

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 rhizomorph 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.

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The authors declare no conflict of interest.

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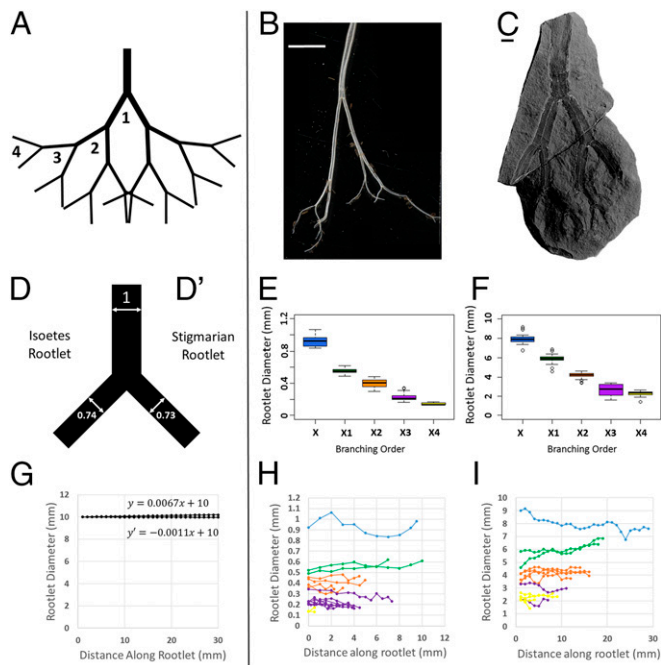


Fig. 1. Stigmarian and *Isoetes* rootlets do not taper but branch dichotomously, decreasing in diameter in a stepwise manner through multiple orders of branching. (A) Cartoon of branched rootlet showing four orders of dichotomous branching with branching orders labeled. (B) *Isoetes* and (C) stigmarian 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 $\sim 25\%$ 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. 1E). 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 $\sim 25\%$ 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. 1C). Furthermore, rootlet diameter decreased by $\sim 25\%$ 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 $\sim 25\%$ 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. 2A), as we observed in the compression fossils described above (Fig. 1C), we hypothesized that there would be more thin rootlets than thick rootlets in coal ball-preserved stigmarian systems (Fig. 2C, 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. 2B), we would expect to observe equal numbers of small- and large-diameter rootlets in a sample of roots preserved in coal balls (Fig. 2C, 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)

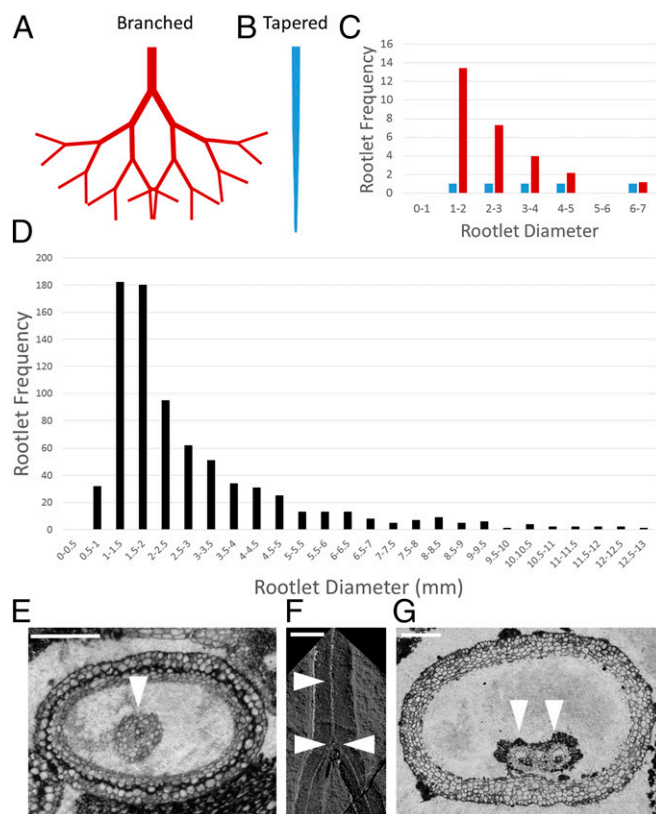


Fig. 2. Measurement of 785 stigmarian rootlets preserved in coal balls indicates that branching was common. Modeling the predicted frequency of rootlet diameters in coal balls based on a branched (A) and tapered (B) rootlets reveals that the two rootlet types have characteristically different frequency 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. 1C; 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).

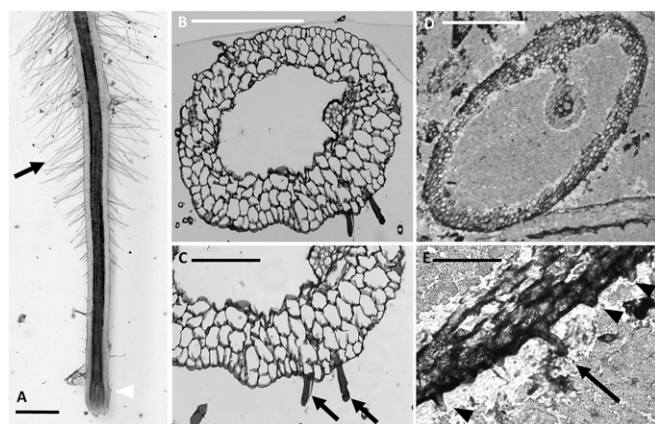


Fig. 3. Stigmarian rootlets possessed root hairs. (A) *Isoetes* rootlets develop root hairs (black arrows in A–E) (note the *Isoetes* rootlet is in the process of branching at the apex—white arrowhead). (B–E) Transverse sections of *Isoetes* (B and enlarged region of B shown in C) and stigmarian (D, 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. 2D). 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. 2D). 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. 3E, white arrowhead); however, above a point of branching, the vascular strand locally bifurcates (Fig. 2F, white arrowheads). Finding rootlets in coal balls containing two vascular strands therefore indicates that

rootlets in coal balls branched (Fig. 2G, white arrowheads). Of the 785 samples observed, twin vascular strands were found in 51 rootlets (Fig. 2G, 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 $\sim 73\%$ 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. 2D). 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. 3E was 13.9 μm in diameter. Root hair diameter of the two *Isoetes* root hairs shown in Fig. 3C 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. S10A). 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. S10B). 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. S10C) (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 $\sim 1,600$ 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

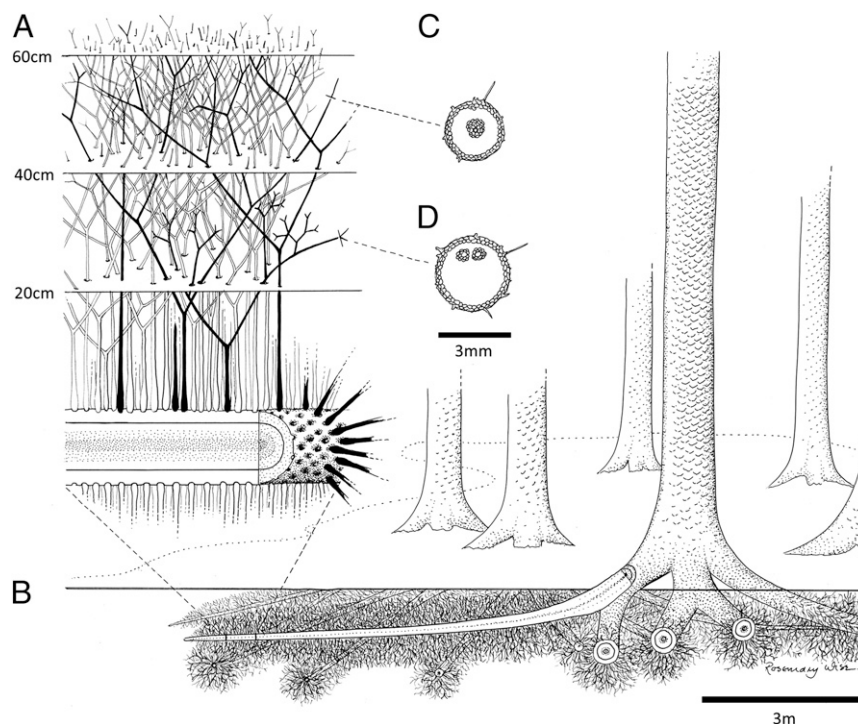


Fig. 4. Reconstruction of stigmarian root systems with highly branched systems of rootlets. (A) Reconstruction of a population of branching rootlets that could grow to over 90 cm in length. (B) Reconstruction of the root plate that comprised rhizomorph axes covered with rootlets producing ~25,600 terminal 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 hixrix* 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 hixrix* 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. 1B and Fig. S1 A–I). *Isoetes* rootlet diameter was measured using Fiji (67) (Fig. 1H 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. 1G) 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. 1C 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. 1G). 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. 2C). 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. 1C) 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. 3C) (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. S7A), and 12 rootlet scars we measured from the poorly preserved apex (Collection No. LL. 15952.471; Fig. S7C) 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. 3A). 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. 3B 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. 3A 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 cereale* L.) root systems was 6,875.4 ft², with root axes contributing 2,554.09 ft² and the root hairs contributing 4,321.31 ft². 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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