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REVIEW

What can lycophytes teach us about plant evolution and development? Modern perspectives on an ancient lineage

Victoria Spencer | **Zoe Nemec Venza** | **Cecily Jill Harrison**

School of Biological Sciences, The University of Bristol, Bristol, UK

Correspondence
Cecily Jill Harrison, School of Biological Sciences, The University of Bristol, Life Sciences Bldg, 24 Tyndall Ave, Bristol, BS8 1TQ, UK.
Email: jill.harrison@bristol.ac.uk

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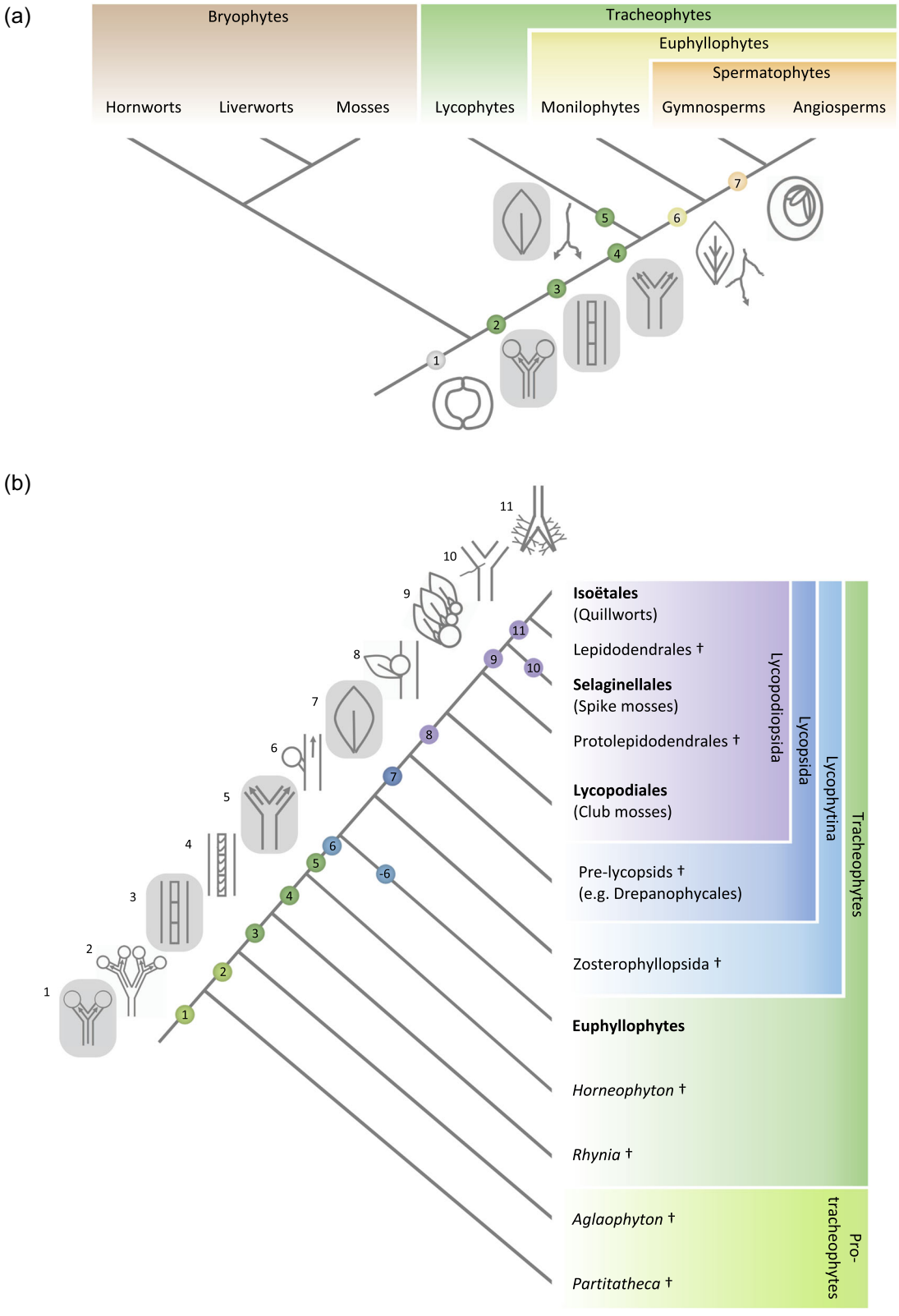
Abstract

All Evo-Devo studies rely on representative sampling across the tree of interest to elucidate evolutionary trajectories through time. In land plants, genetic resources are well established in model species representing lineages including bryophytes (mosses, liverworts, and hornworts), monilophytes (ferns and allies), and seed plants (gymnosperms and flowering plants), but few resources are available for lycophytes (club mosses, spike mosses, and quillworts). Living lycophytes are a sister group to the euphyllophytes (the fern and seed plant clade), and have retained several ancestral morphological traits despite divergence from a common ancestor of vascular plants around 420 million years ago. This sister relationship offers a unique opportunity to study the conservation of traits such as sporophyte branching, vasculature, and indeterminacy, as well as the convergent evolution of traits such as leaves and roots which have evolved independently in each vascular plant lineage. To elucidate the evolution of vascular development and leaf formation, molecular studies using RNA Seq, quantitative reverse transcription polymerase chain reaction, in situ hybridisation and phylogenetics have revealed the diversification and expression patterns of KNOX, ARP, HD-ZIP, KANADI, and WOX gene families in lycophytes. However, the molecular basis of further trait evolution is not known. Here we describe morphological traits of living lycophytes and their extinct relatives, consider the molecular underpinnings of trait evolution and discuss future research required in lycophytes to understand the key evolutionary innovations enabling the growth and development of all vascular plants.

KEYWORDS
clubmoss, Evo-devo, lycophytes, quillwort, *Selaginella*, spikemoss, vasculature

1 | INTRODUCTION

Since plants emerged on land around 470 million years ago (Morris et al., 2018), a series of evolutionary innovations enabled their radiation and occupation of diverse ecological niches across the globe (Figure 1). An ancient evolutionary divergence gave rise to today's dominant vascular plant flora and the bryophyte sister lineage of vascular plants (Puttick et al., 2018). Provascular fossil intermediaries have vastly different forms from either of these groups, showing incremental acquisition of sporophyte branching, differentiated vasculature, and


(a)
Bryophytes
Tracheophytes
Euphyllophytes
Spermatophytes
Hornworts
Liverworts
Mosses
Lycophytes
Monilophytes
Gymnosperms
Angiosperms
1
2
3
4
5
6
7
(b)
1
2
3
4
5
6
7
8
9
10
11
-6
Isoëtales
(Quillworts)
Lepidodendrales †
Selaginellales
(Spike mosses)
Protolepidodendrales †
Lycopodiales
(Club mosses)
Pre-lycopsids †
(e.g. Drepanophycales)
Zosterophyllopsida †
Euphyllophytes
Horneophyton †
Rhynia †
Aglaophyton †
Partitatheca †
Lycopodiopsida
Lycopsida
Lycophytina
Tracheophytes
Pro-tracheophytes

indeterminate growth from a shoot tip (Cascales-Miñana, Steemans, Servais, Lepot, & Gerrienne, 2019; Edwards, 1986; Edwards and Kenrick, 2015; Gensel and Berry, 2001; Harrison, 2017b; Lang, 1937). These traits contributed to the rise of vascular plants and set a platform for all subsequent plant radiations (Figure 1a). Now a diminutive group, lycophytes are the living clade most similar to early vascular plants of the fossil record, but lycophytes once comprised a prolific and abundant part of the biosphere, massively impacting biodiversity, soil production (Kenrick & Strullu-Derrien, 2014), and CO_2 sequestration (Beerling & Berner, 2005; Gensel & Berry, 2001). As such, lycophytes are well placed to elucidate innovations in the ancestors of vascular plants, and answer evolutionary questions about the conservation, convergence, and divergence of developmental processes in plant diversification. The phylogeny and anatomy of lycophytes is well characterised (Figure 1b; Gensel & Berry, 2001; Jernstedt, Cutter, Gifford, & Lu, 1992; Jernstedt & Mansfield, 1985; Kenrick & Crane, 1997; Lu & Jernstedt, 1996), and there has been a recent surge of interest in establishing lycophyte genetic models (Table 1). This review aims to provide a comprehensive overview of developmental and molecular studies in lycophytes for use as a platform to future research.

2 | THE DIVERSIFICATION OF LYCOPHYTES

Modern lycophytes have a widespread distribution and typically grow as understory herbs, but also grow in freshwater (e.g. *Isoëtes lacustris*), in ephemeral pools (e.g. *Phylloglossum drummondii*) or as epiphytes (e.g. *Lycopodium phlegmaria*; Sporne, 1962). The growth forms of lycophytes vary dramatically (Figure 2). Whereas *Selaginella* forms bifurcating prostrate shoots (Figure 2a,b), *Phylloglossum drummondii* has a long thickened stem from which narrow leaves and elongated tubers emerge at the base (Figure 2c) and *Isoëtes* forms a corm with leaves and rooting structures protruding from a central swollen stem (Figure 2d; Gifford & Foster, 1989; Sporne, 1962). While many lycophytes are common in the humid tropics (e.g. *Selaginella kraussiana*), others are well known for their ability to survive desert conditions (e.g. *Selaginella lepidophylla*, the "Resurrection Plant"; Pampurova & Van Dijck, 2014; Zentella et al., 1999). Some species can also tolerate arctic and alpine conditions, particularly in *Lycopodium* (Sporne, 1962; Svensson & Callaghan, 1988). Clearly, the lycophyte radiation involved adaptions to a range of environments, and these enabled lycophytes' persistence among an increasingly dominant angiosperm flora.

The Silurian, Devonian, and Carboniferous eras had vastly different landscapes from today, including lycophyte forests with trees over 30 m tall (Bateman et al., 1998; Gensel & Berry, 2001; Thomas & Watson, 1976). Although some fossils are difficult to place, lycophytes (Lycophytina) comprise two main lineages, the Zosterophyllopsida and Lycopsida (Figure 1b; Gensel & Berry, 2001). Zosterophylls are extinct, but Lycopsida includes living lycophytes and their extinct prelycopsid sister lineages (Figure 1b; Gensel & Berry, 2001; Kenrick & Crane, 1997). Whereas zosterophylls lack leaves and have lateral globose to reniform sporangia borne on sporangial stalks, Lycopsida have shared characteristics such as leaves with a central vascular strand, vasculature with a unique arrangement of tissues, sporangial dehiscence

FIGURE 1 Trait evolution in land plants and lycophytes. (a) Current phylogenetic hypotheses of land plant evolution support bryophytes as a monophyletic sister lineage to tracheophytes (Puttick et al., 2018). Trait innovations likely to have a single origin in the radiation of land plants include stomata to regulate gas exchange and water loss (1), apical branching in the sporophyte (Edwards et al., 2014) (2), specialisation of conducting cells of the xylem (tracheids; Cascales-Miñana et al., 2019) (3), indeterminacy (Coudert et al., 2019) (4), and enclosure of the embryo in a seed (7). Current fossil evidence supports the sequence of trait evolution shown in the figure (Harrison & Morris, 2018). Other innovations such as leaves and roots are thought to have evolved independently at least in the lycophyte lineage (5) and the euphyllophyte lineage (6). Traits highlighted in grey boxes are also represented in part B. (b) Phylogeny showing relationships between extant (in bold; Euphyllophytes, Lycopodiales, Selaginellales, and Isoëtales) and extinct (†; *Partitatheca*, *Aglaophyton*, *Rhynia*, *Horneophyton*, Zosterophyllopsida, Drepanophycales, Protolepidodendrales, and Lepidodendrales) land plant clades (Cascales-Miñana et al., 2019; Gensel & Berry, 2001; Schuettpelz et al., 2016). The last shared common ancestor of vascular plants had branching sporophytes (1; Boyce, 2008; Edwards & Kenrick, 2015), and fossils show a stepwise acquisition of higher order branching (2; Kenrick & Crane, 1997), specialised vascular cells (3; Cascales-Miñana et al., 2019), annular and/or spiral xylem tracheid thickening (4; Cascales-Miñana et al., 2019), shoot meristem indeterminacy (5; Harrison, 2017b), sporangia on short lateral branches (6; Gensel & Berry, 2001), vegetative leaves with a single vascular trace (7; Gifford & Foster, 1989; Gola et al., 2007), and sporangia with a subtending leaf (sporophyll) (8; Sporne, 1962). Lycopodiales and Protolepidodendrales have/had spores that are all the same size (homospory) and no ligule at the base of the growing leaf. The Selaginellales, Lepidodendrales and Isoëtales on the other hand have/had different size spores on the same plant (heterospory) (9) and a small ligule growing at the base of the developing leaf (Sporne, 1962). Selaginellales is the only order that has an angle meristem which has the ability to become a root or a shoot (10; Banks, 2009). Lepidodendrales and Isoëtales both have/had rhizomorphs from which branched rootlets form (11; Hetherington et al., 2016) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Summary of molecular and genetic experiments performed in lycophytes

	Flow cytometry	Genome sequence	Transcriptome	Protoplast extraction	Gene cloning	RT-PCR	In situ hybridisation
Selaginella moellendorffii	1	1, 2	3-10	5, 11	10–14	14-16	6, 16, 17
Selaginella kraussiana	1, 18	18	9, 10, 18		19–21	18, 22	20, 21, 23, 24
Selaginella uncinata					25		25
Selaginella lepidophylla					26, 27		
Selaginella erthropus					26		
Selaginella deticulata					26		
Selaginella remotifolia					28	28	
Selaginella willdenowii			9				
Selaginella selaginoides			9				
Selaginella acathonota			9				
Selaginella wallacei			9				
Selaginella cf. pallescens			9		14	14	
Selaginella apoda			9				
Selaginella martensii			10				
Isoëtes echinospora			29				
Isoëtes drummondii			10, 30				
Isoëtes engelmanii	1						
Isoëtes tegetiformans			9				
Isoëtes yungviensis					31		
Isoëtes hypsophila					31		
Isoëtes sinensis					31	31	31
Isoëtes orientalis					31		
Isoëtes taiwanensis					31		
Phylloglossum drummondii			9, 30				
Huperzia selago			9, 32		32		32
Huperzia serrata			33				
Huperzia myrsinites			9				
Huperzia squarrosa			9				
Huperzia lucidula	1		9		34		
Diphasiatrum digitatum	1		9				
Lycopodium annotinum			9		35, 36	35, 36	
Lycopodium deuterodensum			9				
Dendrolycopodium obscurum			9				
Lycopediella appressa			9				

TABLE 1 (Continued)

	Flow cytometry	Genome sequence	Transcriptome	Protoplast extraction	Gene cloning	RT-PCR	In situ hybridisation
Pseudolycopodiella caroliniana			9				

Note: Table showing each molecular and genetic experiment performed in different lycophyte species. The majority of studies have been performed in *Selaginella moellendorffii*, *Selaginella kraussiana*, *Huperzia selago*, and *Huperzia lucidula* (Wang et al., 2005; Banks et al., 2011; Weng et al., 2005; Zhu et al., 2017; Mello et al., 2019; Frank et al., 2015; Huang and Schiefelbein, 2015; Ferrari et al., 2020; Leebens-Mack, 2019; James et al., 2017; Yin et al., 2009; Moody et al., 2012; Zhang et al., 2019; Kwantes et al., 2012; Kirkbride et al., 2013; Aya et al., 2011; Zumajo-Cardona et al., 2019; Ge et al., 2016; Hirakawa and Bowman, 2015; Harrison et al., 2005; Floyd & Bowman, 2006; Ocheretina et al., 2000; Prigge and Clark, 2006; Floyd et al., 2006; Kawai et al., 2010; Hedman et al., 2009; Zentella et al., 1999; Tanabe et al., 2003; Hetherington et al., 2019; Dixon et al., 2016; Yang et al.,2017; Evkaikina et al., 2017; Luo et al., 2010; Floyd et al., 2014; Svensson et al., 2000; Svensson and Engström, 2002).

and reniform sporangia borne on specialised sporophyll leaves (Figure 1b; Gensel & Berry, 2001; Kenrick & Crane, 1997). However, some fossils appear transitional, lacking some lycopsid features. For example, *Asteroxylon* lacked fully vascularised leaves, and both *Asteroxylon* and *Drepanophycus* lacked sporangial leaves. These species have therefore been placed in a "prelycopsid" clade by some authors (Gensel & Berry, 2001).

After diverging from prelycopsids, lycophytes formed many abundant and species rich lineages. The Protolepidodendrales were present in the early-mid Devonian era and had elaborate leaf shapes, sporophylls that were morphologically similar to vegetative leaves and sporangia with a dehiscence line along their margin (Sporne, 1962). Many Protolepidodendrales had similar growth habits to their modern lycophyte relatives, for instance, the dichotomously branching creeping shoots of *Leclercqia* resemble *Lycopodium* (Bonamo, Banks, & Grierson, 1988). However, some species such as *Chamaedendron multisporangiatum* and *Longostachys latisporophyllus* from the Middle Devonian had tree-like growth habits with distinct vertical shoots and a cone of dichotomously branching roots (Cai & Chen, 1996; Schweitzer & Li, 1996). Lycopsid forests from the early-Late Devonian have been found at Svalbard, where larger lycopsid trees display cormose rooting systems resembling the rooting systems of extant lycophytes in the Isoëtales order (Berry & Marshall, 2015). Perhaps the most successful group of arborescent lycopsids were the Lepidodendrales, which grew a tall upright trunk with a dichotomously branching crown and elaborate dichotomous rooting systems known as rhizomorphs, morphologically resembling shoots (Gensel & Berry, 2001; Sporne, 1962). Rhizomorphs had lateral appendages known as stigmarian rootlets, which were abscised during the plant's development leaving helically arranged circular scars (Taylor, Taylor, & Krings, 2008). It was recently shown that these stigmarian rootlets were highly branched and covered in root hairs, much like the roots of their closest modern relatives, the Isoëtales (Figure 1b; Hetherington, Berry, & Dolan, 2016). By late Devonian and Carboniferous periods, the Lepidodendrales became dominant, and up to 200 species have been recorded (Sporne, 1962). These lycophytes were responsible for massive sequestration of CO_2 from the atmosphere, and produced the majority of coal and oil that fuelled the industrial revolution (Beerling & Berner, 2005).

Despite their previous abundance and success in geological history, lycophytes now represent a small proportion of plant diversity, and it is thought that glaciation events, prolonged drought, and subsequent out-competition by tree ferns and conifers contributed to their demise (Falcon-Lang & Dimichele, 2010). There are three remaining Lycopodiopsida orders, known as the Lycopodiales, Isoëtales and Selaginellales (Figure 1b; Schuettpelz et al., 2016). The Selaginellales comprises 1 family, 1 genus, and 700 species, and cretaceous amber fossils have shown that *Selaginella* was prolific and speciose even during the rise of angiosperms (Schmidt et al., 2020), persisting as the most species diverse lycophyte genus. Lycopodiales comprises 1 family, 16 genera and an estimated 388 species, and Isoëtales comprises 1 family, 1 genus and 250 species (Schuettpelz et al., 2016). Lycopodiales are commonly known as club mosses and fir mosses, the Isoëtales as quillworts and the Selaginellales as spike mosses (Figure 1b). While the Lycopodiales are characterised by equally sized spores (homospory) and a lack of leaf ligules, the Selaginellales and Isoëtales have different sized spores (they are heterosporous) and have a ligule on the adaxial side of leaves (Figure 1b; Sporne, 1962). Four-sided strobili with distinct sporangia types and distributions discriminate Selaginellales from Isoëtales, with Isoëtales sporangia being much larger and more productive (Kenrick & Crane, 1997; Sporne, 1962). Reminiscent of Lepidodendrales, Isoëtales are typically cormose or rhizomatous with small branched rhizoids and have a basal rosette which widens by secondary growth (Figures 1b and 2d; Pigg, 2001), whereas Selaginellales only grow from apical

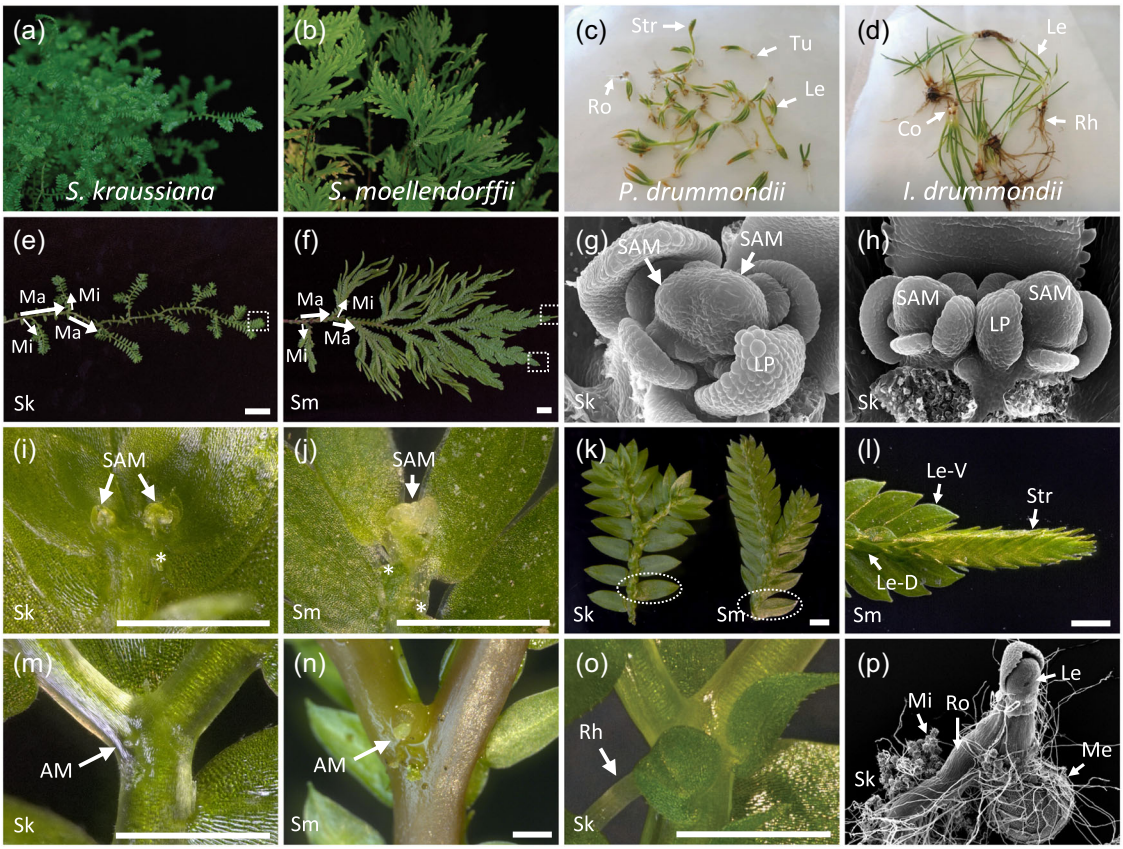


FIGURE 2 Morphologies of extant Lycophytes.(a–d) Four species of lycophyte with diverse growth habits. (a) *Selaginella kraussiana* (Sk) grows as a bifurcating creeping herb. (b) *Selaginella moellendorffii* (Sm) is a herb. (c) *Phylloglossum drummondii* has an elongated reproductive strobilus (Str) that produces spores, long vegetative leaves (Le), short roots (Ro) and swollen tubers (Tu) that all emerge from the base of the plant. (d) *Isoëtes drummondii* has a corm (Co) from which elongated leaves (Le) and short rhizoids (Rh) are produced. (e–p) *S. kraussiana* and *S. moellendorffii* development is well characterised. (**e**) The *S. kraussiana* shoot bifurcates to give shoots a zig-zag morphology, with alternating major (Ma) and minor (Mi) branches. (f) *S. moellendorffii* branches arising from each bifurcation event also grow unequally. White dotted boxes in (e) and (f) show tissue magnified in (k) and (l). (g–j) SAM of *S. kraussiana* (g–i) and *S. moellendorffii* (j). (g) SEM image of a bifurcating *S. kraussiana* SAM surrounded by leaf primordia (LP). (h) SEM image of a *S. kraussiana* shoot apex shortly after bifurcation. (i–j) SAM of *S. kraussiana* (i) and *S. moellendorffii* (j). Dorsal leaves have been removed from positions marked with *. (k) In both *Selaginella* species, leaves are paired and lanceolate, with one large ventral leaf and one smaller dorsal leaf. Leaf pairs in each species are shown in white dotted ellipses. (l) *S. moellendorffii* strobilus (Str), which produces sporangia. Dorsal leaves (Le-D) are small and ventral leaves (Le-V) are larger. (m,n) Angle meristems (AM) before they differentiate into a shoot or rhizophore, emerging at *S. kraussiana* (m) and *S. moellendorffii* (n) branch junctions. (o) Rhizophore (Rh) emerging from the angle meristem of *S. kraussiana*. (p) After fertilisation of an egg, and following embryogenesis, a new sporophyte emerges from the megaspore coat (Me). The sporeling has two embryonic leaves (Le), clear apical basal polarity and a laterally arising root (Ro). Microspores (Mi) are considerably smaller than the megaspore. (e,f) Scale bars = 10mm. (i–o) Scale bars = 1mm. Images in (c) and (d) were kindly provided by Josh Mylne. SAM, shoot apical meristems [Color figure can be viewed at wileyonlinelibrary.com]

meristems or angle meristems found at branch points (Figures 1b and 2g–j,m,n; Banks, 2009).

3 | TRAIT EVOLUTION

3.1 | A single origin for branching

Vascular plants last shared a common ancestor in the Silurian era and have a suite of shared traits that are likely to be ancestral, but other traits have been lost or transformed in different vascular plant lineages (trait divergence), or have similar morphology but multiple independent origins (trait convergence; Figure 1; Harrison & Morris, 2018). All modern vascular plants have retained an ancestral ability to branch (Figure 1a), but branching patterns have diverged during evolution to give plants a range of architectures (Figure 2a–f; Chomicki, Coiro, & Renner, 2017; Hallé, 1986). While angiosperms produce lateral foliar primordia with axillary buds and branches from the main shoot apical meristem (SAM; Domagalska and Leyser, 2011; Q. Wang,

Kohlen, Rossmann, Vernoux, & Theres, 2014), the ancestral pattern of branching in vascular plants involves splitting the shoot tip to generate two new branches (Figure 2g–j). This process is known as bifurcation or dichotomy (Harrison, 2017a; Harrison and Morris, 2018), and dichotomising shoot systems have diversified into a variety of forms in lycophytes. For instance, some zosterophylls such as *Gosslingia* have planar pseudomonopodial growth (Edwards, 1970; Sporne, 1962). Although the molecular regulators of bifurcation are unknown, the cellular dynamics of bifurcation are well characterised in *Selaginella kraussiana* and *Huperzia lucidula*, where the apical cells amplify and are then partitioned to initiate growth of the new branches (Gola & Jernstedt, 2011; Harrison & Langdale, 2010; Harrison, Rezvani, & Langdale, 2007). Daughter branches arising from a bifurcation event can either be the same size and set to the converse angle to the parent branch (isotomous branching), or unequal sizes and/or angles (anisotomous branching). Selaginellales can show weak anisotomy with alternating dominant branches producing a zigzagging architecture (*S. kraussiana,* Figure 2e; Harrison et al., 2007; Jernstedt et al., 1992), or can have strong anisotomy, for instance producing a frond-like structure (*S. moellendorffii,* Figure 2f; Banks, 2009). Branches can also be angled differently in 3D space, to produce prostrate or more scrambling shoots. Unlike the Selaginellaceae, *Isoëtes* forms branches from the central stem organ and has a fan-type structure (Figure 2d; Sporne, 1962). Lycopodiales on the other hand have very diverse branching patterns, from erect stems with leaves and roots branching laterally from the base (*Phylloglossum drummondii;* Figure 2c), to dominant horizontal growth axes with minor vertical side branches (*Lycopodium cernuum*; Sporne, 1962). Lycophytes thus include a range of models suitable for identifying ancestral mechanisms of branching and reveal mechanisms involved in trait divergence.

3.2 | A single origin for vascular transport

Following the evolution of branching, tracheophytes evolved xylem tracheids for water transport (Figures 1 and 3), allowing them to grow taller and probably improve spore spread (Niklas & Kerchner, 1984). While xylem is a defining feature of vascular plants, xylem cell size and shape has diversified throughout the tracheophyte lineage (Figure 3). Tracheary elements of the xylem die to form long and hollow strands to improve water flow and are also thickened with lignin for waterproofing and structural support (Gifford & Foster, 1989). In contrast to vascular plants, bryophytes have large water-conducting cells (WCCs; Figure 3), which can either be perforate or imperforate (hydroids), but lack a lignified cell wall (Ligrone, Duckett, & Renzaglia, 2000). Intermediate vascular structures are found in the fossil record, supporting the stepwise evolution of complex vasculature. For instance, *Rhynia* tracheids are only partially thickened, while *Horneophyton lignieri* tracheids have annular to helical cell wall thickening commonly found in living vascular plants (Figure 3; Cascales-Miñana et al., 2019). Thickenings such as these may have been important for maintaining turgor pressure and increasing plant height, and lycophyte size correlates with increasing vascular complexity in the fossil record. This correlation has been used to model and predict fossil plant heights (Bateman et al., 1998), and the arborescent growth habit of 40-m tall Lepidodendrids would have required elaborate vascular systems to provide water to the canopy (Pittermann, 2010).

3.3 | Diversification of vascular tissue arrangements

In living vascular plants, the procambium produces small protoxylem cells and then larger metaxylem cells to form the primary xylem. There are converse patterns of xylem development in lycophytes and euphyllophytes. While lycophyte root protoxylem develops internally relative to the metaxylem (endarch) and shoot protoxylem develops externally relative to the metaxylem (exarch), euphyllophyte roots are exarch and shoots are endarch to mesarch (protoxylem is surrounded on both sides by metaxylem; Kenrick & Crane, 1997). Euphyllophytes evolved secondary xylem incorporating elongated cells with perforated plates known as vessel elements, which generate wood (Figure 3). Secondary xylem also evolved independently in *Lepidodendron*, conferring the mechanical and conductive properties to support arborescence (Pittermann, 2010). However, an inwards unifacial cambium prevented trunk thickening, possibly limiting height and driving the extinction of *Lepidodendron* in drier climates (Pittermann, 2010). In extinct and extant tracheophytes, the arrangement of the xylem tissue within the stem can be diverse. Lycophytes are typically protostelic (Gola, Jernstedt, & Zagórska-Marek, 2007), having a core of xylem surrounded by phloem and an endodermis, and tissues can be arranged in a lobed central xylem pole (actinostele), numerous xylem poles (plectostele) or a combination of these arrangements (actino-plectostelic, e.g. *Lycopodium clavatum* and *Lycopodium annotinum*; Figure 3; Gola et al., 2007). However, the vascular tissue arrangements of some lycophytes

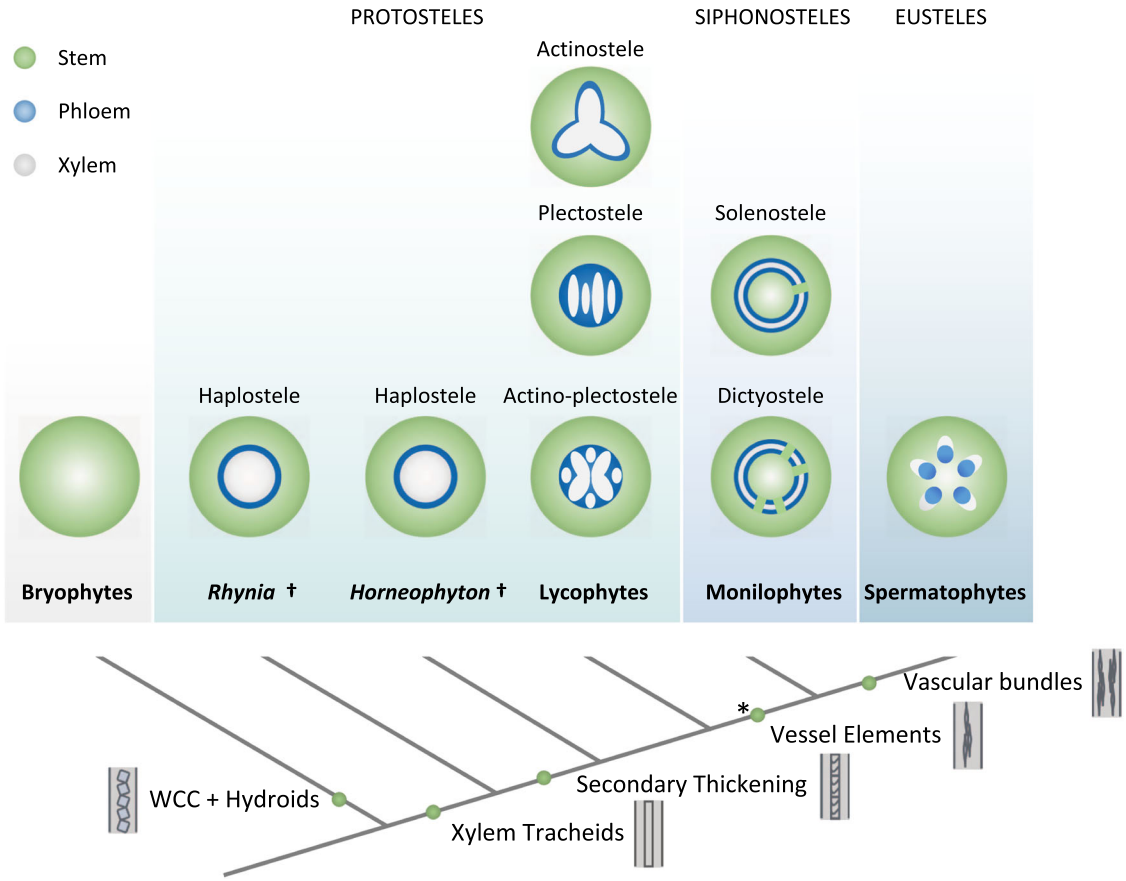


FIGURE 3 Vascular cell and tissue types in land plants. Xylem conformations and phylogeny illustrating the distribution of vascular traits among extinct and extant land plants. Bryophytes do not have xylem but have WCCs known as hydroids, while tracheophytes have xylem tracheids involved in water transport. Fossils such as *Rhynia* have rudimentary xylem tracheids, while *Horneophyton* and extant Lycophyte tracheids have elaborate secondary thickening with lignin. Some euphyllophytes have specialised cells called vessel elements with perforations in the primary cell wall. These are arranged into multiple vascular bundles in spermatophytes. (*) Vessel elements evolved multiple times, including in *Selaginella*, in the *Pteris* fern genus, in Gnetophytes and within angiosperms (Gifford and Foster, 1989). In the diagrams above the phylogeny, the xylem strand (pale grey) is surrounded by phloem (blue) within the stem (green). The xylem strand can be cylindrical (haplostelic, as seen in *Horneophyton* fossils) or lobed (actinostelic, as seen in many club mosses). Alternatively, lycophyte xylem can form many strands within the phloem (plectostelic) or a combination of lobed and multiple strands (actino-plectostelic). Monilophytes have siphonostelic vasculature, in which the xylem has an internal core of parenchyma cells. Monilophytes can have a solenostele, in which only one leaf attachment to the vasculature is evident in a transverse section, or a dictyostele, in which there is evidence of several leaf attachments in a transverse section. Seed plants have a more elaborate eustele, which contains many vascular bundles. WCC, water conducting cells [Color figure can be viewed at wileyonlinelibrary.com]

such as *Selaginella* can be difficult to characterise, as many species can have two or more protosteles (referred to as meristeles), which change their arrangement as the stem develops (Gola & Jernstedt, 2016). In contrast, monilophytes are siphonostelic, having a parenchymatous pith inside the xylem core. Ferns differ from seed plants as the stele has interruptions in the main vascular cylinder at points of leaf insertion known as leaf gaps (solenostelic or dictyostelic), while seed plants can have many vascular bundles around the pith (eustelic; Figure 3; Gifford and Foster, 1989). Clearly, vasculature is common to all tracheophytes but cell type and vascular architectures are highly diverse (Gola & Jernstedt, 2016; Matsunaga, Cullen, & Tomescu, 2017). Such differences should be taken into account in selecting models for vascular evolution and development.

3.4 | The origin of indeterminacy and diversification of shoot apical meristems

A third putative synapomorphy of vascular plants is the capacity to grow and continuously produce new organs and tissues from the shoot apex, a trait known as indeterminacy (Figure 1; Harrison, 2017b). Fossil vascular plants from the late Silurian to early Devonian have

dichotomously branching sporophytes with each branch terminating in sporangia, suggesting that indeterminate shoot growth evolved after vasculature and involved the displacement of terminal sporangia onto short lateral branches (Figure 1b; Boyce, 2008; Edwards, 1986; Edwards, Morris, Richardson, & Kenrick, 2014; Edwards, Richardson, Axe, & Davies, 2012; Harrison, 2017b; Lang, 1937). The production of similar forms in moss mutants (T. A. Bennett et al., 2014; Ortiz-Ramírez et al., 2016) suggests that the earliest vascular plants may have had similar meristematic activities to moss sporophytes, comprising an apical cell and an intercalary proliferative zone subtending sporangia (Coudert, Novák, & Harrison, 2019; Puttick et al., 2018). Early vascular plants could have grown upwards by displacement from a proliferative intercalary zone, rather than by downwards displacement from the shoot apical cells as in euphyllophytes (Coudert et al., 2019; McKim, 2019). While there is no evidence of apical organisations in coalified fossils, silicified shoot apices of the early lycopsid, *Asteroxylon mackiei*, have been preserved (Kidston & Lang, 1917). These show the primordia of leaf-like appendages emerging (Kerp, Wellman, Krings, Kearney, & Hass, 2013), but cannot be used to determine the number of meristematic cells. It is also not possible to infer ancestral apical organisations among lycophytes because living lycophytes have diverse apical organisations comprising a single initial cell or a multicellular meristem. Early anatomical studies in *Selaginella* reported meristems with one or two initial cells, each with two, three, or four cutting faces (Barclay, 1931; Dengler, 1983; Hagemann, 1980; Imaichi & Kato, 1989; Williams, 1931). A more recent clonal sector analysis in *S. kraussiana* showed that two transiently acting apical initials generate the major axis of the shoot (Harrison & Langdale, 2010; Harrison et al., 2007; Jones & Drinnan, 2009). In contrast to Selaginellaceae, Lycopodiales, and *Isoëtes* meristems are multicellular (Philipson, 1990). In a study of the *Huperzia lucidula* meristem surface, the shape of cell packets has been interpreted as the result of four active initials that can be replaced during growth (Gola & Jernstedt, 2011). These meristem types are also distinct in terms of plasmodesmatal networks: Selaginellaceae have a high plasmodesmatal density like ferns, while Lycopodiaceae and Isoëtaceae have lower plasmodesmatal densities, comparable with multicellular seed plant meristems (Imaichi & Hiratsuka, 2007). Although developmental changes involved in the origin of indeterminacy remain obscure, the variation among modern lycophytes and distinction between lycophyte and single stem-celled monilophyte meristems suggest that multicellular shoot meristems evolved by convergence in lycophyte and euphyllophyte lineages (Harrison, 2017b).

3.5 | Multiple origins of leaves

Similarly to meristems, lycophyte leaves had a separate evolutionary origin from euphyllophyte leaves (Figure 1a), and the latter were gained independently in several lineages (Tomescu, 2009). Generally, extant lycophyte leaves have a single vascular trace running along the centre of the blade and the vascular strand connects directly to the protoxylem poles in the stem (Gola et al., 2007). In contrast, euphyllophyte leaves have branched venation whose development is controlled by auxin flow, and there are leaf gaps in the main xylem where the leaf attaches to the stem (Berleth, Mattsson, & Hardtke, 2000). Lycophyte leaf phyllotaxy is species-specific, and can vary from spiral to whorled to opposite, arising independently of xylem patterns in the stem (Gola et al., 2007; Webster, 1992). According to the enation theory, lycophyte leaves arose by progressive elaboration of epidermal outgrowths, into which vascular strands later entered (Bower, 1935). Consistent with this notion, fossils such as *Asteroxylon* have only partially vascularised leaves (Kidston & Lang, 1920), and leaves in the living lycophyte *S. kraussiana* arise from two adjacent epidermal cells growing to form opposite pairs with a small dorsal and larger ventral leaf per pair (Figure 2k; Harrison et al., 2007). During juvenile development, *S. kraussiana* leaves are spirally arranged, and leaf development is plastic, responding to hormonal cues (Sanders & Langdale, 2013). In adult plants, the number of leaves per internode is consistent, with major branches having eight pairs per internode and minor branches having six pairs, and a further pair develops at each branch point (Harrison et al., 2005). Strobili have opposite and decussate leaf pairs as in *S. moellendorffii* (Figure 2l). While *S. kraussiana* and *S. moellendorffii* leaves are lanceolate (Figure 2k–l), other lycophytes have distinctive leaf shapes, such as the needle-like structures of *Isoëtes* (Figure 2d; Sporne, 1962).

3.6 | Origins and diversification of roots

Devonian fossils suggest that roots also evolved independently in lycophytes and euphyllophytes (Figure 1a), and all extant lycophyte roots branch isotomously, and have root hairs and a root cap (Hetherington & Dolan, 2017). Despite high conservation of morphology, lycophyte roots develop from varied structures and cell types, such as basal meristem cells in *Isoëtes*, the internal cell layers of *Selaginella* rhizophores, and superficial cells of tubers in some *Lycopodium* and *Phylloglossum* species (Hetherington & Dolan, 2017). The early Devonian lycophyte *Asteroxylon mackiei* had roots that lacked root hairs, an endodermis and a root cap, but

instead had an intact epidermis that covered the root apex (Hetherington & Dolan, 2018). This suggests that endodermis and root caps also evolved independently in lycophytes and euphyllophytes, perhaps reflecting multiple geological transitions to a more soil-like substrate as land plants became dominant and changed the soil geochemistry (Hetherington & Dolan, 2018). As in lycophyte shoots, roots have diverse meristem organisations (Fujinami et al., 2017). Root meristems in the Selaginellaceae have an apical cell with multiple cutting faces, similar to the single pyramidal apical cell of monilophyte roots. In contrast, the Isoëtaceae and Lycopodiaceae have multicellular root meristems of three main types. Type I meristems in *Lycopodium* have a non-layered group of initial cells with low mitotic activity, much like the Quiescent Centre (QC) of seed plants. *Lycopodiella* and *Huperzia* instead have Type II meristems characterised by layers of epidermal initial cells. The initials produce an epidermal covering similar to *A. mackiei* fossils, but separating the root proper from the external root cap (Fujinami et al., 2017; Hetherington & Dolan, 2018). Finally, Type III root meristems have a layer of initial cells which form both the epidermis and the root cap, as seen in Isoëtaceae (Fujinami et al., 2017). Further studies are needed to determine how these diverse root meristem structures evolved in lycophytes.

3.7 | Origin of a unique lycophyte organ, the rhizophore

While shoots and roots have evolved as similar solutions to plants' sessile habit and resource requirements in diverse lineages, at branch junctions *Selaginella* spp. have a unique organ known as the rhizophore (Figures 1b and 2m–o). Rhizophores are similar to roots in lacking chlorophyll and stomata and having gravitropic development, but are also similar to shoots in lacking root hairs and root caps, so their identity has been debated (Jernstedt & Mansfield, 1985; Jernstedt et al., 1992; Kawai, Tanabe, Soma, & Ito, 2010; Mello, Efroni, Rahni, & Birnbaum, 2019; Webster, 1992; Wochok & Sussex, 1974, 1976). Rhizophores develop from angle meristems (Figure 2m–n), which are typically positioned on both the dorsal and ventral sides of each branch point in *Selaginella* (Banks, 2009). The number of angle meristems at each branch point varies between species, and the identity of angle meristems shows plasticity, sometimes attaining shoot fate over rhizophore fate. In *S. moellendorffii* the dorsal angle meristem most often produces a shoot, while the ventral angle meristem most often becomes a rhizophore (Mello et al., 2019). In contrast, *S. kraussiana* has a single dorsal angle meristem that typically forms a rhizophore (Otreba & Gola, 2011). Rhizophores are often referred to as "root bearing organs" as they produce roots once near or in contact with the soil, in contrast to the roots that are constitutively formed from the developing sporeling (Figure 2p; Banks, 2009). Since rhizophores produce the only roots in the adult plant, they are critical for anchorage and acquiring water.

Angle meristem plasticity may be regulated by long range auxin transport (Mello et al., 2019; Williams, 1937; Wochok & Sussex, 1975), but as yet no mechanism has been identified. A recent study investigating the vascularisation of the rhizophore in nine species found three main patterns (Matsunaga et al., 2017). The vasculature can diverge from the centre of the stele bifurcation, or from the stele of the main stem close to the stele bifurcation point, or from the stele of the side branch, in what is often known as K- or H-branching. Interestingly, in all cases vascular strands arch backwards from the direction of the main vascular strands, perhaps reflecting redirection of auxin from basipetal transport in the stem to acropetal transport in the growing rhizophore (Matsunaga et al., 2017). However, the location and direction of auxin transport in vascular development are yet to be established in rhizophores or bifurcating shoots. The *Selaginella* rhizophore could be a useful model to study how whole organ identity is determined.

3.8 | Ancient divergence in reproductive strategies

Changes in plants' reproductive strategy to reduce reliance on water for life cycle completion were also important in land plant diversification, and distinct patterns of life cycle progression typify plant groups. Phylogenetic mapping of extant spore characteristics suggests that the last common ancestor of land plants had a single type of spore (homospory), but the evolution of specialised spores of different sizes (heterospory) has been a repeating trend in plant evolution. Among extinct and living lycophytes, the Lycopodiales and Protolepidodendrales are homosporous and the Selaginellales, Lepidodrendrales, and Isoëtales are heterosporous with large female megaspores and small male microspores (Figures 1b and 2p; Sporne, 1962). This suggests that heterospory evolved in the lycophyte lineage after the divergence of the Protolepidodendrales. All lycophyte spores are produced in sporangia which arise from reproductive leaves (sporophylls) in *Isoëte*s or on specialised reproductive shoots known as strobili in *Selaginella* and some *Lycopodium* spp. (Figure 2l; Gifford & Foster, 1989). A study in *Selaginella* found that species have

diverse locations on the strobilus of large megasporangia and small microsporangia. The megasporangia can either be basally located, distributed along two sides of the strobilus, or the whole strobilus can have mega- or micro-sporangia (Horner & Arnott, 1963). After release from sporangia, the spores germinate to form mega- (female) and micro- (male) gametophytes which are largely contained within the spore wall (endospory). These gametophytes develop archegonia and antheridia respectively, and then eggs and sperm. After fertilisation and embryogenesis, the sporophyte emerges from the megaspore coat (Figure 2p), a characteristic shared with seed plants but not homosporous ferns or bryophytes (Linkies, Graeber, Knight, & Leubner-Metzger, 2010). In common with other vascular plants, but in contrast to bryophytes, the gametophyte life cycle stage of living lycophytes is transient and the sporophyte stage is dominant and free living. However, *Aglaophyton, Rhynia* and *Horneophyton* fossils from the Rhynie chert have life cycles that are distinct from both bryophytes and vascular plants with free-living sporophytes and gametophytes, so the ancestral pattern of life cycle progression in land plants is ambiguous (Taylor, Kerp, & Hass, 2005). Nevertheless, lycophyte models will help to elucidate the molecular evolution of traits such as heterospory, sporophyte dominance, and endospory in vascular plants.

4 | GENOMIC AND GENETIC RESOURCES FOR LYCOPHYTES

4.1 | Genomics and transcriptomics

The phylogenetic position and ancient evolutionary divergence of the lycophyte lineage offer exciting opportunities to identify the genetic basis of conservatism, convergence and diversification in trait evolution. Due to the advent of low-cost and high-quality sequencing methods, genomic resources for lycophytes have expanded significantly in the last ten years, lowering the feasibility threshold for reverse genetic approaches. The first lycophyte sequence datasets were a BAC gDNA library and an EST library from *S. moellendorffii* (W. Wang et al., 2005; Weng, Tanurdzic, & Chapple, 2005). This species was selected on the basis of C values showing that, at 88-127 Mbp/1C, *S. moellendorffii* has the smallest genome among *Huperzia lucidula* (5585 Mbp/1C), *Diphaiastrum digita* (2670 Mbp/1C), *Isoëtes engelmanii* (1710 Mbp/1C), and *S. kraussiana* (211-240 Mbp/1C; Table 1; W. Wang et al., 2005). An annotated *S. moellendorffii* genome was published by Banks et al., (2011), and there are RNASeq and DNASeq datasets from *S. kraussiana* (Table 1; Ge et al., 2016). Several lycophyte transcriptomes such *Selaginella martensii* and *Isoëtes echinospora* have recently been published (Hetherington, Emms, Kelly, & Dolan, 2019; James et al., 2017; Leebens-Mack, 2019) and lycophyte transcriptomes from *Isoëtes drummondii* and *Phyllloglossum drummondii* are also available on request (Table 1; Dixon, Harrison, Hetherington, Mylne, & Zhang, 2016). Such genomic and transcriptomic data are important for analyses of gene copy number and the identification of developmental gene orthologs and will facilitate future evo-devo studies by providing templates for primer design. Evolutionary changes in genome architecture have also been investigated. It was shown that more recent Long Terminal Repeat Retrotransposons (LTR-RT) insertion events in lycophytes correlated with smaller genome sizes, typically found in heterosporous species (Baniaga & Barker, 2019). Future studies will reveal how genome expansion and contraction contributed to important developmental innovations.

4.2 | Gene expression analyses

Many developmental genes have been isolated from lycophytes, and reverse transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR (qRT-PCR), transcriptomic and in situ hybridisation analyses have been used to infer gene expression patterns (Table 1). For example, Frank et al. (2015) isolated apical cells, the core meristem region and leaf primordia from *S. moellendorffii* by laser microdissection and performed RNASeq on each tissue type to study differential gene expression across the shoot apex. Similarly, Mello et al. (2019) compared the transcriptomes of roots, stems, leaves, and rhizophores in *S. moellendorffii* to investigate rhizophore identity, and similar sampling was used in *S. kraussiana* to study WOX evolution in land plants (Ge et al., 2016). *Huperzia selago* shoot tips were used in an RNASeq experiment to investigate leaf evolution (Evkaikina et al., 2017). More recently, a gene expression atlas for *S. moellendorffii* has been produced and made available on the eFP Browser and CoNekT-Plants databases, generating a useful resource for in silico expression analyses (Ferrari et al., 2020). Using different developmental stages, time points, and heat stress conditions, the latter paper also demonstrates that coexpression data of genes present in the same tissues could be used to predict functional gene modules, such as lignocellulose biosynthesis in the cell wall which is conserved across vascular plants (Ferrari et al., 2020). Protocols for tissue scale resolution of gene expression patterns (e.g. RNA in situ hybridisation) are available for *S. kraussiana*, *S. uncinata*, and *S. moellendorffii*, and have been used to indicate the functions of a range of developmental gene

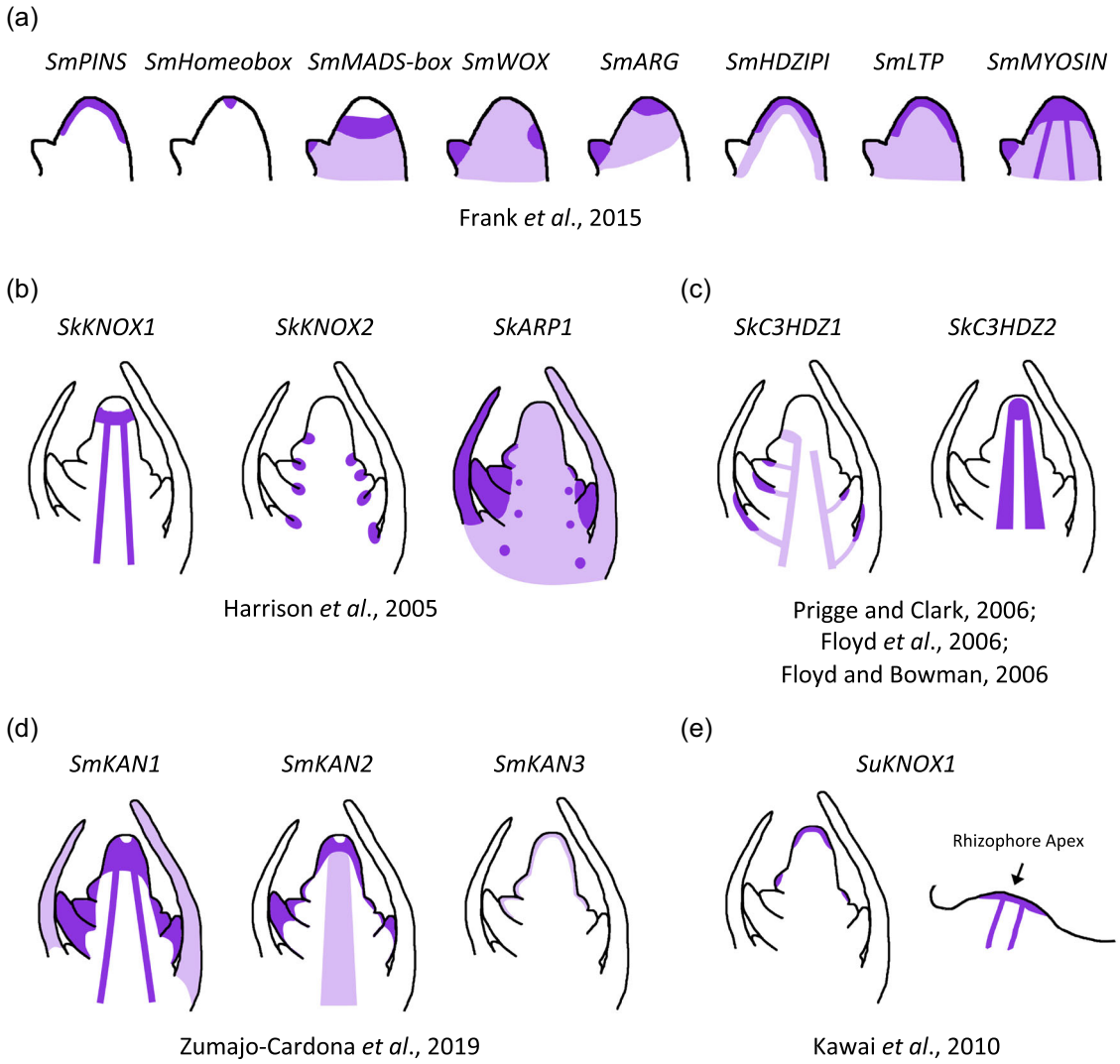


FIGURE 4 Schematics summarising the expression patterns of key developmental genes in Lycophytes. (a) *SmPINS*, *SmHomeobox*, *SmMADs-box*, *SmWOX*, *SmARG*, *SmHDZIPI*, *SmLTP*, and *SmMYOSIN* genes are expressed in the *Selaginella moellendorffii* shoot tip (Frank et al., 2015). (b) *SkKNOX1* and *SkKNOX2* in *Selaginella kraussiana* show distinct expression patterns in the shoot apex. *SkKNOX1* is expressed in a band underneath the apical cells and in the developing vascular strands, while *SkKNOX2* is expressed in the internodes. *SkARP1* expression is diffuse in the whole meristematic region, but is stronger in leaf primordia and leaf vascular traces (Harrison et al., 2005). (c) *SkC3HDZ1* is expressed in the *Selaginella kraussiana* shoot tip in provascular strands and in the adaxial side of leaf primordia. *SkH3HDZ2* signal is present immediately underneath the apical cells and in the developing provascular strands (Floyd and Bowman, 2006; Floyd et al., 2006; Prigge & Clark, 2006). (d) *SmKAN1* and *SmKAN2* expression was detected in *Selaginella moellendorffii* developing vasculature, leaf primordia and in the apical dome, with the exception of the apical cells. *SmKAN3* was expressed only faintly in the outer layer of the shoot tip (Zumajo-Cardona et al., 2019). (e) *SuKNOX1* expression in the *Selaginella uncinata* shoot apex and rhizophore apex. Expression was absent in root meristems (Kawai et al., 2010) [Color figure can be viewed at wileyonlinelibrary.com]

families such as KNOX, ARP, HD-ZIPIII, PIN, WOX, and KANADI (Figure 4; Floyd & Bowman, 2006; Floyd, Zalewski, & Bowman, 2006; Frank et al., 2015; Harrison et al., 2005; Kawai et al., 2010; Prigge & Clark, 2006; Zumajo-Cardona, Vasco, & Ambrose, 2019). In using a combination of phylogenomic and transcriptomic approaches and expression localisation tools, great advances in understanding the evolution of plant developmental gene families have been made.

4.3 | Genetics

Methods to interrogate gene functions currently remain limiting in lycophytes, but an in vitro method for crossing *S. kraussiana* was published in 1979, involving induction of sporogenesis, megaspore culture and sperm released from microgametophytes (Webster, 1979). This method was used to establish the dominance of the *S. kraussiana aurea* mutation (Harrison et al., 2007;

Webster & Tanno, 1980), which confers a golden-green colour in heterozygotes. The recent advent of new methods for transformation in ferns (Muthukumar, Joyce, Elless, & Stewart, 2013; Plackett, Huang, Sanders, & Langdale, 2014), and development of DNA-based and DNA-free nucleoprotein genome editing methods (Ferenczi, Pyott, Xipnitou, Molnar, & Merchant, 2017; Kim et al., 2017; Mallett, Chang, Cheng, & Bezanilla, 2019) bring great promise for future genetic analyses in lycophytes.

5 | ANALYSES OF GENE FUNCTIONS IN TRAIT EVOLUTION

5.1 | Roles of auxin and PINs in development, including branching and vascular formation

The phytohormone auxin integrates many signalling pathways to mediate *Arabidopsis* development (Biedroń & Banasiak, 2018; Xiong & Jiao, 2019). Importantly, auxin is able to pass freely into cells, but it is then acidified and therefore unable to pass out of the cell (Rubery & Sheldrake, 1974). PIN FORMED (PIN) membrane proteins transport auxin out across the plasma membrane and can be positioned to create a local Polar Auxin Transport (PAT) stream through a tissue (Gälweiler et al., 1998). In a moss sister lineage to vascular plants, disruption of polar auxin transport and PIN function causes developmental changes that make sporophytes branch and look similar to protracheophyte fossils, potentially implicating PINs in the origin of vascular plant branching architectures (T. A. Bennett et al., 2014). The PIN gene family diversified independently in lycophyte and euphyllophyte lineages (T. Bennett et al., 2014). There are six homologs in *S. moellendorffii*, *PINR-PINU* and *PINUß* (T. A. Bennett et al., 2014), but while *PINS* is expressed at the shoot apex (Figure 4a; Frank et al., 2015), the functions of *Selaginella* PINs are unknown. All lycophyte PINs have a central intracellular loop domain typical of the canonical euphyllophyte PINs, which are localised to the plasma membrane, unlike noncanonical PINs which can be ER-localised (T. A. Bennett et al., 2014). A GFP-tagged SmPINR expressed under the *AtPIN2* promoter in *Arabidopsis* localised to the plasma membrane but not the base of cells, consistent with the notion that lycophyte PINs are canonical, and providing indirect evidence that *PINR* may not be polarly localised in the same way as *Arabidopsis* canonical PINs (Y. Zhang, Xiao, Wang, Zhang, & Friml, 2019). Expression of the DR5:GFP auxin response reporter (Ulmasov, Murfett, Hagen, & Guilfoyle, 1997) in *S. moellendorffii* protoplasts supports functional conservation of auxin signalling pathways in lycophytes (Mello et al., 2019). Auxin and its transport regulate many aspects of *Selaginella* development, including root branching, vascular formation in leaves, shoot indeterminacy, initial cell function, phyllotaxy, leaf development, and rhizophore identity and outgrowth (Fang, Motte, Parizot, & Beeckman, 2019; Mello et al., 2019; Sanders & Langdale, 2013). However, unlike *Arabidopsis*, exogenously applied auxin does not directly affect root branching (Fang et al., 2019). Since auxin and PIN-mediated auxin transport are important regulators of many aspects of development in all land plants so far assayed (Harrison, 2017a), future analyses of PIN function in lycophytes are likely to give insights into the evolution of plant architecture.

5.2 | Gene regulatory networks for vascular development and meristem identity

The CLAVATA pathway is a further key regulator of *Arabidopsis* development, comprising small diffusible CLE peptides in TDIF and CLV3 classes, their receptors and downstream targets such as WUSCHEL (Fletcher, 2018). In the *Arabidopsis* vascular cambium the WUS-like proteins WOX4 and WOX14 promote proliferation to regulate the girth of the shoot, and TDIF CLEs act through the PXY receptor-like kinase to promote WOX expression (Etchells, Provost, Mishr, & Turner, 2013; Etchells & Turner, 2010; Hirakawa, Kondo, & Fukuda, 2010). *Selaginella moellendorffii* has four TDIF-encoding genes (Whitewoods et al., 2018), and in another study a SkCLE-TDIF peptide was exogenously applied to the developing vasculature of *A. thaliana* and *S. kraussiana* to test conservation of TDIF functions in vascular development. This inhibited vascular development in *A. thaliana*, but not *S. kraussiana*, implying that roles for TDIF peptides might not be conserved (Hirakawa & Bowman, 2015).

In *Arabidopsis* shoot apical meristems, the WUSCHEL transcription factor promotes meristem identity, and CLV3 peptides repress meristem identity acting through one or more Leucine Rich Repeat-Receptor Like Kinase (LRR-RLK) receptors of CLV1, CLV2, CRN, or RPK2 receptor classes (Clark, Running, & Meyerowitz, 1995; Ito & Fukuda, 2006; Jeong, Trotochaud, & Clark, 1999; Kondo et al., 2006; Laux, Mayer, Berger, & Jürgens, 1996; Müller, Bleckmann, & Simon, 2008; Ogawa, Shinohara, Sakagami, & Matsubayash, 2008; Somssich, Je, Simon, & Jackson, 2016). *Selaginella moellendorffii*

has eleven CLV3-encoding genes, three CLV1-like receptors and one RPK2-like receptor (Whitewoods et al., 2018), however the roles of these genes and receptors in lycophytes are unknown. With respect to WOX genes, *S. moellendorffii* has four genes and *S. kraussiana* has eight genes (Ge et al., 2016; Segatto, Thompson, & Freitas, 2016). A recent phylogeny segregates WOX genes into T1WOX, T2WOX, and T3WOX clades (Wu, Li, & Kramer, 2019). The T1WOX clade comprises sequences from all major land plant lineages and their charophyte sister lineages. T2 and T3 WOXs, respectively, comprise seed plant and vascular plant sequences, and lycophyte sequences are sister to the T3 and T2 + T3WOX clades. In situ analyses in *S. moellendorffii* showed that an *SmWOX* gene is expressed in meristematic tissue and developing leaves (Figure 4a; Frank et al., 2015) and RNASeq analyses in *S. kraussiana* have shown that four WOX genes are expressed throughout the plant, one is expressed at the rhizophore tip and three genes showed undetectable expression. A homolog of *WOX5*, which is root-specific in *Arabidopsis*, showed ubiquitous expression (Ge et al., 2016), suggesting that lycophyte WOX genes may not function in rooting, and that root-specific WOX functions evolved in euphyllophytes (Liu & Xu, 2018). In conjunction with data from a moss suggesting that CLAVATA and T1WOXs affect unrelated developmental processes (Sakakibara et al., 2014; Whitewoods et al., 2018), these data point to lineage specific roles for TDIF peptides, their receptors and WOXs, shaping an interesting case study of gene regulatory network evolution.

5.3 | *KNOX* genes have conserved roles in promoting meristem indeterminacy

In common with PINs and CLV3, KNOX transcription factors are likely conserved regulators of indeterminacy in the last common ancestor of vascular plants. This inference comes from outgroup comparison of moss KNOX functions to vascular plant KNOX functions. In *Arabidopsis*, Class I KNOX genes are expressed throughout the proliferative region of shoot tips except in leaf primordia (Long, Moan, Medford, & Barton, 1996), and KNOX proteins activate cytokinin biosynthesis to promote proliferation (Hay & Tsiantis, 2010; Jasinski et al., 2005; Yanai et al., 2005). Class I KNOX proteins similarly promote proliferation by activating cytokinin biosynthesis in an intercalary proliferative zone of developing moss sporophytes, leading to the hypothesis that indeterminacy arose following sporangium displacement from the shoot tips, and consequent juxtaposition of initial cells and a subtending proliferative region in the last common ancestor of vascular plants (Coudert et al., 2019; Harrison & Morris, 2018). Among lycophytes, *S. kraussiana* has two Class I KNOX genes. *SkKNOX1* is most strongly expressed in the proliferative region underneath the apical cells and *SkKNOX2* is expressed in internodes (Figure 4b; Harrison et al., 2005). Coupled with in situ hybridisation and immunolocalisation analyses in a range of ferns showing KNOX expression in proliferative meristematic regions (Ambrose and Vasco, 2016; Bharathan et al., 2002; Harrison et al., 2005; Sano et al., 2005), these data suggest that KNOX function in promoting indeterminacy is conserved and arose in the last common ancestor of vascular plants.

5.4 | Divergent pathways for leaf initiation and patterning

In *Arabidopsis*, KNOX proteins also regulate entry into leaf development pathways, and downregulation of meristematic *KNOX* expression is an early marker of leaf initiation, contrasting with a complementary pattern of *ARP* gene expression. While downregulation of Class I KNOX and upregulation of ARP expression also marks leaf initiation in *S. kraussiana* (Figure 4b; Harrison et al., 2005), a *Huperzia selago* shoot tip transcriptome yielded KNOX but not ARP transcripts (Evkaikina et al., 2017), suggesting that KNOX and ARP genes could have divergent roles in leaf development across lycophytes (Harrison et al., 2005). In ferns, KNOX expression appears persistent in the leaf primordia, and KNOX and ARP homologue activities can overlap in leaf primordia (Bharathan et al., 2002; Harrison et al., 2005; Sano et al., 2005). These differences in KNOX function among lycophytes and between vascular plant lineages are likely to reflect independent leaf origins.

Other pathways for leaf development that are well characterised in *Arabidopsis* also appear to have divergent roles in lycophytes. For instance, HD-Zip gene clades which regulate shoot apical meristem initiation, xylem development, leaf initiation, and leaf polarity (*REV*/*PHV*/*PHB* clade) or phloem development (*CNA*/*AthB8* clade) in *Arabidopsis* arose in a seed-plant specific gene duplication (Prigge et al., 2005), and lycophyte HD-Zips diversified independently (Floyd & Bowman, 2006; Prigge & Clark, 2006). *Selaginella moellendorffii* and *S. kraussiana* each have two HD-Zip genes, and one *S. kraussiana* HD-Zip has expression patterns consistent with roles in meristem function and xylem development while the other has patterns consistent with roles in leaf initiation, polarity and phloem development (Figure 4c; Floyd & Bowman, 2006; Floyd et al., 2006; Prigge & Clark, 2006). While HD-Zips show adaxial expression in

Arabidopsis and *S. kraussiana*, KANADIs regulate abaxial identity in *Arabidopsis* and show abaxial expression. A recent study found three *S. moellendorffii* KAN genes, which form a sister group to fern KANs. *EhyKAN1*, *2*, and *3* were all expressed on the abaxial side of maturing leaves in the horsetail, *Equisetum hyemale* (Zumajo-Cardona et al. 2019). However, in *S. moellendorffii*, *SmKAN1* and *SmKAN2* were uniformly expressed in all stages of leaf development (Figure 4d; Zumajo-Cardona et al., 2019). In combination, the data above point to a permissive gene regulatory environment for the evolution of vascular plant leaves, consistent with a high level of homoplasy in leaf evolution (Tomescu, 2009).

5.5 | Similar molecular regulators of root development were independently recruited

The independent origin of roots in lycophytes and euphyllophytes calls into question the recruitment of distinct or homologous molecular regulators for root development (Augstein & Carlsbecker, 2018). The meristematic and elongation/differentiation zones were isolated from *Selaginella moellendorffii* roots and their transcriptomes compared to six vascular plant root tissues (Huang & Schiefelbein, 2015). Transcriptomic analyses showed that the root transcriptome of *S. moellendorffii* is similar to euphyllophyte roots, suggesting that there was either an ancestral root development mechanism in the vascular plant ancestors, or that highly similar pathways were recruited convergently to root development in lycophytes and euphyllophytes (Ferrari et al., 2020; Huang & Schiefelbein, 2015). Homologues of the lateral root cap initial-promoting gene *FEZ* were also found in *S. moellendorffii*, despite the proposed independent evolution of the root cap in the two lineages (Augstein & Carlsbecker, 2018; Hetherington & Dolan, 2018; Huang & Schiefelbein, 2015). While the conservation of lycophyte root morphology suggests that there may have been a single origin of roots in lycophytes, the divergent patterns of root organogenesis in lycophytes suggests otherwise. Genetic studies would determine if lycophyte roots are homologous, or if pre-existing organs such as leaves acquired root-like characteristics and associated genetic signatures convergently (Hetherington & Dolan, 2017). A transcriptomic approach recently showed that the short rootlets of *Isoëtes echinospora* are more transcriptionally similar to *Selaginella* and *Arabidopsis* roots than leaves, addressing a longstanding question about the identity of rhizomorphic lycopsid rootlets (Hetherington et al., 2019). It will be important to identify genetic similarities between other root types and the organs on which roots are borne to identify conservation, convergence, and divergence in root evolution.

5.6 | Molecular identity of the rhizophore

Shoots, roots, and leaves comprise the basic organ systems of today's vascular plant flora, and while pathways for a few key developmental gene families have conserved roles in regulating meristem functions, pathways for lycophyte root and leaf evolution have been recruited convergently or divergently into development. In contrast to these exemplars, the innovation of a unique organ system within lycophytes offers the opportunity to explore the genetic basis of lineage-specific morphological novelty and to address a longstanding question in biology. To this end, the molecular identity of the rhizophore was first investigated by comparison of 2D gel electrophoresis patterns between rhizophore, stem, root, and leaf samples, showing a strong similarity between rhizophores and stems (Jernstedt & Mansfield, 1985). More recent transcriptomic comparisons instead suggest that the RNASeq profile of the rhizophore is distinct but more similar to roots than shoots, sharing many genes involved in cytokinin signalling (Mello et al., 2019). More specifically, in situ hybridizations showed that Class I *SuKNOX1* expression marks shoot, root, and rhizophore tips in *S. uncinata*, consistent with a general role for KNOX genes in promoting proliferation (Figure 4e; Kawai et al., 2010). Despite inconsistencies between these studies, it is clear that the rhizophore is a unique organ with some similarity to both roots and shoots.

5.7 | Reproductive transitions

Following vegetative growth, most lycophytes produce specialised reproductive shoots which bear sporangia and are known as strobili. Similar vegetative to reproductive phase transitions in *Arabidopsis* and other flowering plants are well characterised at the molecular level, involving a microRNA-mediated mechanism. The expression of microRNAs miR156 and miR157 in shoot meristems slowly declines through time, lifting repression of SQUAMOSA PROMOTER BINDING PROTEIN (SBP/SPL) transcription factor expression to switch leaf and meristem identity during phase change (Fouracre & Scott Poethig, 2019; Poethig, 2013). All land plants are thought to have miR156, (Poethig, 2013), and a miRNA-SPL module regulates phase transition in *Marchantia* (Tsuzuki et al., 2019), suggesting that miRNA-SPL

control of phase change may be conserved. In *Arabidopsis*, reproductive meristem identity is also controlled by the LEAFY transcription factor, and LEAFY activates MADS-box gene expression in inflorescence and floral meristems to specify floral organ identity. Roles for LEAFY in regulating the reproductive transition are likely to be an innovation of seed plants as LEAFY homologue expression precedes MADS expression in *Welwitschia* cone development (Moyroud et al., 2017), but LEAFY homologues control apical proliferation in a fern (Plackett et al., 2018) and zygote proliferation in a moss (Tanahashi, 2005). A study of five *Isoëtes* species showed that *LEAFY* is expressed in proliferating vegetative and reproductive tissue, however this expression is diffuse and not localised to meristems (Yang, Du, Guo, & Liu, 2017). Links between LEAFY and MADS-box function are so far unclear in nonseed plants, and LEAFY has divergent DNA binding capacities in mosses and hornworts and liverworts and vascular plants, suggesting that downstream targets are unlikely to be conserved (Sayou et al., 2014).

5.8 | Sexual organ development

Land plant MADS proteins fall into MIKC^C and MIKC* clades based on the length of their K domain (Thangavel and Nayar, 2018). Whereas seed plant sexual organ development is spatially regulated by MIKC^C class genes, fern and moss MIKC^C genes are expressed in reproductive and vegetative tissues of the sporophyte as well as in gametophytes (Hasebe, Wen, Kato, & Banks, 1998; Koshimizu et al., 2018). In the lycophytes *Lycopodium annotinum* and *Selaginella remotifolia*, RT-PCR showed that MIKC^C genes are expressed in strobili and vegetative tissues such as shoot apices of the sporophyte (Svensson and Engström, 2002; Tanabe, Uchida, Hasebe, & Ito, 2003). Thus, in nonflowering plants, MIKC^C genes have diverse roles in both sporophytes and gametophytes. Interestingly, MIKC* genes also regulate sporophyte and gametophyte growth in ferns and bryophytes (Koshimizu et al., 2018; Kwantes, Liebsch, & Verelst, 2012). However, RT-PCR experiments showed that MIKC* genes are expressed exclusively in the strobili of *L. annotinum* (Svensson, Johannesson, & Engström, 2000), and in *S. moellendorffii* and *S. pallescens* are highly upregulated in the microsporangia (Kwantes et al., 2012). While these RT-PCR experiments showed broad expression patterns (e.g. whole strobili), they were unable to resolve the precise stage at which these genes are expressed (e.g. sporophylls, microsporangia, megasporangia, and gametophytes), but this will be important in revealing how spatial characteristics such as the location of mega- and microsporangia in *Selaginella* reproductive development arise (Horner & Arnott, 1963; Horner & Beltz, 1970). Generally, however, MIKC* genes seem to be important in gametophyte development in all land plants, with further roles in the bryophyte and fern sporophytes. Functional characterisation of these genes in lycophytes will be important to resolve the ancestral role of MADs genes in sporophyte versus gametophyte development.

5.9 | Life cycle progression

Reproductive phase transitions are indicators of life cycle progression in general, and insights into the molecular regulation of life cycle progression have emerged from a wide range of plant groups, involving homeodomain proteins of KNOX and BELL classes. A single KNOX and BELL protein differentiate mating strains of the unicellular alga *Chlamydomonas*, and after mating, these proteins dimerise to activate zygotic development and meiosis. Changes in expression and delayed meiosis may have contributed to the origin of embryos and the emergence of the sporophyte generation in land plant life cycles (Lee, Lin, Joo, & Goodenough, 2008). KNOX proteins duplicated in a streptophyte ancestor of land plants (Frangedakis, Saint-Marcoux, Moody, Rabbinowitsch, & Langdale, 2017) and BELL proteins can dimerise with Class II KNOX proteins in a moss, with both protein classes regulating gametophyte/sporophyte life cycle stage identity (Sakakibara et al., 2013). A Class I KNOX gene regulates proliferation in moss sporophytes, and as previously discussed, KNOX genes were likely important for the evolution of sporophyte indeterminacy in vascular plants (Coudert et al., 2019). Unveiling the functions and interactions of type I and II KNOX genes, and KNOX and BELL genes in lycophytes will be important in understanding the evolution of sporophyte life cycle stage dominance in vascular plants.

6 | CONCLUSIONS AND FUTURE DIRECTIONS

6.1 | Molecular data will reveal conservation, convergence, and divergence in gene function

Here we summarise current thoughts about trait conservation, convergence, and divergence in vascular plant evolution, and highlight the importance of appropriate models and sampling strategies for evolutionary inferences. If a trait is highly conserved, then taking a few representative models from each major land plant lineage may be sufficient. However, some basic developmental processes have massively diversified in plants to produce a

variety of forms. *Arabidopsis* is often used to typify angiosperm development, but genetic studies must build on new genomic resources (DePamphilis et al., 2013; L. Zhang et al., 2020) to check for conservation, convergence, and divergence across the angiosperms, between angiosperms and gymnosperms and between gymnosperms and their monilophyte sister lineages (Guan et al., 2016; Li et al., 2018; Marchant et al., 2019; Plackett et al., 2014; Wan et al., 2018). Similarly, while one lycophyte species is useful for looking at broadly conserved processes, appropriate sampling must be used to investigate divergence or convergence across the lycophytes and land plants. Clearly, understanding the phylogeny and molecular regulators of development of each plant lineage is likely to change hypotheses about trait loss and gain through time (Delaux et al., 2019; Harrison, 2017b).

6.2 | What have we learnt from studies in lycophytes?

To date, several key findings have been made by morphological comparisons and preliminary genetic studies. It is clear from the fossil record and extant species that shoot branching, vasculature, and shoot indeterminacy were acquired sequentially and are conserved in vascular plants, while roots, root caps, leaves, and complex meristem structures evolved independently in lycophytes and euphyllophytes (Harrison & Morris, 2018). Genetic studies corroborate these findings, as Class I KNOX genes appear conserved regulators of indeterminacy (Coudert et al., 2019; Harrison et al., 2005), but genetic regulators of leaf development such as KANADI, and HD-ZIP genes are differentially expressed in lycophytes and euphyllophytes (Floyd & Bowman, 2006; Harrison et al., 2005; Prigge & Clark, 2006; Zumajo-Cardona et al., 2019). These studies revealed a propensity for the independent recruitment of some gene families to similar developmental processes in different lineages. Coupled with expression patterns from RNASeq analyses and in situ hybridisation (Frank et al., 2015; Mello et al., 2019), these studies have been critical for connecting genetics to the evolution of vascular plant development.

6.3 | What are the next steps to advance lycophyte research?

To progress further, modern imaging and molecular techniques such as confocal live imaging, CRISPR gene editing and RNASeq should be routinely used in lycophytes. Although expression patterns can indicate gene activities, they do not show gene function. For this, mutant phenotype analysis is needed, but no transformation method has yet been established in lycophytes. Protoplasts have been extracted from *S. moellendorffii* leaves and roots and successfully transformed (Mello et al., 2019; Yin, Richter, Börner, & Weihe, 2009), but no regeneration protocol has yet been reported. This is the biggest challenge for future lycophyte research, but new transformation techniques bring significant potential to answer questions about how vascular plants evolved.

6.4 | What can we learn from future studies in lycophytes?

The genetic basis of some of the key evolutionary innovations such as shoot and root branching, tracheid development and thickening, apical cell identity, root and shoot meristem diversification, sporophyte dominance and heterospory could be identified using lycophytes. Molecular studies could also help to reveal how analogous organs such as lycophyte and euphyllophyte leaf blades evolved. They could also address broad evolutionary and developmental questions, such as how plant tissues maintain developmental plasticity, how genetic circuits are remodelled during evolution and how the rates at which genes and genomes duplicate in eukaryotic lineages differ. The recent increase in molecular studies of lycophytes bring us a step closer to unveiling the evolutionary path that lead to the incredible diversity and success of vascular plants, and together with modern fossil analysis will unravel how land plants evolved and shaped today's ecosystems.

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ORCID

Cecily Jill Harrison http://orcid.org/0000-0002-5228-600X

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