 1C).

There is clear zonation of cell sizes in active root meristems of growing roots; meristematic cells are relatively small and vary in size by ~2-fold as dividing cells go through the cell cycle. Then cells expand as they differentiate. Consequently, there is a gradient from the smaller cells of the promeristem to the larger cells in the differentiating tissues. In contrast, there is no cell size gradient in inactive meristems where cell size abruptly increases from the small inactive initials, which abut much larger differentiated cells close to the apex (Figures 2A, 2B, and 2D).The distribution of cell areas in the differentiating ground tissues of *R. carbonica* indicated that there was a gradual increase and a roughly 2-fold difference in cell area (see heatmap in Figures 2C and 2D; there is a 2-fold difference in cell size between blue [<300 μm^2] and turquoise cells [300–600 μm^2] throughout the majority of the body of the root), typical of actively growing root apices. These data indicate that *R. carbonica* is the first and only example of a root fossilized during active growth, which has preserved the cellular organization of the meristem.

Comparison of the cellular organization of the different regions of the root apex indicates that the cellular dynamics in *R. carbonica* conform to that observed in extant root meristems. The root apices of all extant roots are covered by a protective cap. Root caps are typically tapered (they are thinner in proximal positions than in distal positions) because older cell layers are sloughed off as the root grows through the soil. *R. carbonica* is

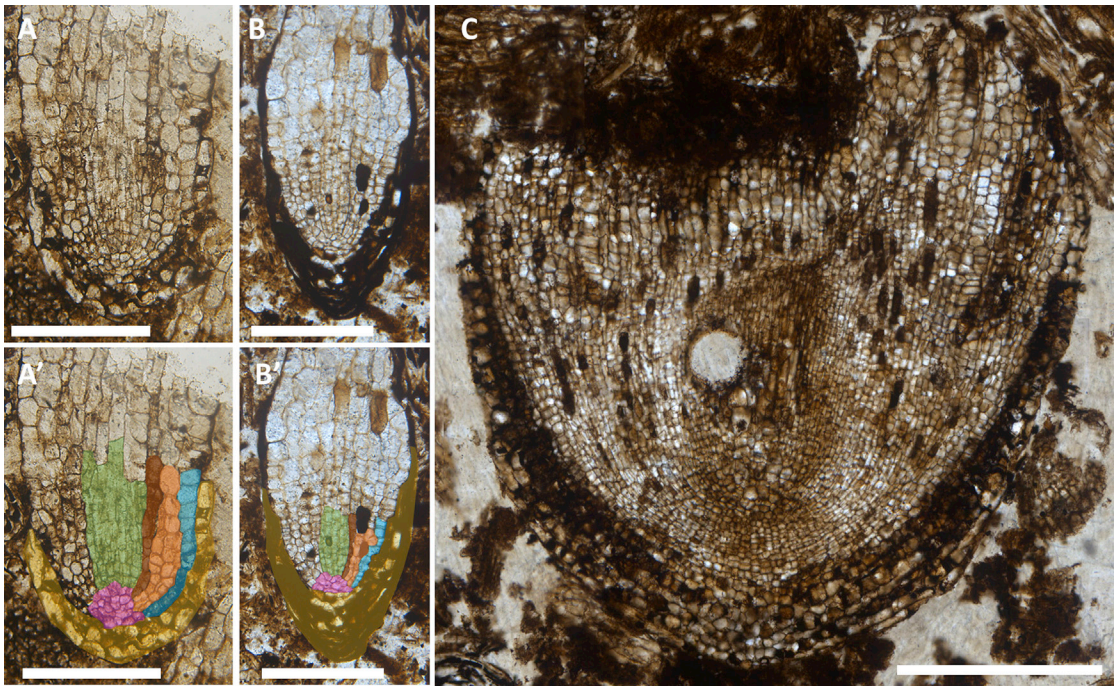

Figure 1. Two New Fossil Root Apices from the Carboniferous Period

(A and A′) Thin section 76, shown by permission of the Oxford University Herbaria. (A) *Apex 76.1* tentatively assigned to *Lyginopteris oldhamia*. (A′) *Apex 76.1* is shown with the main tissue types color coded (yellow, root cap; blue, epidermis; pink, differentiated cells at the position of the promeristem; orange, cortex; brown, endodermis; and green, procambium).

(B and B′) Thin section R646, shown courtesy of the Manchester Museum, The University of Manchester. (B) *Lyginopteris oldhamia* root apex discovered by Stopes and Watson [3]. (B′) *L. oldhamia* is shown overlaid with colors to represent the major tissues types as shown in (A′).

(C) Thin section 81, shown by permission of the Oxford University Herbaria. Holotype of the root apex of *Radix carbonica* (produced by the assembly of a series of continuous images of the root apex).

Scale bars, 200 μm (A and B) and 500 μm (C).

covered by a protective root cap that tapers rapidly, indicating that cells were sloughing, typical of roots of extant species [1] (Figure 3A, yellow). The promeristem is the group of cells in a growing root that gives rise to all tissues [1], and it is identified in *R. carbonica* as the region where the files of cells of fundamental tissues converge (Figure 3A, pink). The *R. carbonica* promeristem is large, consisting of 138 cells arranged in 10–15 tiers when imaged in the longitudinal plane of section (Figures 3A, pink, and 3B, pink and lilac). It comprises two morphologically distinct pools of initials (Figure 3B, pink and lilac) (see Supplemental Information), and initials give rise to many mature cell files, meaning that discrete initials for each cell layer do not exist. The *R. carbonica* promeristem is different from all extant vascular plant root meristems (see Supplemental Information), because of its large size and the spatial organization of cells that are arranged in more than ten tiers of initials. The distal promeristem (Figure 3B, lilac) of *R. carbonica* takes the form of a regular block of cells; a similar organization of the promeristem is found in almost all extant gymnosperms [12–23]. However, the structure of *R. carbonica* differs from that of extant gymnosperm root meristems in two ways.

The first feature that distinguishes *R. carbonica* from extant gymnosperm root meristems is the discrete root cap that is not continuous with the distal promeristem in *R. carbonica* (Figure 3B, lilac). In extant gymnosperms, it is not possible to distinguish a boundary between the promeristem and the root cap [12–23]. However, in *R. carbonica*, the promeristem is broad and not continuous with the root cap, which is discrete from other tissues. Furthermore, within this broad promeristem there are large numbers of anticlinal cell divisions (marked in yellow on Figure 3C), which lead to the loss of the columnar organization of cell files between the promeristem and the cap. While some gymnosperm promeristems are columellar [14–21], and anticlinal division occurs in the promeristem of others [19, 20, 22, 23], numerous anticlinal divisions within a columellar promeristem have not been described in any species. No similar organization with broad promeristem and discrete root cap has been described in any root meristem to date (see Supplemental Information).

The second feature that marks *R. carbonica* as distinct from the meristems of extant gymnosperms is the position of the Körper-Kappe boundary [1, 13, 24, 25] (see Supplemental Information for an extended description of the Körper-Kappe theory). The boundary between the Körper and Kappe complex is a highly conserved feature of all extant gymnosperms. In gymnosperms the Körper complex contains the vascular tissue and, in some cases, a small number of layers of the ground tissues [13–16] (Figures 4A and 4C, red). The Kappe complex, on the other hand, makes up the majority of the tissues (remainder of the ground tissues, epidermis, and root cap) of the root meristem

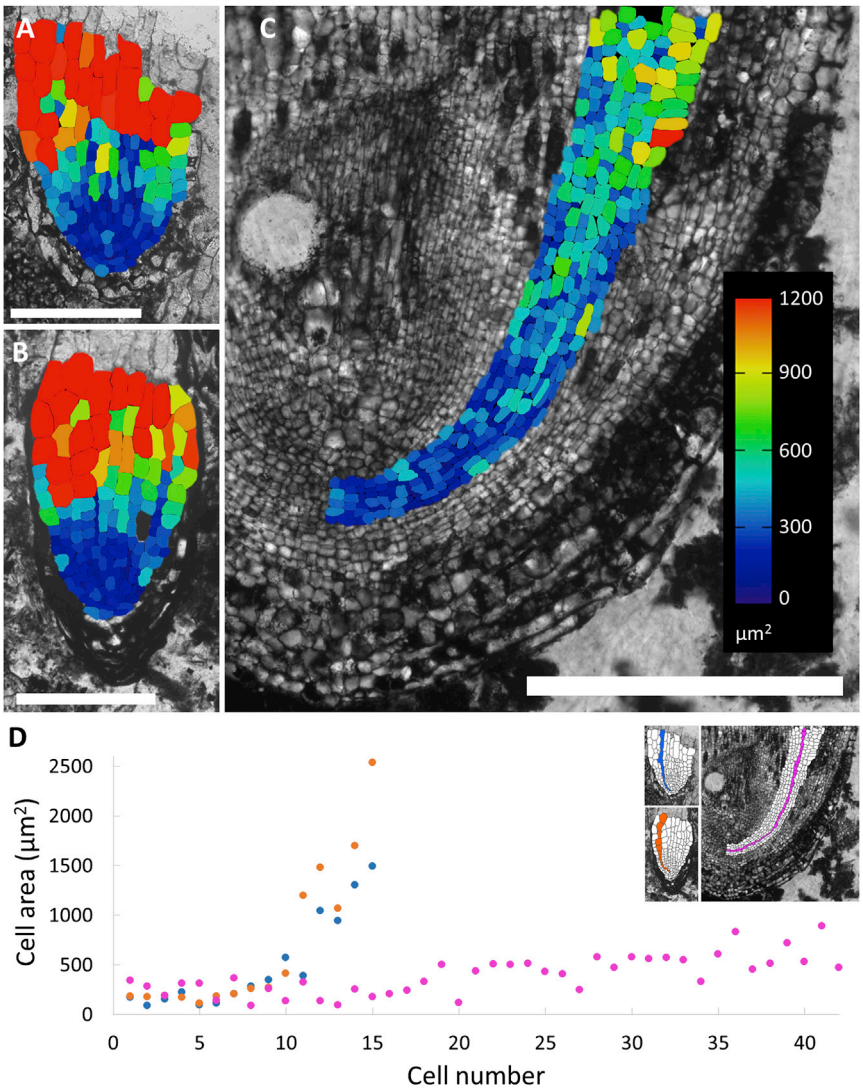

Figure 2. *R. carbonica* Is the First Fossil of an Active Meristem in a Growing Root

(A–C) Cell surface area heat plots show (A) *Apex 76.1* (Figure 1A), (B) *L. oldhamia* [3] (Figure 1B), and (C) *R. carbonica* (Figure 1C). Scale bars, 200 µm (A and B) and 500 µm (C).

(D) Cell area increase along a single-cell file in *Apex 76.1* (blue), *L. oldhamia* [3] (orange), and *R. carbonica* (pink). Note the gradual increase in cell size within the ground tissue of *R. carbonica* compared to *Apex 76.1* and *L. oldhamia*.

[13–16] (Figures 4A and 4C, blue). The Körper-Kappe boundary is, therefore, located very close to the junction between the provascular tissues and the ground tissue (Figures 4A and 4C). However, the Körper-Kappe boundary is markedly different in *R. carbonica*. The *R. carbonica* Körper complex constitutes the stele and almost all the ground tissue (Figure 3C, red; Figure 4D, red). The Kappe complex (Figure 3C, blue shading; Figure 4D, blue) comprises the root cap and the cell file abutting the root cap interpreted as the epidermis. Therefore, the position of the Körper-Kappe boundary of *R. carbonica* is structurally different from all extant gymnosperm root meristems (Figure 4).

R. carbonica is the only root meristem that has been preserved in which the patterns of cell division in the active apex can be elucidated. This allowed us for the first time to compare the organization of cells in the promeristem of an extinct Carboniferous root with the organization of cells in root meristems of extant plants. The organization of stem cells and differentiating cells suggests that the same general cellular dynamics in the self-renewing populations and their derivatives occurred in *R. carbonica* as in extant root meristems. However, the discrete root cap, zone of anticlinal cell divisions in the group of columella-like promeristem, and the position of the Körper-Kappe boundary mark *R. carbonica* as structurally distinct from all other previously described meristems (Figure 4).

Using the organization of cells in the promeristem and the meristem as criteria, Schüepp [13] identified nine classes of vascular plant root meristems. There were five classes of meristems in non-angiosperm tracheophytes (lycophytes, monilophytes, and gymnosperms [26]) combined. He identified seven classes in angiosperms, of which four were angiosperm specific. The evolution of novel meristem types [13, 28, 29] in angiosperms has, therefore, been associated with their rise to dominance. The discovery of the organization of stem cells and their derivatives in *R. carbonica* demonstrates that the diversity of developmentally distinct root meristem types that existed before the origin of angiosperms [27] (Figure 4E) but are now extinct was greater than previously described. It also shows that extant root meristem organization represents a subset of the diversity that has existed since roots first evolved.

EXPERIMENTAL PROCEDURES

The 139 thin sections of Carboniferous coal balls from the Oxford University Herbaria and the University of Oxford Natural History Museum were inspected for root meristems. The original *L. oldhamia* root apex described by Stopes and Watson [3] and Weiss [7] also was re-examined courtesy of the Manchester Museum, The University of Manchester (Thin section R646). Meristems were imaged with an Olympus BX50 microscope and quantitatively characterized using Fiji [30]. To quantitatively characterize the cell shape, cell area, and cell division pattern of *R. carbonica*, a line drawing was made of the 988 cells that constitute the distal portion of the apex (Figure 2C) and the 405 cells representing the development of the ground tissues (Figures 3B and 3C). Line drawings also were made of the cells in the distal portion of *Apex 76.1* and *L.oldhamia* apices (Figures 2A and 2B).

SUPPLEMENTAL INFORMATION

Supplemental Information includes a Supplemental Discussion and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.04.072.

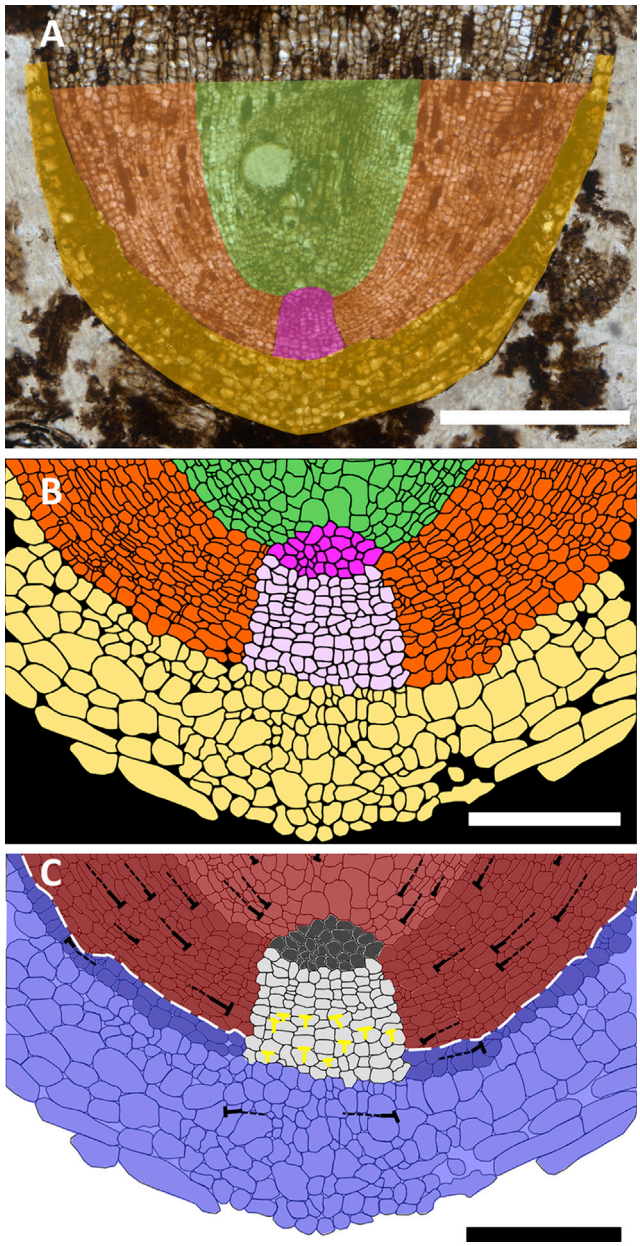

Figure 3. *R. carbonica* Has a Unique Cellular Organization

(A) *R. carbonica* (Figure 1C) is overlaid with colors representing the four major tissues found within the apex (yellow, root cap; pink, promeristem; orange, ground tissues and epidermis; and green, procambium).

(B) Line drawing of the apical portion of the *R. carbonica* holotype (Figure 1C) is color coded to represent the major tissue types as in (A), except the promeristem is further divided (pink, proximal promeristem; and lilac, distal columella-like promeristem).

(C) Same line drawing as in (B) (blue, Kappe complex; red, Körper complex; and gray, promeristem). Black Ts indicate T-divisions in both complexes with dashed lines showing the cell files that make the vertical stroke of the T. White dashed line marks the boundary between the Körper-Kappe complexes. Yellow Ts mark positions of anticlinal cell divisions within the central columella-like region of the promeristem.

Scale bars, 500 μm (A) and 200 μm (B and C).

AUTHOR CONTRIBUTIONS

A.J.H. and L.D. designed the project. A.J.H. carried out the analyses with assistance from J.G.D. A.J.H., J.G.D., and L.D. wrote the manuscript.

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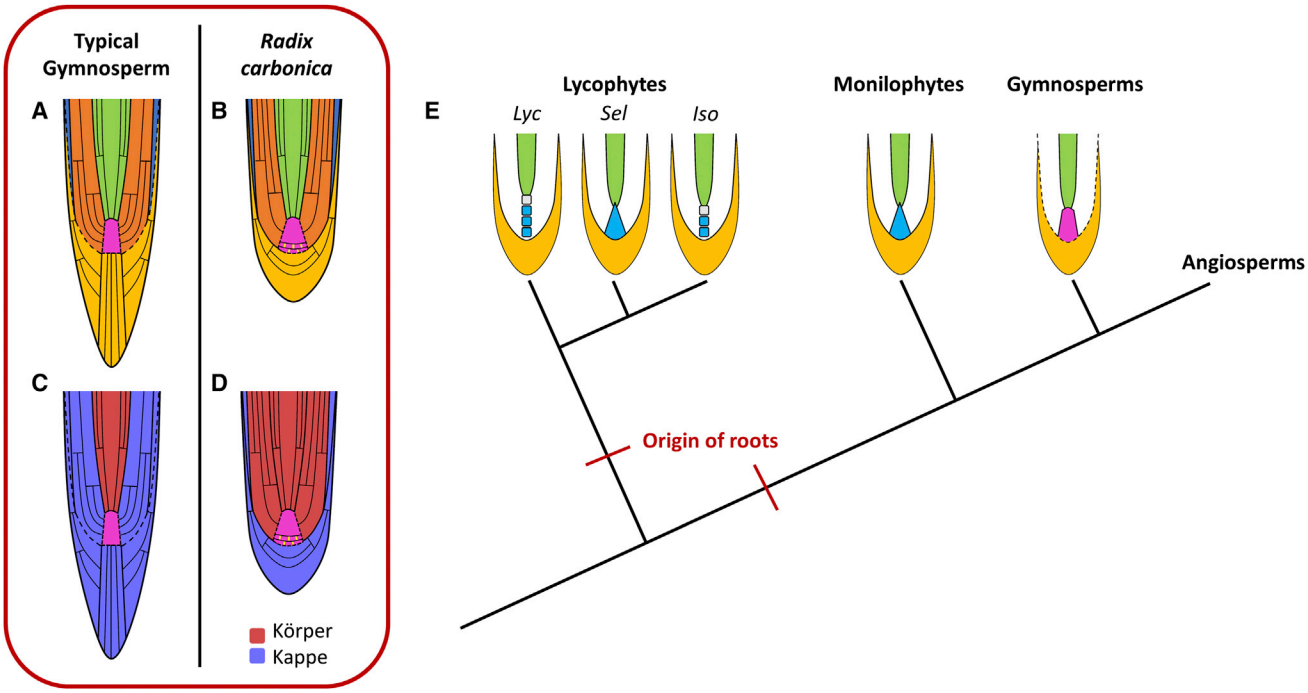

Figure 4. *R. carbonica* Is Distinct from All Extant Root Meristems

Schematic diagrams show the cellular organization of a typical gymnosperm (A and C) and *Radix carbonica* meristem (B and D).

(A and B) Schematics are color coded for the major tissue types within the meristem (yellow, root cap; pink, promeristem [yellow lines in the *R. carbonica* promeristem indicate the positions of anticlinal cell divisions within the promeristem]; orange, ground tissue; blue, epidermis; and green, procambium).

(C and D) Same schematics as in (A) and (B) but color coded to mark the position of the Körper- (red) Kappe (blue) complexes. Note the difference in the Körper-Kappe boundary between *R. carbonica* (D) and the gymnosperm meristem (C).

(E) A simplified vascular plant cladogram [26], showing the two hypothesized origins of roots [27], and schematics of lycophyte, monilophyte, and gymnosperm root meristems. *Lyc* stands for the Lycopodiales that typically have multicellular promeristems consisting of either three or four tiers of initials. *Sel* stands for the Selaginellales that typically have a single initial cell (apical cell). *Iso* stands for the Isoetales that typically have multicellular promeristems consisting of either two or three tiers of initials. Monilophyte root meristems typically have a single initial cell (apical cell). Gymnosperm root meristems have multicellular promeristems consisting of a zone of common initials for all tissues, or common initials for all non-vascular tissues and a separate set for all vascular tissues.

For a detailed review of meristem types, see Supplemental Information.

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