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Reconstructing spore evolution recalibrates the timescale of land plant diversification

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The origin and evolution of land plants precipitated fundamental changes to the Earth system and enabled the diversification of life on land. Their early evolution is documented by a rich fossil record of sporopollenin-walled spores that is difficult to interpret because the evolution of plant spore characteristics is unresolved. We employ a suite of ancestral state estimation methods to elucidate the pattern of spore character evolution among land plants. We find that the ancestral embryophyte underwent monoplastidic meiosis and produced unenveloped spores with a three-layered sporoderm. We infer that trilete spores (that developed in tetrahedral tetrads), traditionally considered a feature of vascular plants, were produced by the crown-embryophyte. We recalibrate the molecular timescale of early land plant evolution on the basis of a reinterpretation of the fossil record in this light. We find that crown-embryophytes originated in the Late Ordovician (457–450 Ma) and that both crown-tracheophytes (432–428 Ma) and crown-bryophytes (445–429 Ma) diverged in the early Silurian.

The evolutionary origin of land plants precipitated fundamental changes to the Earth system, altering biogeochemical cycles through alterations of atmospheric composition, carbon sequestration, weathering and soil formation, as well as creating niches for animals and fungal symbioses[1–5]. However, the timing of these events remains unclear because of the heterogeneity of the rock record in which the fossil record is entombed and, consequently, the poorly informative nature of fossil calibrations of molecular clock analyses that have resulted in estimates for the antiquity of embryophytes that range from the late Mesoproterozoic to the early Devonian (1,040–407 million years ago (Ma))[6–17] (Fig. 1).

While the macrofossil record of early land plants is patchy, the fossil record of their dispersed spores is more continuous and predates the first unequivocal land plant megafossil, *Cooksonia*, by approximately 40 million years (Myr)[18–25]. The fidelity of the fossil spore record stems from their protective wall, which is composed of the highly recalcitrant biopolymer sporopollenin[26], and their accumulation in fluvial and marine sedimentary sequences, providing reliable samples of floral diversity from broad spatial areas over geologic history[21].

Irregular polyad palynomorphs lacking regular geometric cell arrangements that are potentially terrestrially derived first appear in the middle Cambrian[27–31]. They have controversially been termed cryptospores using a definition based on terrestrial origin rather than biological affinity. The current consensus is that these were produced by non-embryophyte streptophyte algae that potentially included close relatives or ancestors of the embryophytes[20,32].

The first definitive appearance of regular polyad palynomorphs is in terrestrial deposits of the Darriwilian (Middle Ordovician). Their affinity to embryophytes was initially based on their morphological similarity to the permanent tetrads and dyads produced by some living bryophytes and their occurrence in terrestrial deposits[33]. They were subsequently reported in situ from exceptionally preserved eophyte plants from younger deposits[34]. Trilete spores first appear sporadically and rarely in the Late Ordovician, becoming continuous and increasingly dominant from the late Llandovery (early Silurian) until they dominate late Silurian spore assemblages[35–37]. Trilete spores are widely attributed to vascular plants[35,38,39], but they are also produced by some extant bryophytes[33,39–41]. Despite the fidelity of the spore fossil

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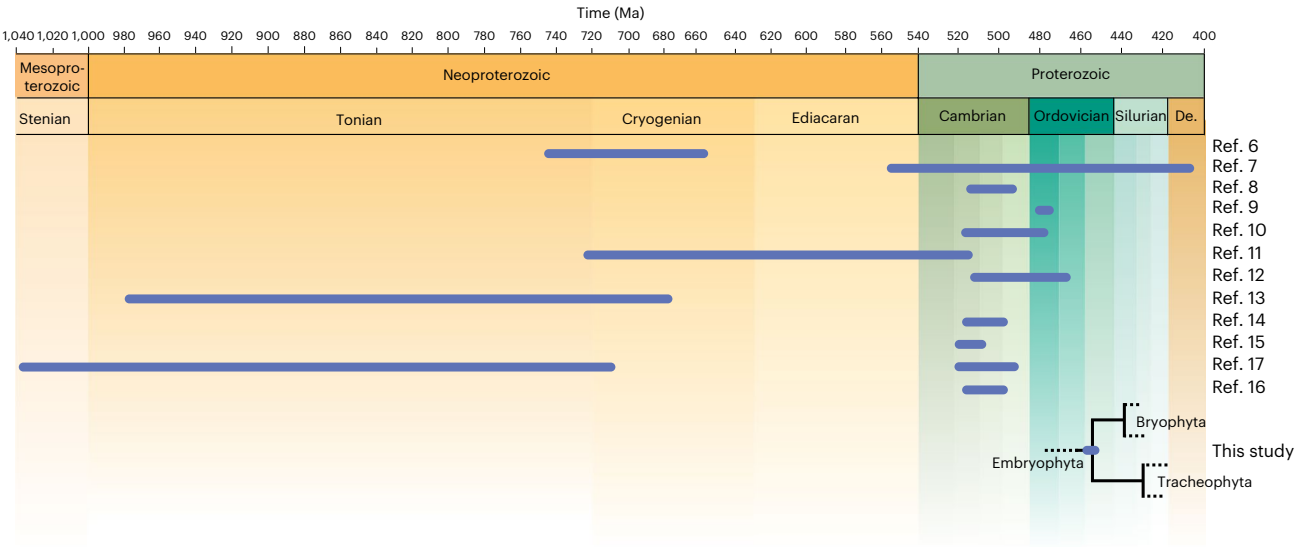


Fig. 1 | A comparison of the inferred age of crown-Embryophyta between the present study and previous molecular clock analyses. The blue bars represent the reported confidence intervals from each study[6–17]. Note the change in scale between the Proterozoic and Neoproterozoic. Some studies present multiple clock analyses, in which cases we have selected the most relevant or the focal analyses of such studies. De., Devonian.

record, its interpretation is challenging. Mature spore fossils are usually found dispersed from their parent sporophyte, rendering their taxonomic affiliation problematic. Meanwhile, in situ fossil spores are usually immature and may possess different ultrastructural features from mature palynomorphs. Taphonomy can also result in the loss of informative ultrastructural characters; thus, many fossil spore morphologies cannot be assigned to extant groups with confidence[35,42]. Furthermore, the phylogeny of spore characters is unclear, not least since the spore fossil record has traditionally been rationalized in light of the now widely rejected phylogenetic framework of bryophyte paraphyly[5,43] (but see ref. 44).

Here our aim is to establish a new timescale for the evolution of land plants based on the recalibration of molecular clock analyses, following a reinterpretation of the spore fossil record. This is achieved through probabilistic reconstruction of the pattern of spore character evolution within the framework of bryophyte monophyly[14,45,46], revealing the sequence in which spore characters evolved as well as providing a basis for reinterpreting the spore fossil record. On this basis, we establish new fossil calibrations that we employ in determining a new evolutionary timescale for early land plant evolution using a relaxed molecular clock. The results of our analyses indicate that trilete spores (that developed in tetrahedral tetrads) are characteristic of total-group embryophytes, and a molecular clock analysis recalibrated in light of this suggests a Late Ordovician origin of crown-embryophytes and a mid-Silurian origin of crown-tracheophytes.

Results

Ancestral state estimation

To establish the sequence of spore evolution, we compiled a dataset composed of 27 discrete characters describing spore morphology, spore ontogeny and life history of the parent sporophyte, for 27 extant and 11 extinct genera, representative of the principle clades of bryophytes and tracheophytes and their outgroups. A full list of characters, character states, discussions of the scores provided for each character and references used to generate those scores is provided in Supplementary Tables 1–3. Ancestral state estimation (ASE) was based on three timetrees, one containing only extant taxa[14] and two further trees that also included fossil taxa and differed in the phylogenetic position of eophytes (Fig. 2). The inclusion of fossil taxa was based on phylogenetic position as resolved in cladistic studies[47–49], with tip ages based on the minimum age of each fossil taxon (Methods) and attachment times established arbitrarily according to the mean age of the extant lineage from which they branch. To control for competing methods of ASE, we used (1) parsimony[50], (2) a maximum likelihood equal-rates model[51,52], (3) a maximum likelihood all-rates-different model[53] and (4) Bayesian Markov chain Monte Carlo-based stochastic character mapping[54,55].

Generally, ancestral state estimates were consistent across different analytical conditions and robust to the inclusion of fossil taxa; they are summarized in Fig. 3. The ancestral embryophyte was robustly inferred to have released monad spores derived from tetrads, which were unenveloped and possessed a trilete aperture (Fig. 4), with a sporoderm consisting of three layers: an outer exospore, an inner exospore of granular or amorphous sporopollenin and an endospore. The deposition of sporopollenin on white-line-centred lamellae was found to be the ancestral mode of exospore formation in embryophytes. This ancestral sporophyte is inferred to have been homosporous and exhibited monoplastidic meiosis, and to have possessed a parietal or secretory tapetum, which contributed to the formation of the exospore. Otherwise, a series of discrete character losses and gains with respect to this ancestral spore morphotype characterizes the pattern of spore evolution across the land plant clade. Mosses are recovered to have independently gained persistent spore mother cell walls and an extra-exosporal layer (perispore). In liverworts, the aperture is inferred to have been lost independently and a parietal tapetum of one cell layer gained. Cellular elaters were gained along the stem-lineage, and patterned exospore precursors appear in the Haplomitriopsida. An extra-exosporal layer derived from a persistent spore mother cell wall and cellular elaters were gained in stem-hornworts, but the tapetum was lost. The origins of the paraexospore and heterospory are recovered in the lineage leading to *Selaginella* and *Isoetes*. Me. Mesoproterozoic; Ne., Neoproterozoic; Ca., Cambrian; Or. Ordovician; S., Silurian; Ca., Carboniferous; Pe., Permian; Tr., Triassic; Ju., Jurassic; Cr., Cretaceous; Pa., Palaeogene; N., Neogene.

Polyplastidic meiosis and an amoeboid tapetum are estimated as ancestral to euphyllophytes. Within the ferns, *Equisetum* possesses a unique form of elater in the spore wall and uniquely gained an additional sporoderm layer termed the epispore. A perispore was present in the ancestor of all remaining ferns, and an endospore

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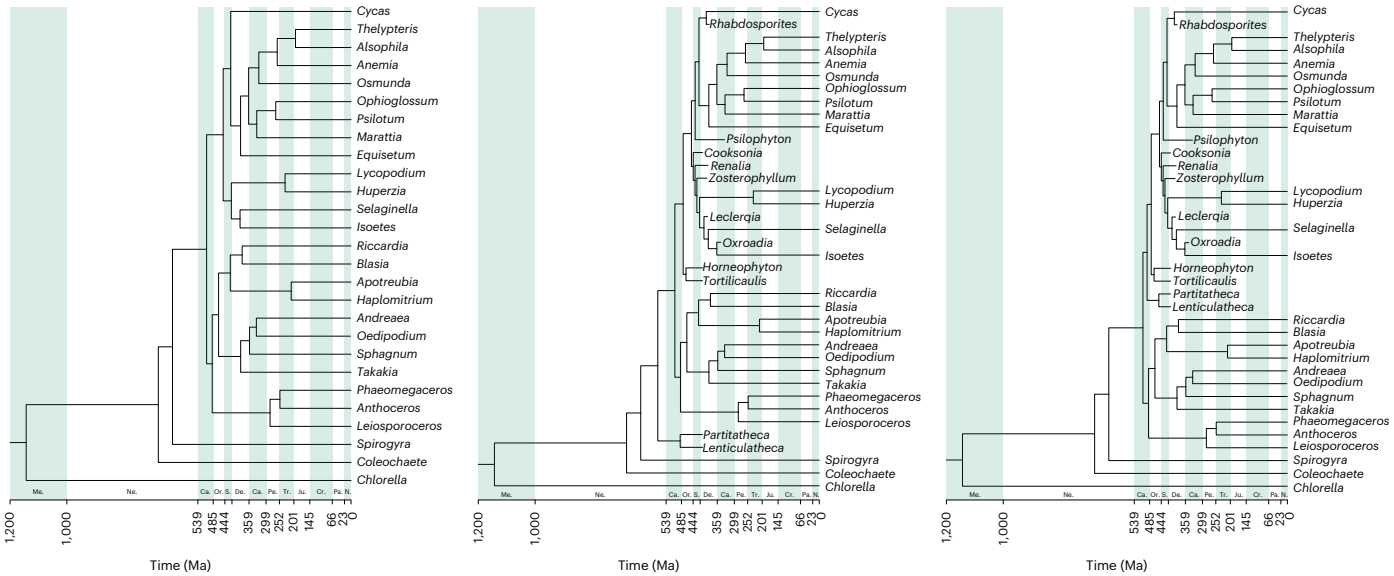


Fig. 2 | Timetrees used to perform ASE analyses. Left: Timetree consisting of only extant taxa used in this study. Node ages are positioned on the mean age estimates derived in refs. 14,92. Middle: Timetree with fossil genera inserted. Here the eophytes *Partitatheca* and *Lenticulatheca* are placed as stem-embryophytes. Right: Timetree with fossil genera inserted. Here the eophytes *Partitatheca* and *Lenticulatheca* are placed as stem-tracheophytes.

layer that develops at germination was present in the ancestor of all leptosporangiate ferns.

Although many estimated node states were consistent across different analyses, some variation between analytical conditions and phylogenetic hypotheses was encountered (Fig. 4). The majority of analyses suggest that the ancestral embryophyte shed unenveloped spores, although the all-rates-different model, performed on both phylogenies that include eophytes, suggests that the ancestral embryophyte spore was enveloped. Under the all-rates-different model, endospore precursors are inferred to have been present in the ancestral embryophyte and then lost independently in the mosses and tracheophytes. Under equal rates, however, this character was lost independently in the liverworts and hornworts. Parsimony suggests that orbicules were gained in the euphyllophyte stem-lineage, but the inclusion of fossils instead results in inference of a series of repeated independent gains of this character among the tracheophytes. Finally, under parsimony, elaters are inferred to have been lost independently in mosses from the ancestral bryophyte, whereas marginal likelihood analyses suggest independent gains in the liverworts and hornworts.

Fossil calibrations

The most important conclusion drawn from this series of ancestral state estimates (Supplementary Figs. 1–56) is that the ancestral embryophyte spore was a trilete monad, a finding that is robust to all the different analytical models used across all three phylogenetic arrangements (Figs. 3 and 4). To be present in the ancestor of all embryophytes, the trilete spore must therefore be considered a character of total-group Embryophyta and must have evolved among stem-embryophytes. Consequently, fossil cryptospores cannot be crown-Embryophyta. These fossils have previously been used to constrain divergence time estimates within the embryophyte clade[8,10,14], and this calibration approach requires reappraisal. The new fossil calibrations defined for this study are provided below, and a full list of all fossil calibrations used in the divergence time estimation study is provided in Supplementary Table 4.

The age constraint on the divergence of Zygnematophyceae and Embryophyta (crown-Anydrophyta) is revised to a minimum of 449.65 Ma on the basis of the occurrence of the earliest unequivocal trilete spore, *Ambitisporites avitus*, from level 551.0 m in Core 56 from the Qusaiba-1 core, Qasim Formation, northern Saudi Arabia[18,36]. The constraint on the divergence of Bryophyta and Tracheophyta (crown-Embryophyta) is revised to 459.1–426.9 Ma maximally on the basis of the oldest possible trilete spores, from Dalby Limestone from the Borenshult-1 borehole drilled in the vicinity of Motala, east of Lake Vättern in central Sweden[56], and minimally on the earliest occurrence of a total-group tracheophyte, which is *Cooksonia* cf. *pertoni* from the Homerian Cloncannon Formation of Borrisnoe Mountain, County Tipperary, Ireland[22,23]. We did not use the Dalby Limestone spores to establish a minimum constraint because we are concerned that the trilete mark in the figured specimens might be an artefact of compression. Finally, the divergence of Lycopodiophyta and Euphyllophyta (crown-Tracheophyta) is constrained to 431.2–420.7 Ma on the basis of the same record of *Cooksonia* cf. *pertoni* and the earliest secure record of crown-Tracheophyta, which is *Zosterophyllum* sp. from beds equivalent to the Bathurst Island and Stuart Bay Formations, Ludlow (Silurian), exposed 14 km north of Polar Bear Pass research station, eastern Bathurst Island, Arctic Canada[57]. A full justification of these calibrations, following best practice[58], is provided in Supplementary Table 4. A relaxed molecular clock analysis using these revised calibrations estimates crown-embryophytes to have originated in the Late Ordovician (457–450 Ma) and both crown-tracheophytes (432–428 Ma) and crown-bryophytes (445–429 Ma) to have diverged in the early Silurian (Fig. 5).

Discussion

Spore character evolution

Despite recent changes in our understanding of the fundamental relationships among land plant clades[5], some of our inferences of spore character evolution are unchanged from previous studies of sporogenesis. These characters include sporopollenin deposition along white-line-centred lamellae being plesiomorphic in embryophytes[59,60], the presence of endospore and exospore layers in the ancestral spore[61] and the independent loss of apertures in liverworts[62]. However, our results encompass a number of novel findings. The most important is that the ancestral embryophyte spore is recovered as a trilete monad. As a character of total-group Embryophyta, the appearance of trilete spores can be used to constrain the divergence of the embryophyte clade rather than the first appearance of regular, tetrahedral tetrad cryptospores. Monoplastidic meiosis appears to be ancestral

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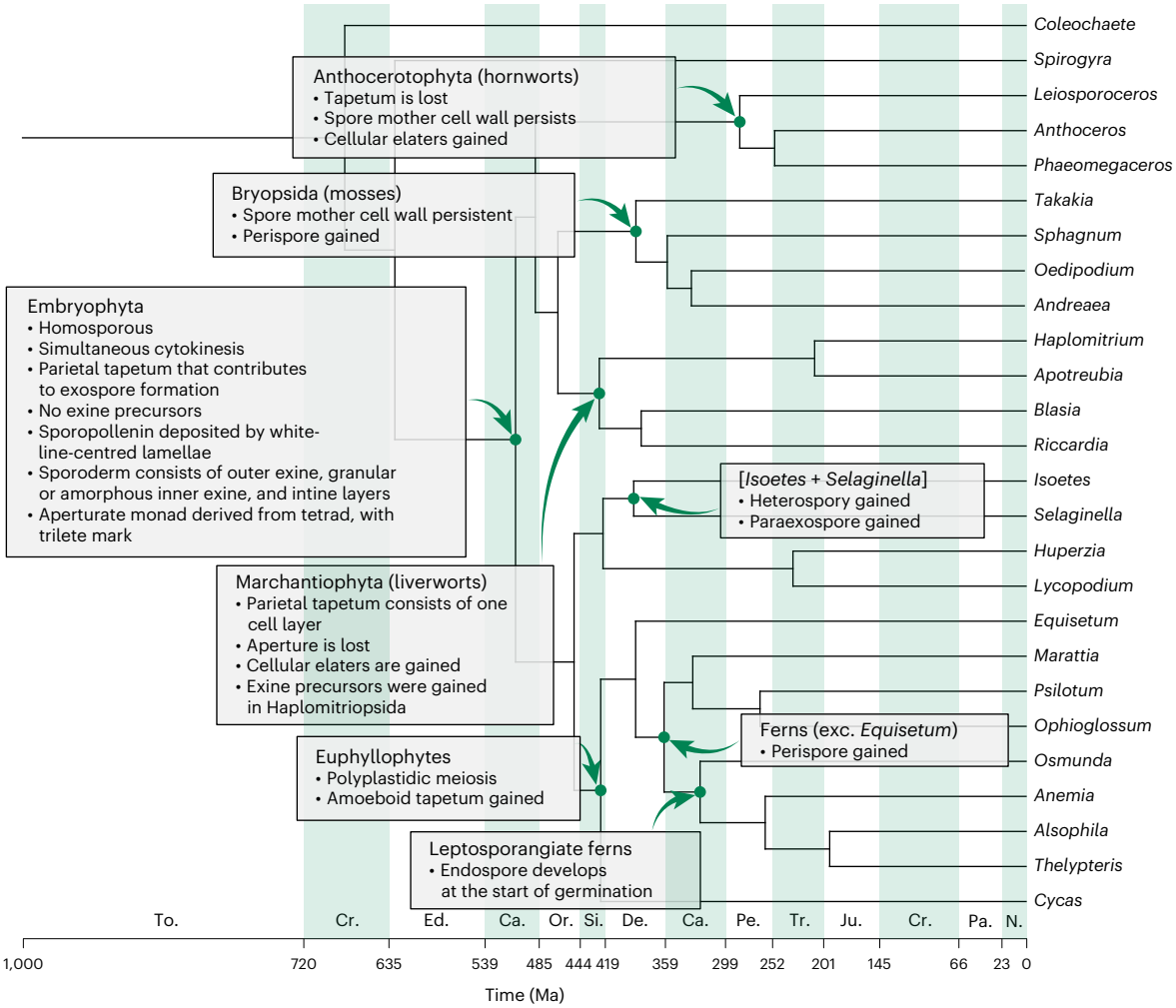


Fig. 3 | Summary of ASE analyses superimposed on the phylogeny of the extant taxon sample used in this study. Key character gains and losses at nodes of interest as inferred via ASE are summarized.

to all embryophytes rather than just bryophytes[61], and the ancestral embryophyte spore possessed a sporoderm consisting of an inner exospore, an outer exospore and an endospore, rather than containing a single-layered exospore or perispore[62]. The perispore, an extra-exosporal layer (sometimes also referred to as the perine), is consistently reconstructed as absent in the ancestral embryophyte[62].

Although many results were recovered across all analytical conditions, some character state histories were inferred differently depending on the assumed model of evolution used or the inclusion of fossil specimens in the analysis. ASE includes a range of approaches that aim to infer patterns of character evolution, across traits that can be described using continuous or discrete data. Despite the breadth of methodologies available, and some evidence that likelihood-based methods deliver more accurate results than parsimony[63,64], there remains little consensus as to which methods produce results with the highest levels of accuracy or precision[65,66]. A recent simulation study demonstrated that the equal-rates model was the most accurate in the greatest number of simulations, and that the all-rates-different model frequently overestimated transition rates between character states[66]. It is likely that the unique reconstructions resolved under the all-rates-different model are driven by the proximity of *Partitatheca*, an eophyte that produced enveloped dyads, to the embryophyte node. Given that the phylogenetic position of *Partitatheca* is not well evidenced, we used the equal-rates model as a benchmark for the assessment of ASEs[66] and sought consensus between ancestral states inferred under different analytical conditions.

The above leads us to the caveat that while debate over the phylogenetic relationships of extant plant taxa is generally agreed[5] (but see ref. 44), this is not the case for fossil early land plants. For example, one of our timetrees places the eophytes as stem-tracheophytes, while other researchers have suggested that the eophytes are stem-embryophytes[42] (Fig. 2). The position of the eophytes has yet to be formally tested using a cladistic analysis, and it will be interesting to see where they are situated in the phylogeny and whether this affects the importance of traditional cryptospores in studies such as ours.

The timescale of land plant evolution

Although molecular clock studies have provided estimates for the divergence of crown-embryophytes ranging from the Mesoproterozoic[13,17] to the Early Ordovician[12], there has been convergence on a late Cambrian to Early Ordovician origin in recent studies[10,14] (but see ref. 13) (Fig. 1). Harris et al.[14] commented on the sensitivity of the embryophyte node to different calibration strategies, a finding that is borne out in our analysis. The use of revised fossil calibrations results in a much later age estimate for crown-Embryophyta (450–457 Ma; Sandbian-Katian, Late Ordovician; Fig. 5), the mean of which is 55 Myr younger than the equivalent estimate, using the same molecular dataset, in Harris et al.[14]. Although most dates are congruent with this previous study, our revised calibrations result in younger and more precise estimates for the divergence of the deepest nodes in land plant phylogeny. This is most apparent in the divergence of crown-embryophytes, estimated to have occurred during a narrow 7-Myr interval in the Late

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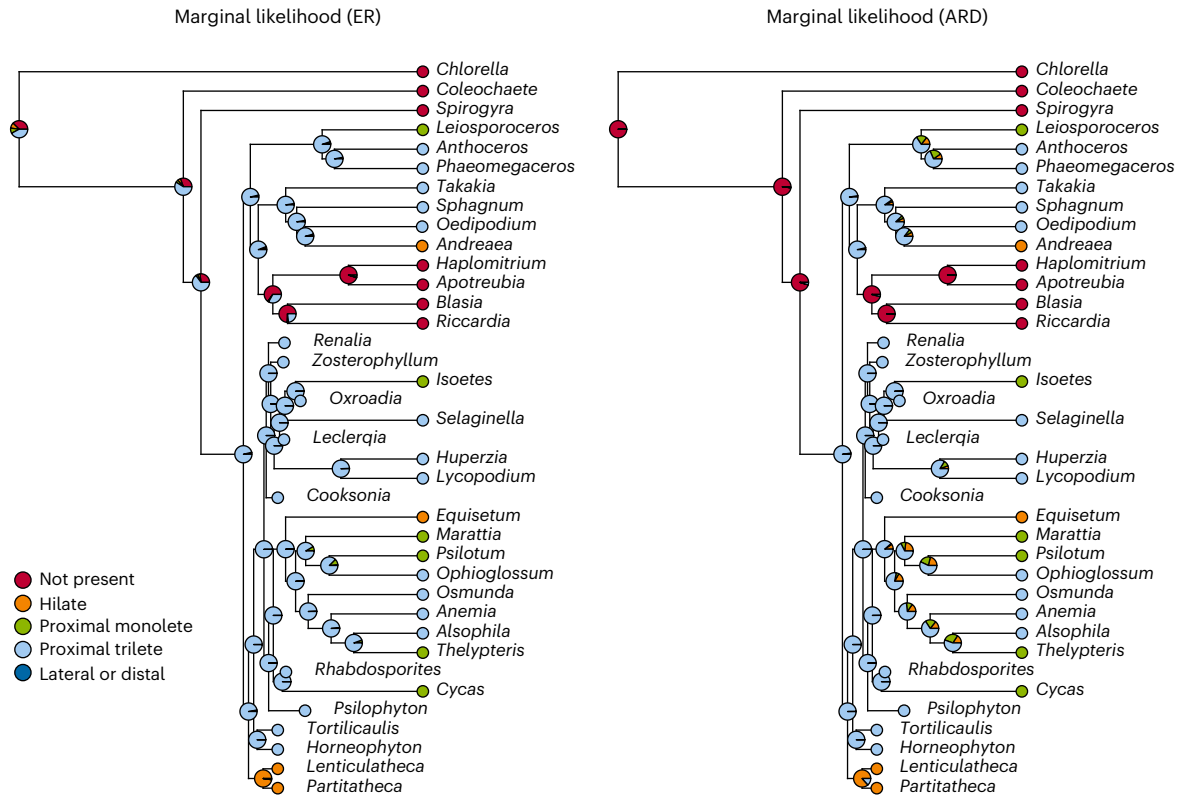


Fig. 4 | Example ASE output for character 21 (aperture type), performed on the timetree with eophytes placed as stem-tracheophytes. Left: Marginal likelihood analysis performed under the equal-rates (ER) model of evolution. Right: Marginal likelihood analysis performed under the all-rates-different (ARD) model of evolution. Tip labels represent the state scored for each extant taxon. Node labels represent the probability that each node represents each character state.

Ordovician (Fig. 5). The divergence estimates of the tracheophyte, euphyllophyte, lycophyte, bryophyte, setaphyte, liverwort and moss crown clades are also much younger and more precise in this analysis (Fig. 5 and Extended Data Figs. 1–3). The Late Ordovician estimate for the origin of crown-embryophytes brings the findings of molecular clock studies into closer accord with traditional interpretations of the fossil record. Previous estimates of a Cambro-Ordovician origin of crown-embryophytes implied that there was a gap of up to 80 Myr between the genetic divergence of the lineage and the first appearance of unequivocal land plants in the fossil record[14,22,25]. In light of this analysis, the mismatch between palaeontological and molecular clock estimates is reduced to as little as 19 Myr. Our results further suggest that the divergence of crown-tracheophytes was coincident with the earliest known specimens of *Cooksonia*, a stem-tracheophyte[8,22,67]. This convergence between our molecular timescale and the fossil record may suggest that our study is a better approximation of the true timescale than previous studies. Previous molecular clock studies differ in that they are based on different molecular data and implement different methodological assumptions including the underlying topology, partition strategy and clock model[6–17]. However, these studies differ most importantly in terms of how they interpret and implement the fossil record, which has the most influential impact on the ensuing timescale[5,11,68]. We find confidence in our timescale because it integrates formal interpretation of the spore fossil record of early land plants, which dominates the first tens of millions of years of the land plant fossil record[20]. Doubtless, however, this will not be the final attempt to estimate the timescale of early land plant evolution, and nor should it, as future studies explore the impacts of different assumptions and interpret the underlying data in different ways.

In the interim, it is pertinent to consider the implications of our revised timescale of early land plant evolution for hypotheses that seek to explain their impact on the Earth system. The initial diversification of land plants has been implicated in global climate shifts through the Ordovician. The gradual decline of CO_2 levels exhibited through the Ordovician has been connected to Late Ordovician glaciations associated with a marine mass extinction[2]. On the basis of modelling the weathering potential of the moss *Physcomitrium patens*, the diversification of non-vascular plants was found to have sufficiently amplified silicate weathering to result in modelled atmospheric CO_2 levels consistent with those necessary to cause this period of glaciation[2]. Land plant diversification has also been implicated in the increase in Palaeozoic atmospheric oxygen to modern levels by 400 Ma through increases in global carbon burial[69]. These models are predicated on a diversification of non-vascular land plants from 470 Ma based on the diversity of regular tetrad cryptospores in the fossil record[70,71]. Other models have suggested a key window of opportunity for early plant diversification at around 470 Ma, followed by a period of increasing aridity and cooling, which reduced the habitability of the Earth surface for plants, aligned with the appearance of vascular plant adaptations for water acquisition and retention[72]. Our revised timescale for land plant diversification (Fig. 5) may require reassessment of the assumptions under which this modelling was undertaken. Our timescale estimates crown-embryophyte diversification within the interval 457–450 Ma and the appearance of crown-tracheophytes 428–432 Ma (Fig. 5), discordant with many climate and biogeochemical models that have assumed diversification of crown-tracheophytes on the basis of the first appearance of unequivocal trilete spores in the Late Ordovician, ~450 Ma[36,37,69]. However, many of the characteristics of crown-embryophytes and crown-tracheophytes would have been established within their respective stem-lineages. ASE may aid in constraining the uncertainty associated with the capacity of

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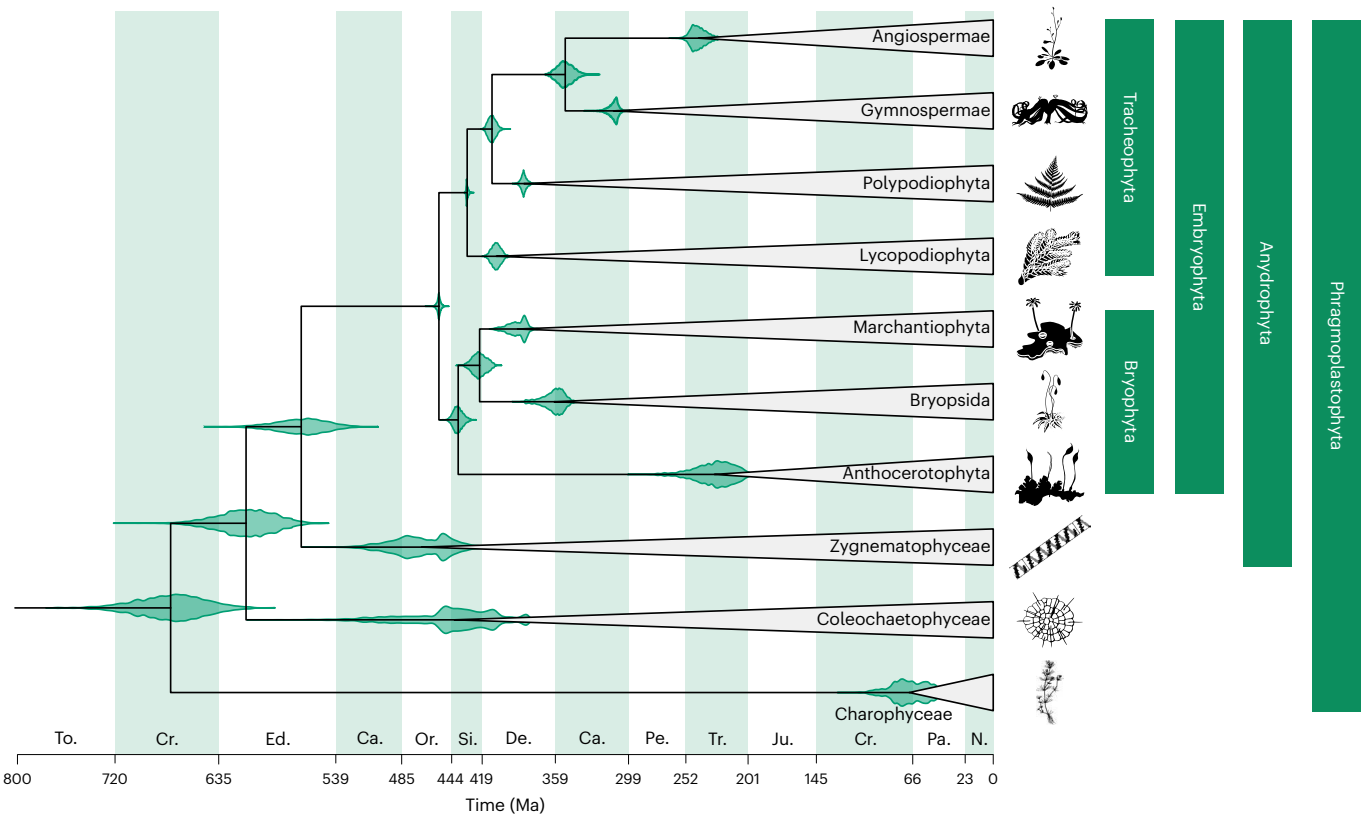


Fig. 5 | Time-scaled phylogeny of Phragmoplastophyta, with posterior distributions of divergence time estimates superimposed on node positions. Nodes are positioned on the mean age estimates. Credit: Zygnematophyceae icon, Matt Crook, PhyloPic under a Creative Commons license CC BY-SA 3.0; Charophyceae icon, Guillaume Dera, PhyloPic under a Creative Commons license CC0 1.0.

stem-embryophytes for early land floras to exert sufficient effects on global nutrient flux to trigger the magnitude of carbon drawdown required to effect the end-Ordovician glaciations[71,73].

Conclusions

The ancestral land plant spore was a naked monad with a trilete mark, a finding that is robust to different evolutionary models and phylogenetic hypotheses. This finding also has important implications for how we constrain studies of the rate and tempo of land plant evolution. The first appearance of trilete spores can be used as a soft maximum constraint on the divergence of the embryophyte clade. Recalibrating a molecular clock in this way reveals that the land plants originated 457–450 Ma in the Late Ordovician, some 55 Myr later than previously thought. It is uncontroversial to say that the evolution of land plants fundamentally transformed the surface of the Earth, and that their terrestrialization enabled other kingdoms of life to flourish on land. Our findings suggest that further efforts are required to fully understand the impact that the earliest land plants had on the evolution of the Earth system.

Methods

Character matrix construction and scoring

As noted in the main text, the evolution of plant spore characteristics is unresolved. This is largely a consequence of the considerable difficulties in identifying and scoring suitable characters in extant plants and applying these to fossil plants and their in situ and dispersed spores. The reasons for this include the following: (1) certain groups of land plants have relatively few detailed studies of sporogenesis (for example, hornworts), and the taxa reported on often are not phylogenetically well distributed; (2) since modern electron microscope studies of sporogenesis commenced in the 1960s, character reporting has varied, meaning that for most taxa we know only a subset of their character states; (3) the aforementioned problem has been compounded by the development of widely varying terminologies, with the result that it is often very difficult to interpret previous reports, and there is considerable confusion over the terminology and scoring of character states; and (4) as is usual in biology, exceptions (with respect to character states) are often reported, making generalizations and conjectures difficult. We have attempted to take a pragmatic approach to overcome these difficulties. This has involved (1) careful selection of taxa whose spore development is well known, while attempting to maintain adequate phylogenetic coverage among the few taxa selected for each plant group; and (2) scoring where possible from primary reports but using generalizations and conjectures (based largely on review articles) to iron out the ambiguities reported in the literature and thus reduce the proportion of unscored characters in the matrix.

We compiled a morphological dataset composed of 27 discrete characters describing spore morphology, spore ontogeny and life history of the parent sporophyte. The data matrix, character definitions, scoring and discussion, as well as the literature sources used in scoring, are presented in Supplementary Tables 1–3. Previous studies of spore character evolution have been performed on narrower samples of extant land plant diversity and under the phylogenetic assumption that bryophytes are a paraphyletic array of early-diverging land plants, often assuming that liverworts most closely resemble the ancestral land plant body plan[43,59,61,74,75]. In this study, taxa were selected from across the main clades of living land plants to investigate whether the current working phylogenetic assumption of bryophyte monophyly affects the estimation of ancestral character states.

A total of 27 extant genera and 23 extinct genera comprise the total taxon sample. The extant sample consists of 24 genera representing members of the major divisions of land plant diversity (3 hornworts, 4 liverworts, 4 mosses, 4 lycophytes, 4 eusporangiate ferns,

4 leptosporangiate ferns and 1 spermatophyte) and 3 algal genera representing successive outgroups to Embryophyta (including the Zygnematophyceae, sister taxon to Embryophyta). Published data on sporogenesis is generally limited for all of these groups. For example, regarding the bryophytes, there are published data on only 3 hornwort taxa, ~40 liverwort taxa and ~25 moss taxa, and most of these reports have incomplete character coverage. We selected taxa that are well known in terms of sporogenesis characters while also attempting to use the few representative taxa for each group to cover phylogeny without being biased towards early divergent forms or model taxa.

The fossil taxon sample consisted of 11 plant megafossil taxa of known phylogenetic affinity and 12 dispersed spore taxa. The former consist primarily of fossil tracheophytes; this reflects both a general paucity of unequivocal bryophyte fossils occurring before the Devonian and the small number of early land plant fossil taxa that yield in situ spores that have been studied ultrastructurally[76]. The dispersed palynomorph taxon includes examples of Middle Ordovician and younger 'traditional' cryptospores (permanently fused tetrads and dyads and hilate cryptospores derived from dyads) and more controversial Cambrian 'cryptospores' from the Bright Angel Shale. Fossil genera were chosen to be positioned close to the internal nodes of the extant phylogeny and for sufficient availability of published ultrastructural data from transmission electron microscopy studies that could be used to populate the character matrix.

Ancestral state estimation

ASE was based on three different phylogenetic timetrees (Fig. 2). The first contained the extant taxon sample based only on the phylogeny recovered by a previous molecular phylogenetic study[14]. Node ages in this timetree were fixed to the mean divergence times estimated in ref. 14. The taxon sample used in this study did not match exactly, so four taxa (*Anemia*, *Ophioglossum*, *Riccardia* and *Apotreubia*) were grafted on using divergence time estimates from ref. 77 instead. Simulation studies have shown that the inclusion of fossil taxa is important to return more accurate results by breaking long branches and increasing the number of tips[66,78]. Thus, two further timetrees were constructed, both of which contained fossil genera that can be placed phylogenetically. These trees differed only in the placement of the eophytes *Lenticulatheca* and *Partitatheca*, representing their unresolved position as either stem-tracheophytes or stem-embryophytes[34,39]. As yet, there is no consensus understanding of the phylogenetic position of the diversity of early land plant megafossils within an updated framework of bryophyte monophyly. The position of each fossil taxon in the phylogeny was therefore taken from published cladistic studies[47–49], tip ages were defined as the minimum date of the scored specimen based on published ages[34,39,79–88], and their divergence times were calculated to bisect the relevant branch of the extant phylogeny according to the insertion point of the fossil taxon.

The ancestral character states for all characters were then estimated across these three phylogenetic hypotheses under four different analytical conditions to understand the effect of fossil taxon inclusion and evolutionary model, given that there is little agreement about the best conditions under which to perform ASE[65,66]. Probabilistic node states were calculated in the following ways: (a) node states were estimated under maximum parsimony using the function ancPropStateMat in the R package paleotree v.3.4.7 (ref. 50), (b) node states were calculated under maximum likelihood applying the equal-rates model of evolution using the ancr function in phytools v.2.4.4 (refs. 51,52), (c) maximum likelihood was used to calculate node states under the all-rates-different model[53] using the ancr function and (d) stochastic character mapping[54] was used to generate ancestral states under a Bayesian Markov chain Monte Carlo approach, using the make.simmap function in the R package phytools, itself an extension of the ace function in the R package ape v.5.8.1 (ref. 55). The algorithm used the equal-rates model of evolution and ran for 10,000 generations (sampling every 10). The resulting sample of character maps was used to generate the posterior probability distribution for node states throughout each phylogenetic hypothesis. The results of all ASE analyses are provided in Supplementary Figs. 1–56 and summarized in Figs. 3 and 4.

Divergence time estimation

To assess the effect of newly derived fossil calibrations on the origin of the embryophyte clade, divergence time estimation was carried out using amino acid sequence and tree topology data published in ref. 14. Fossil calibrations for key nodes were defined as equal probability distributions between minimum and maximum constraints, with a 2.5% probability of either the minimum or the maximum being exceeded. Most fossil calibrations were borrowed from previous studies[10,14,89], while calibrations for the divergence of Embryophyta from the streptophytes, the divergence of Embryophyta into Tracheophyta and Bryophyta, and the divergence of Tracheophyta into Euphyllophyta and Lycopodiophyta were defined anew. In defining these calibrations, best practice as outlined in ref. 58 was followed, and the full calibrations are provided in Supplementary Table 4. Branch lengths were initially estimated using codeml v.4.10.7 (ref. 90). The divergence time estimation was performed using the analytical conditions published in ref. 14 using the normal approximation method in v.4.10.7 (ref. 90). The Markov chain Monte Carlo run was first performed without data to assess the effective prior and then performed with sequence data for 1,250,000 generations (sampling every 250) to achieve convergence with a 25% burn in. Convergence was assessed in Tracer v.1.7.2 (ref. 91).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The source data used in this study are available via data.bris at https://doi.org/10.5523/bris.my0fgbqr45ir2b4tva1tachu7.

Code availability

The code and software command files used in this study, along with the outputs, are available via data.bris at https://doi.org/10.5523/bris.my0fgbqr45ir2b4tva1tachu7.

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Author contributions

J.W.C. and P.C.J.D. conceived and designed the study. P.C.J.D. supervised the project and obtained the funding. J.H., C.H.W. and A.M.W. compiled the phenotypic dataset. J.H. and J.W.C. performed the analyses. J.H. produced the original draft of the paper. All authors contributed to the paper and approved the final version.

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Competing interests

The authors declare no competing interests.

Additional information

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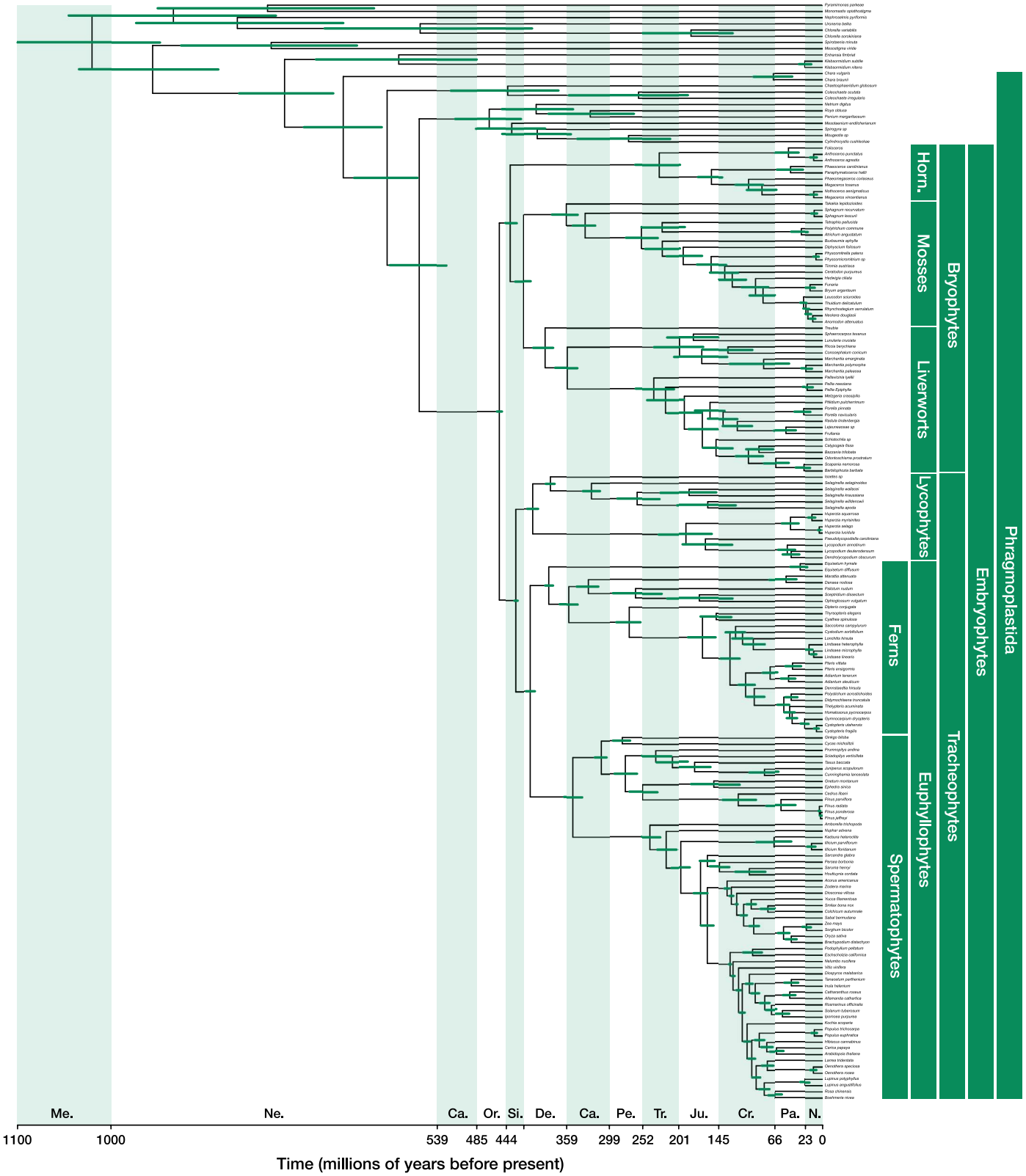
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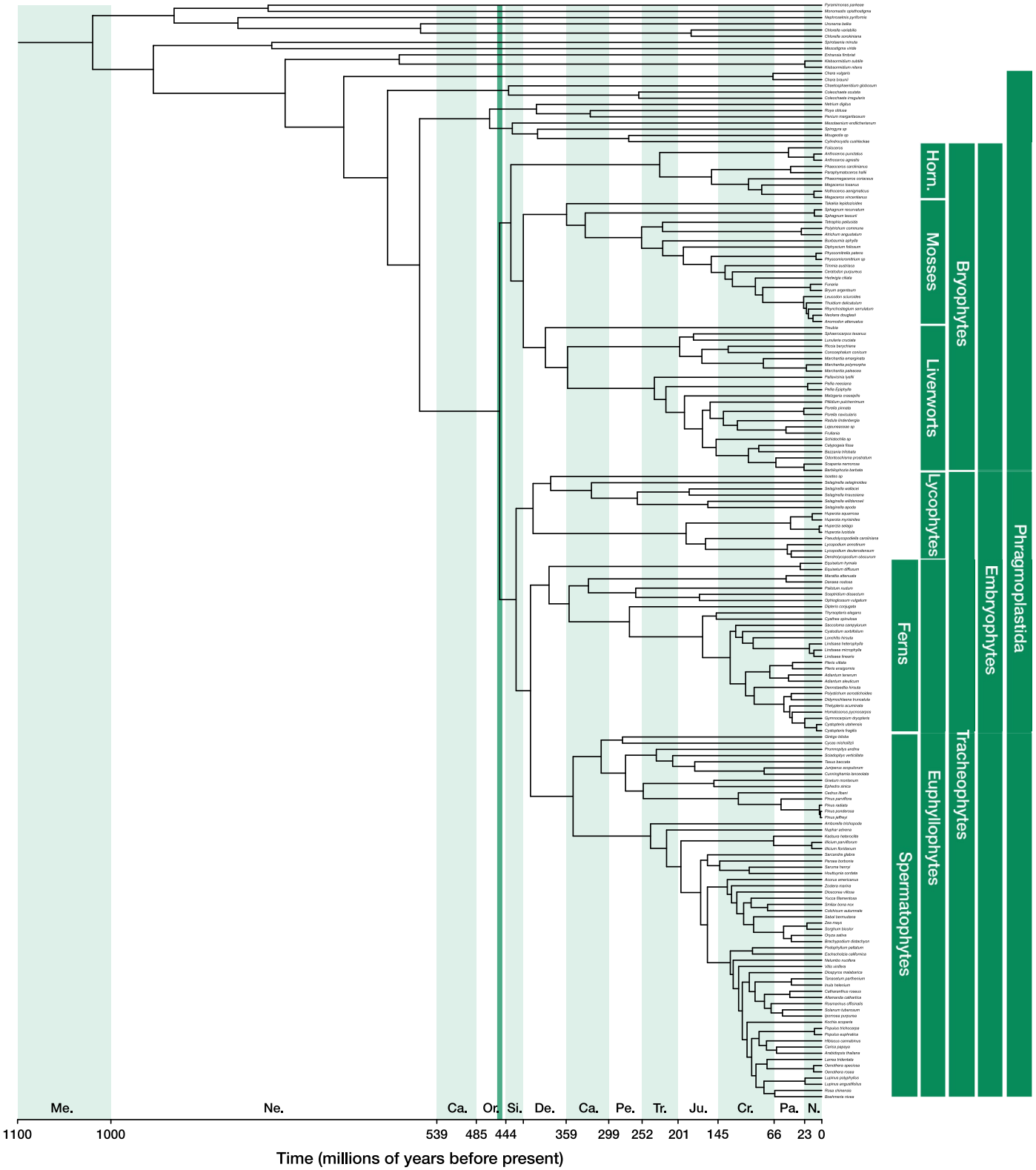
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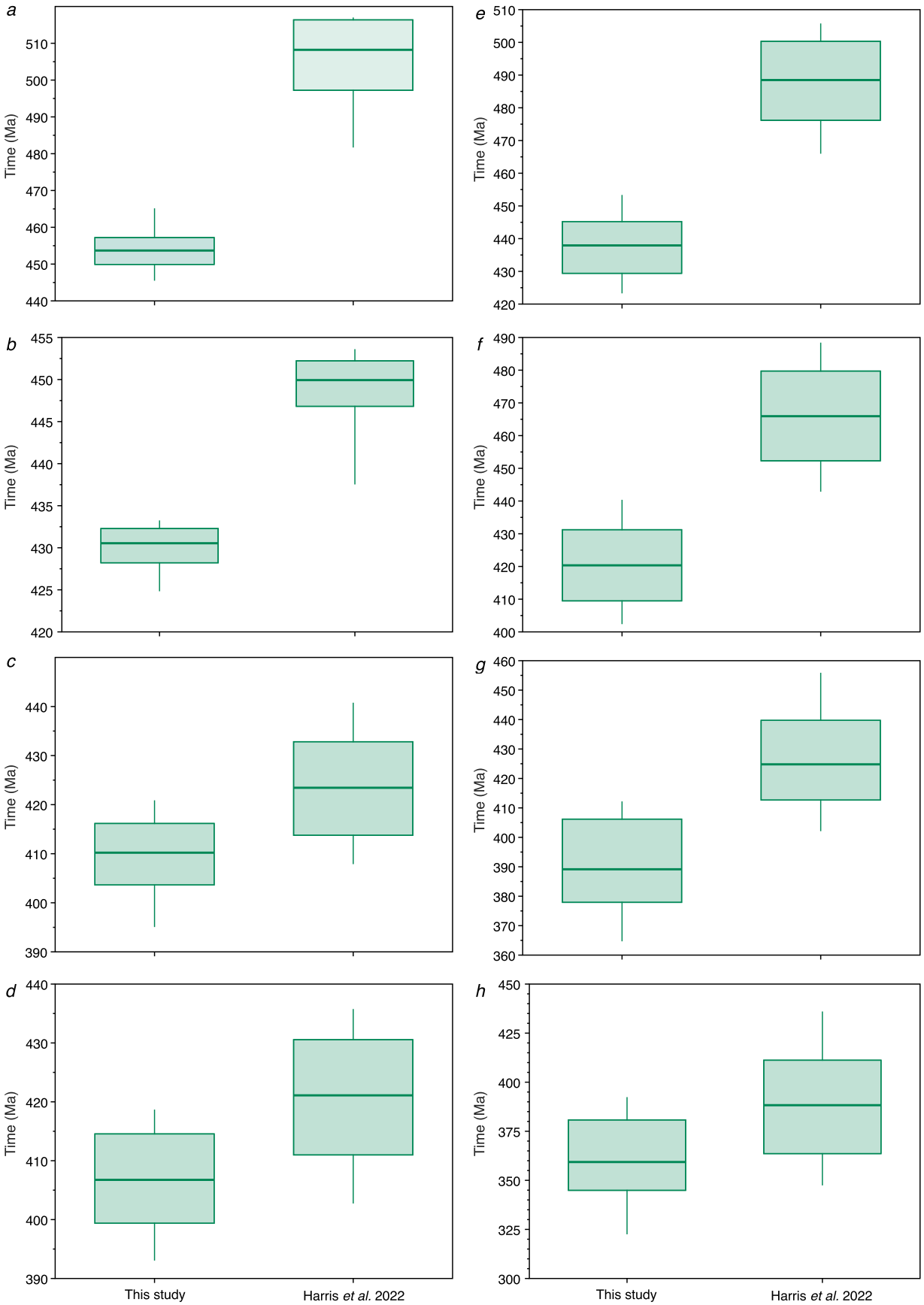
Extended Data Fig. 1 | Divergence time estimates for nodes in the Viridiplantae in millions of years before present, as inferred using a molecular clock model and 68 fossil calibrations, based on Harris et al.[14]. Nodes are positioned on the mean age estimate, and the green bars represent the 95% highest posterior density distribution.

https://doi.org/10.1038/s41477-026-02432-3



Extended Data Fig. 2 | Divergence time estimate for the divergence of crown-Embryophyta in millions of years before present, as inferred using a molecular clock model and 68 fossil calibrations. Nodes are positioned on the mean age estimate, and the green bar represents the width of the 95% highest posterior density distribution for the divergence of node 184, crown-Embryophyta.

https://doi.org/10.1038/s41477-026-02432-3



Extended Data Fig. 3 | Comparison between divergence time estimates derived in this study and those of[44], produced in Tracer[91]. Boxes represent the 95% highest posterior density intervals, whiskers represent the full range of divergence time estimates sampled in each run, the central line in each box is placed on the mean age estimate. **a:** Node 184, crown-Embryophyta. **b:** Node 185, crown-Tracheophyta. **c:** Node 186, crown-Euphyllophyta. **d:** Node 272, crown-Lycophyta. **e:** Node 285: crown-Bryophyta. **f:** Node 286, crown-Setaphyta. **g:** Node 287, crown-Marchantiophyta. **h:** Node 310, crown-Bryopsida.

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Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
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☒	☐ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☐	☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Not applicable.
Data analysis	The ancestral state estimation was conducted using the R libraries paleotree (Bapst 2012. Methods Ecol. Evol. 3: 803-807), phytools (Revell 2012 Methods Ecol. Evol. 3: 217-223), and ape (Paradis, E. & Schliep, K. 2019 Bioinformatics 35, 526–528); the molecular clock analysis was conducted using the PAML package (Yang 2007 MBE 24: 1586-1591).

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The source data, code and software command files used in this study, along with the outputs, are available via data.bris at: https://data.bris.ac.uk/data/dataset/XXXXX

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	Not applicable
Reporting on race, ethnicity, or other socially relevant groupings	Not applicable
Population characteristics	Not applicable
Recruitment	Not applicable
Ethics oversight	Not applicable

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	The study entailed assembly of a dataset characterising the nature of the spores in lineages of living and fossil land plants. These data were used to infer the evolution of spore characteristics in early land plant evolution. The results of this analysis were used to reinterpret the fossil record of land plant spores, the results of which were used to inform calibrations for a molecular clock analysis.
Research sample	Literature-based sampling of spore characteristics among the principle lineages of land plants.
Sampling strategy	Representatives of each of the fundamental lineages of land plants and their streptophyte algal relatives were sampled.
Data collection	Literature-based survey.
Timing and spatial scale	Not applicable.
Data exclusions	No data were excluded from analysis.
Reproducibility	We used MCMC-based methods to infer ancestral states and our evolutionary timescale.
Randomization	Representatives of each of the fundamental lineages of land plants and their streptophyte algal relatives were sampled.
Blinding	Representatives of each of the fundamental lineages of land plants and their streptophyte algal relatives were sampled.

Did the study involve field work? ☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Plants

Seed stocks	Not applicable
Novel plant genotypes	Not applicable
Authentication	Not applicable