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Epidermal patterning and stomatal development in Gnetales

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• **Background and Aims:** The gymnosperm order Gnetales, which has contentious phylogenetic affinities, includes three extant genera (*Ephedra*, *Gnetum*, *Welwitschia*) that are morphologically highly divergent and have contrasting ecological preferences: *Gnetum* occupies mesic tropical habitats, whereas *Ephedra* and *Welwitschia* occur in arid environments. Leaves are highly reduced in *Ephedra*, petiolate with a broad lamina in *Gnetum* and persistent and strap-like in *Welwitschia*. We investigate stomatal development and prepatterning stages in Gnetales, to evaluate the substantial differences among the three genera and compare them with other seed plants.
• **Methods:** Photosynthetic organs of representative species were examined using light microscopy, scanning electron microscopy and transmission electron microscopy.
• **Key Results:** Stomata of all three genera possess lateral subsidiary cells (LSCs). LSCs of *Ephedra* are perigene cells derived from cell files adjacent to the stomatal meristemoids. In contrast, LSCs of *Gnetum* and *Welwitschia* are mesogene cells derived from the stomatal meristemoids; each meristemoid undergoes two mitoses to form a 'developmental triad', of which the central cell is the guard mother cell and the lateral pair are LSCs. Epidermal prepatterning in *Gnetum* undergoes a 'quartet' phase, in contrast with the linear development of *Welwitschia*. Quartet prepatterning in *Gnetum* resembles that of some angiosperms but they differ in later development.
• **Conclusions:** Several factors underpin the profound and heritable differences observed among the three genera of Gnetales. Stomatal development in *Ephedra* differs significantly from that of *Gnetum* and *Welwitschia*, more closely resembling that of other extant gymnosperms. Differences in epidermal prepatterning broadly reflect differences in growth habit between the three genera.

Key words: *Ephedra*, Gnetales, *Gnetum*, prepatterning, stomatal development, *Welwitschia*.

INTRODUCTION

Among at least 15 ancient seed-plant lineages, only five, including Gnetales, have surviving species; the remainder are known only as fossils. The five extant seed-plant lineages display considerable disparity in their respective extant taxon numbers. Angiosperms are the most diverse, with ~17 020 genera and 352 000 species, many of them resulting from relatively recent radiations (figures based on the Plant List, 2013). The four gymnospermous lineages that include living representatives are conifers (~74 genera/383 species), cycads (10/308), *Ginkgo* (1/1) and Gnetales (3/112).

The order Gnetales represents a useful case study for exploring stomatal development because it encompasses only three extant genera (*Ephedra*, *Gnetum*, *Welwitschia*) that are morphologically highly distinct, and each represents an ancient lineage: Ickert-Bond *et al.* (2009) estimated a Jurassic divergence date of ~165 Ma between the lineage leading to crown-group *Ephedra* and the lineage leading to *Welwitschia* and *Gnetum* (see also Ickert-Bond and Renner, 2016). These three genera differ radically from each other in many respects but share some common features, notably the possession of vessels in the secondary xylem, which is an unusual feature outside the angiosperms (Carlquist, 2012; Ickert-Bond and Renner, 2016). Early Cretaceous fossils indicate that the gnetalean lineages were formerly more diverse in their vegetative structure, including leaf morphology (e.g. Rydin *et al.*, 2003; Kunzmann *et al.*, 2009, 2011; Yang *et al.*, 2015). Among the three extant genera, *Ephedra* (~50 species) consists of highly branched rambling shrubs or scrambling climbers from arid regions of Eurasia and the Americas, *Gnetum* (~40 species) includes trees, shrubs or climbers from the moist tropics of America, Africa and Asia, and *Welwitschia* is represented by a single extant species endemic to the deserts of south-western Africa (Kubitzki, 1990; Hou *et al.*, 2015; Ickert-Bond and Renner, 2016). Genomic tools for Gnetales have recently been extended by publication of a genome sequence for *Gnetum montanum* (Wan *et al.*, 2018), making it a potentially useful model for evo-devo studies.

The photosynthetic organs of the three genera are strikingly disparate in morphology. Leaves of *Ephedra* are reduced to pairs or whorls of minute scale-like structures borne at the nodes and often fused into a short tubular sheath; plants of this genus rely primarily on their thin branching stems for photosynthesis (Fig. 1A, B). Leaves of *Gnetum* are also borne in opposite pairs but resemble those of many angiosperms in possessing a short petiole and an entire lamina that is broad and elliptical in outline with a central midrib (Fig. 1E, F); they increase in size by means of both a marginal meristem and plate meristem activity (Rodin, 1967; Tomlinson and Fisher, 2005). The reticulate venation of *Gnetum* consists of several major secondary veins that form

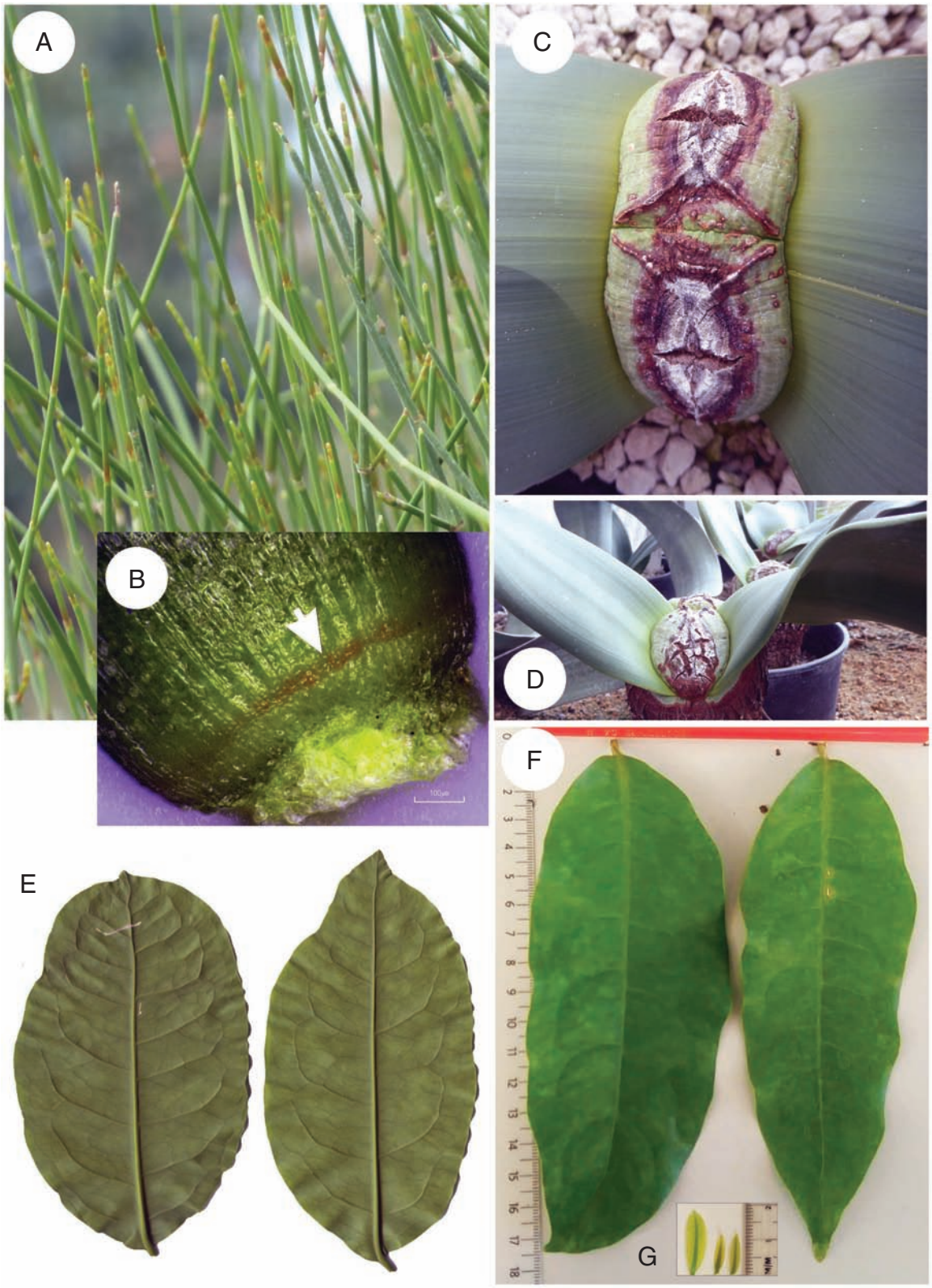

FIG. 1. Vegetative parts of plants of Gnetales. (A) *Ephedra fragilis*, tips of growing photosynthetic shoots. (B) *Ephedra* sp., detail of stem base with scale leaves removed to reveal meristematic 'diaphragm' region (arrow). (C, D) *Welwitschia mirabilis*, leaf bases. (E–G) *Gnetum gnemon* leaves. (E) Abaxial views of two mature leaves. (F) Adaxial views of two mature leaves with scale. (G, inset) Youngest leaves examined, shown at same scale as in (F).

closed submarginal loops (brochidodromous venation *sensu* Hickey, 1973; see also Tomlinson and Fisher, 2005). *Welwitschia* bears only a single opposite pair of extremely long-lived, linear, strap-like leaves with parallel venation (Fig. 1C, D); these leaves grow from a highly localized basal meristem.

Across land plants, stomatal traits represent a suite of characters that is potentially informative not only in reconstructing phylogenies but also in understanding structural evolution. Contrasting stomatal patterns observed in different major groups of land plants are genetically determined and the relevant gene families are well conserved (Peterson *et al.*, 2010; Rudall *et al.*, 2013). Yet despite considerable work on stomatal development in angiosperms, especially among highly derived model taxa such as *Arabidopsis*, comparative data on gymnosperms are relatively sparse.

Our aims in this paper are to characterize and compare stomatal structure, development and patterning in representative species of the extant genera of Gnetales, in the context of evo-devo studies of stomata and comparative studies in a range of seed plants, both living and extinct (e.g. Rudall *et al.*, 2012, 2013, 2017; Rudall and Knowles, 2013; Cullen and Rudall, 2016). Our ultimate goal is to use these data in combination with other comparative studies of stomata to evaluate the evolution of the stomatal complex across seed plants. No previous detailed study of stomatal development in Gnetales has included electron microscopy or observations on prepatterning and development prior to the guard mother cell (GMC) stage. Based primarily on the work of Takeda (1913*a*, *b*) and Florin (1931, 1933, 1934), morphological cladistic analyses of seed plants (e.g. Doyle and Donoghue, 1986; Doyle, 1996, 2006; Nixon *et al.*, 1994; Rothwell and Serbet, 1994; Hilton and Bateman, 2006) have consistently scored stomata of *Ephedra* as haplocheilic/anomocytic but those of *Gnetum* and *Welwitschia* as syndetocheilic/paracytic; this relatively profound (and potentially developmentally dictated) difference merits further attention. Stomatal terminology used throughout the remainder of this paper follows Payne (1979) and Rudall *et al.* (2013).

Relationships among seed-plant lineages

Of at least 15 ancient seed-plant lineages, only five remain extant (Doyle, 1996, 2006; Hilton and Bateman, 2006). Given that DNA preservation in pre-Quaternary fossils is insufficient for phylogenetic analyses (e.g. Parducci *et al.*, 2017), our understanding of relationships among the seed-plant lineages is based partly on molecular analyses of only the extant taxa (e.g. Graham and Iles, 2009; Ran *et al.*, 2018) and partly on morphological analyses that include fossils (e.g. Doyle, 2006; Hilton and Bateman, 2006). These contrasting approaches have produced highly conflicting results, even among analyses using different types of molecular data, in which long branches subtend both Gnetales and angiosperms (Rydin *et al.*, 2002; Mathews, 2009; Zhong *et al.*, 2010; Ran *et al.*, 2018). The analyses yield little confidence about the broader relationships among the various seed-plant lineages and the phylogenetic placement of Gnetales remains especially controversial. Gnetales have been placed as sister to a diverse range of taxa, including angiosperms, Pinaceae, conifers, all other gymnosperms and all other seed plants. The morphological analysis of Nixon *et al.* (1994) even suggested a paraphyletic Gnetales, with *Gnetum* and *Welwitschia* as a sister pair closely related to angiosperms and *Ephedra* more distantly related, though they noted that trees with a monophyletic Gnetales clade are only two steps longer. Thus, none of these placements is conclusive. Based on reproductive morphology, Mundry and Stützel (2004) emphasized a possible relationship between Gnetales and extinct Cordaitales, the putative sister group to conifers. However, 'Gnetifer' trees, in which a monophyletic Gnetales are sister to conifers, are currently widely favoured (e.g. Coiro *et al.*, 2018).

MATERIALS AND METHODS

All material was collected from plants growing in the living collections at the Royal Botanic Gardens, Kew (RBGK), listed here with their accession numbers (Table 1). For *Ephedra*, young growing green stems were cut into short sections for processing. Leaves of *Gnetum gnemon* were collected at successive growth stages from stage 1 (1–2 cm long; Fig. 1G) to stage 5 (17–20 cm long; Fig. 1E, F). Although leaves are approximately similar within each pair, general leaf morphology is surprisingly variable in size and shape, even along the same branch. Thus, although we determined a series of leaf stages, leaf size was not always a reliable determinant of stomatal stage. For *Welwitschia*, small squares were removed from different locations along one of the two leaves.

For light microscopy (LM) and scanning electron microscopy (SEM), material was fixed in formalin acetic alcohol (FAA), For transmission electron microscopy (TEM), material was fixed in Karnovsky's fixative (2 % v/v paraformaldehyde and 2.5 % v/v glutaraldehyde in 0.05 M phosphate buffer). For bright-field LM using sectioned material, FAA-fixed leaves were transferred through an ethanol series, followed by an ethanol–LR-White resin series, then embedded in LR-White resin (London Resin Co., Basingstoke, UK) using a vacuum oven at 60 °C. Semi-thin sections were cut using a Reichert-Jung Ultracut ultramicrotome and a glass knife before mounting on glass slides. Samples were stained with toluidine blue and imaged using a Leica DM6000B light microscope. For differential interference contrast (DIC) microscopy, material was cleared using a modified version of Herr's clearing fluid (lactic acid/chloral hydrate/phenol/clove oil/Histoclear, 2:2:2:2:1 by weight) and examined using a Leitz Diaplan photomicroscope.

For SEM, material was transferred through an ethanol series before drying in a Supercritical Autosamdri 815B critical point dryer. Dried samples were mounted onto Cambridge stubs and coated with platinum using a Quorum Q150T sputter coater.

TABLE 1. *List of species examined and techniques used*

Species	RBGK accession number	Techniques used
Ephedra chilensis C.Presl.	1967–25610	LM, SEM, TEM
E. equisetina Bunge	1996–2544	LM, SEM, TEM
E. fragilis Desf.	2000–4381	LM, SEM, TEM
E. gerardiana Wall.	1995–3617	LM, SEM, TEM
E. likiangensis Florin	1988–844	LM, DIC, SEM, TEM
E. sp.	1965–65601	LM, SEM, TEM
Gnetum gnemon L.	1998–514	LM, SEM, TEM
Welwitschia mirabilis Hook.f.	2010–1271	LM, DIC, SEM, TEM

Samples were examined and imaged using a Hitachi S-4700 SEM at 2 kV.

For TEM, Karnovsky's-fixed leaves were washed in phosphate buffer, pre-stained using osmium tetroxide and washed again in phosphate buffer. Samples were embedded in LR-White acrylic resin as for LM. Ultrathin sections were cut using a Reichert-Jung Ultracut ultramicrotome with glass and diamond knives and collected using formvar-coated copper grids. Grids were stained with uranyl acetate and lead citrate solution. They were imaged using a Hitachi H-7650 TEM.

RESULTS

Stomata of Ephedra *(Figs 2–4)*

Stems of *Ephedra* are more or less circular in transverse section, possessing axial ribs. Pairs of scale leaves inserted at each node are congenitally fused at their bases, at least in the species examined (Fig. 2A, H). Short papillae are present on the ribs of the stems. Stomata are located in linear cell files in intercostal regions (between veins) along the long axis of the stem (Fig. 2B–G). Stomata are also present on both abaxial and adaxial surfaces of the scale leaves that are inserted at each node, at least in the apical and central regions of the leaves, though they are sparse on the leaf bases and absent from lateral regions.

Mature stomata are weakly paracytic with perigene lateral subsidiary cells (LSCs). They are deeply sunken so that the guard cells are not visible in surface view (Figs 2B and 3A). The outer stomatal opening is oval or rectangular and formed by both polar and lateral neighbour cells; mature pore length in both *E. gerardiana* and *E. likiangensis* is ~20 µm. There is a thick cuticle, and the surface is encrusted with epicuticular wax. Guard cells (GCs) are oriented with their long axes parallel to the stem axis; mature GC length observed here ranged from ~40 µm in *E. gerardiana* to ~25 µm in *E. equisetina*. Each stoma is flanked by a pair of axially oriented cells in the lateral adjacent cell files; the contents of these lateral cells are sometimes more granular than those of surrounding epidermal cells, which are highly tanniniferous (dark cell contents in Fig. 3C). Mature GCs contact the LSCs on their outer walls and mesophyll cells on their inner walls (Fig. 3F). The GC walls are thickened at their poles, central regions and regions where the two cells contact each other.

Stomata are derived from an intercalary meristem above each node (arrowed in Fig. 1B). Asymmetrical mitoses in cell files result in cells of alternating sizes; the smaller cells are meristemoids (Fig. 4). The meristemoids form GMCs directly, without undergoing further division. Following GMC formation but before GC differentiation, oblique divisions in lateral neighbouring cells adjacent to the GMC result in perigene LSCs that are only weakly modified relative to other pavement epidermal cells, at least initially. At this stage, the LSCs differ from adjacent pavement epidermal cells primarily by their oblique walls (Fig. 4D–F). As the epidermis thickens anticlinally and the GCs become sunken, the LSCs enlarge and become crescent-shaped, ultimately overarching the GCs.

Stomata of Gnetum *(Figs 5–7)*

In *Gnetum gnemon*, stomata are restricted to intercostal regions (between veins) on the abaxial surface of the leaf (Fig. 1E, F). In older leaves, intercostal stomata maintain a fairly regular pattern, typically oriented in cell files that are either parallel or perpendicular to each other rather than scattered in a chaotic (random) arrangement (Fig. 5). This regular pattern sometimes becomes slightly disrupted as the leaf enlarges and the epidermis undergoes further mitoses; the subsequent mitoses are mostly symmetrical, resulting in chains of cells, but occasionally asymmetrical and oriented obliquely to the rest (e.g. Fig. 7B).

Mature stomata in *Gnetum* are paracytic with mesogene LSCs (Fig. 5). Each mature stomatal complex consists of a pair of GCs and at least one pair of LSCs. Twinned stomata are common, in which the GCs share adjacent walls; they are formed when a meristemoid undergoes a secondary division and both of the resulting cells become GMCs. In mature stomata, the GC walls opposite the pore are thickened. The GCs of mature stomata are ~25 µm in length in *G. gnemon*, exhibiting little size range in intercostal regions (e.g. Fig. 5J). The cuticle is relatively thick and epicuticular wax is present (Fig. 5C).

Early-formed large stomata are located over the veins or close to them and are often aligned with the veins; these early stomata remain larger than the others and are often slightly raised above the leaf surface (Fig. 6B). During epidermal development, only the youngest leaf stages examined (Fig. 1G) showed epidermal patterning prior to GMC development. In these young leaves, intercostal protodermal cells are mostly rectangular in shape, aligned with others to produce quartets of four cells each, predominantly arranged in tetragonal tetrads (Fig. 6). Epidermal cells over the veins and leaf margins are more elongated and occur in approximately linear files.

Intercostal stomatal initiation occurs rapidly at an early stage (Fig. 7), followed by a series of divisions in subsidiary cells. In intercostal regions, the quartets of cells continue to divide perpendicularly to each other, always along their longest axis, and mostly symmetrically (Fig. 7C). At this stage, asymmetrical divisions oriented at an acute angle to the others are rare. Typically, within each quartet of cells, one cell (occasionally two cells) divides along its longest axis to form a meristemoid and a mesogene LSC (which often subsequently divides again, as in Fig. D1). In cases of two mitoses within a single quartet, they are oriented perpendicular to each other, depending on which axis is longest (Fig. 7C1). The meristemoid then divides again in the same plane, forming a row of three cells (a triad) arranged with their longest walls adjoining each other. The resulting central cell forms a further meristemoid that either acts directly as a GMC, so that the resulting daughter cells form an equal pair of guard cells, or undergoes further symmetrical divisions in the same plane, resulting in a chain of cells, of which one or more may form a GMC. The LSC either directly forms a subsidiary cell or divides again parallel to the guard cells. Occasionally, larger subsidiary cells divide perpendicularly to the guard cells (Fig. 7C1).

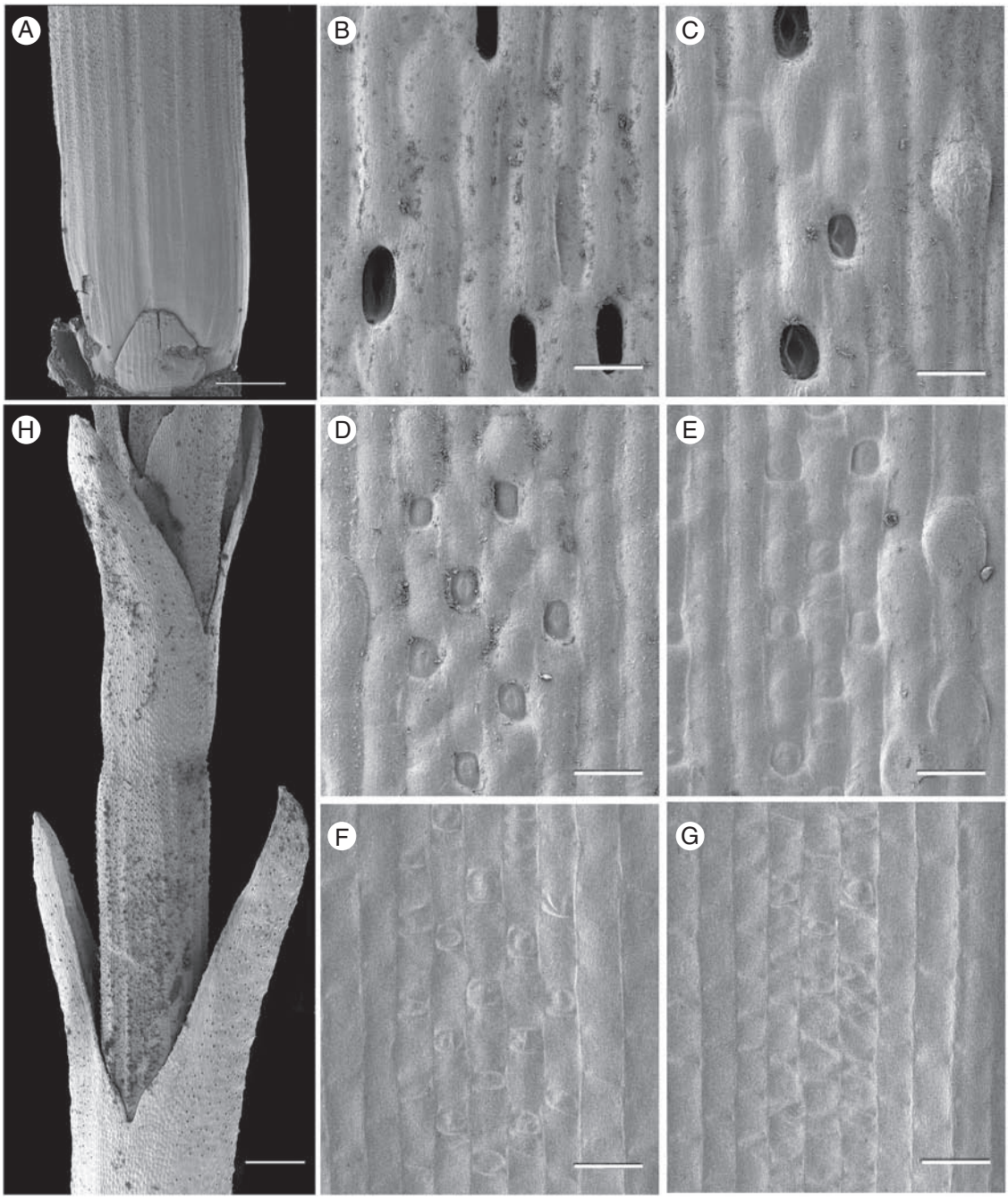

FIG. 2. *Ephedra likiangensis* (SEM). (A) Stem internode with scale leaves removed from node, revealing an axillary bud (at base). (B–G) Details of surface of stem shown in (A) from top to bottom, showing series of stomatal developmental stages across a single internode, from mature sunken stomata in (B) to meristemoids in (G). (H) Internode with congenitally fused pairs of scale leaves at each node. Scale bars: (A, H) = 500 µm; (B–G) = 20 µm.

Stomata of Welwitschia *(Figs 8 and 9)*

In the xeromorphic leaves of *Welwitschia mirabilis*, the epidermis is similar on both surfaces (Fig. 8E), with a thick outer cell wall and a thick cuticle encrusted with fine crystals (Fig. 8D) and overlain with dense epicuticular wax. The crystalline region extends over the LSCs into the stomatal pores, but not into the sunken GC walls (Fig. 8D).

Stomata are present in intercostal regions on both surfaces. Stomata are formed in cell files from an intercalary meristem at the leaf base, located close to the point of leaf insertion and below the region exposed to light. Mature stomata are sunken below the surface so that the guard cells are not visible in surface view (Fig. 8B). The outer stomatal opening is very narrow and formed by both polar and lateral neighbour cells, which together overarch the pore; mature pore length is ~20–30 µm (Fig. 8A, B). The GCs are oriented with their long axes parallel to the leaf veins; mature GC length is ~40 µm. Each stoma is flanked by a pair of axially oriented LSCs in the same cell file (Fig. 8F–H).

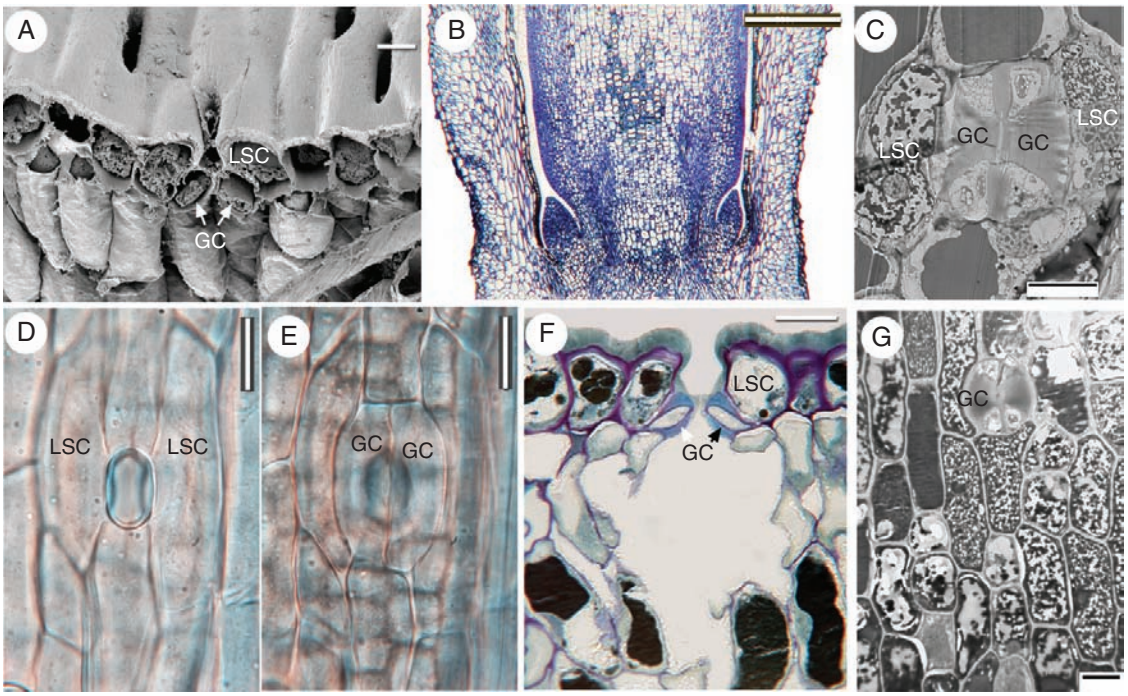

FIG. 3. Mature stomata of *Ephedra*. (A) *E. likiangensis*: transverse section of epidermis through stoma (SEM). (B) *E. equisetina*: longitudinal section of stem node. (C) *Ephedra* sp. 65601: mature stoma (TEM). (D, E) *E. likiangensis*: optical LM sections through the same epidermal region, showing surface pore in (D) and guard cells in (E). (F) *Ephedra* sp. 65601: transverse section of stoma showing substomatal cavity in mesophyll. (G) *Ephedra* sp. 65601: TEM of mature epidermis with stoma. Scale bars: (A) = 10 μm; (B) = 500 μm; (C, G) = 10 μm; (D–F) = 20 μm. GC, guard cell; LSC, lateral subsidiary cell.

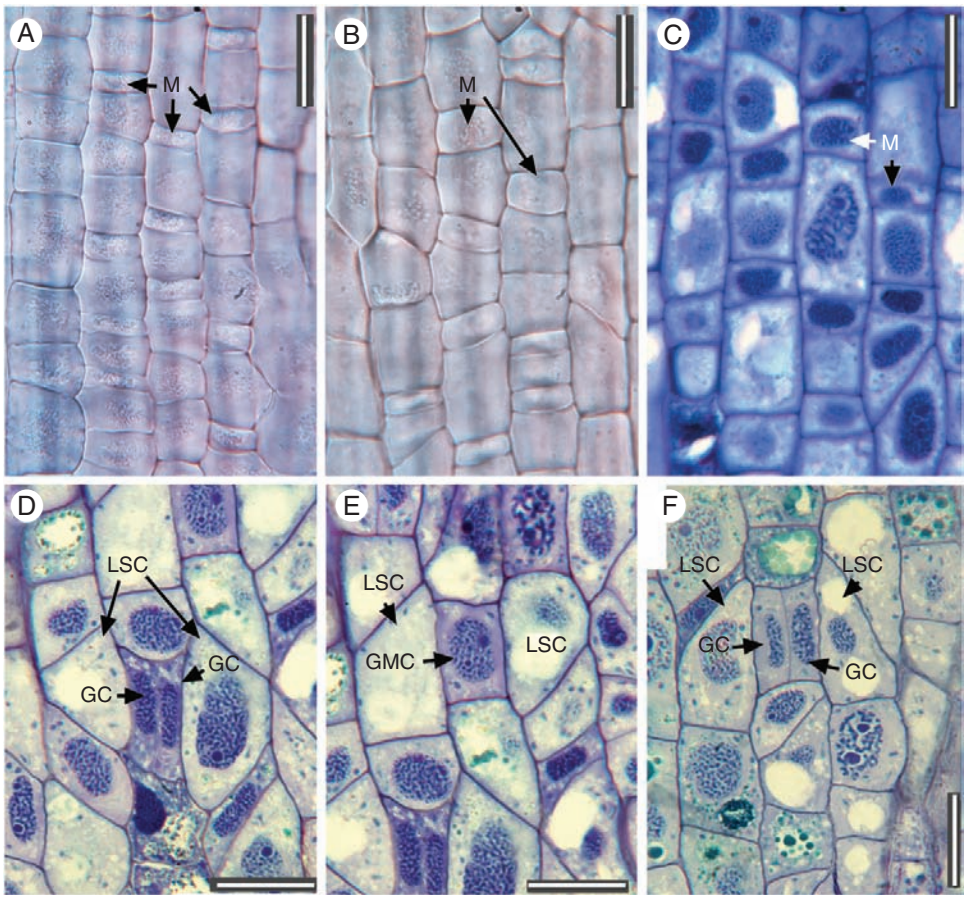

FIG. 4. Stages of stomatal development in cell files in growing stems of *Ephedra* (LM). (A, B) *E. gerardiana*. (C, E, F) *E. equisetina*. (D) *E. likiangensis*. Scale bars = 20 μm. GC, guard cell; GMC, guard mother cell; LSC, lateral subsidiary cell (with characteristic oblique walls); M, meristemoid.

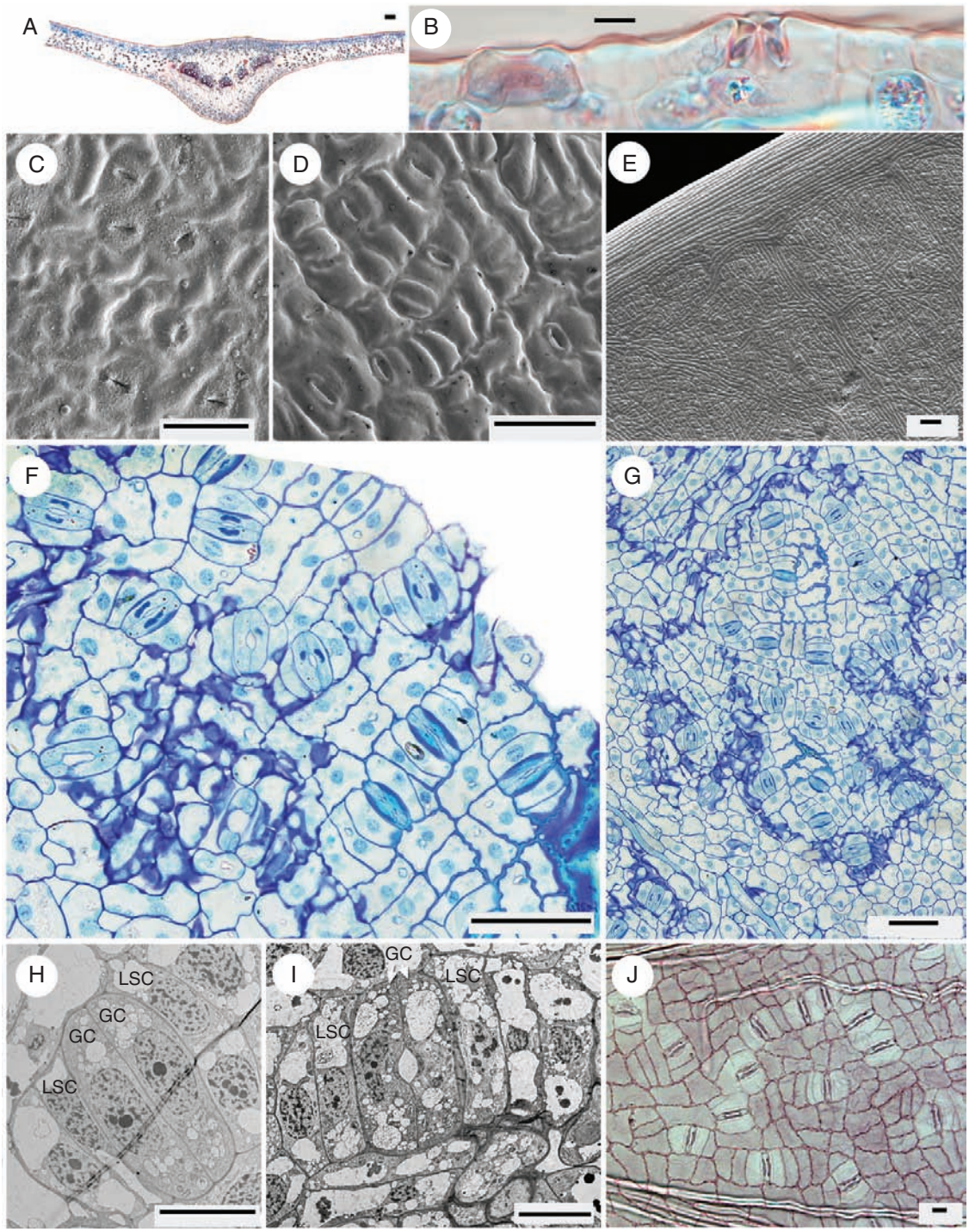

FIG. 5. *Gnetum gnemon*. (A) Transverse section of leaf (from a slide in Kew's microscope slide collection). (B) Detail of abaxial epidermis in transverse section, showing two stomata, one perpendicular to the other. (C) Abaxial surface of mature leaf encrusted with surface waxes; stomatal guard cells slightly raised (SEM). (D) Abaxial surface of younger leaf showing regular stomatal orientation (SEM). (E) Abaxial leaf surface showing margin and areoles between veins (SEM). (F, G) Abaxial epidermis of mature leaf showing mostly regular stomatal orientation. (H, I) Mature stomata showing lateral subsidiary cells adjacent to guard cells (TEM). (J) Abaxial epidermis of mature leaf (from a slide in Kew's microscope slide collection). Scale bars: (A, E) = 100 µm; (B, H–J) = 10 µm; (C, D, F, G) = 50 µm. GC, guard cell, LSC, lateral subsidiary cell.

Mature GCs contact the lateral cells on their outer walls and mesophyll cells on their inner walls (Fig. 8A, C, D). In paradermal section, GC walls appear thickened in central regions and around the pore (Fig. 8F, H).

During stomatal development (Fig. 9), following GMC formation, the meristemoids undergo two successive mitoses to form a triad of cells that spans the width of the cell file. The central cell forms a GMC and undergoes symmetrical division

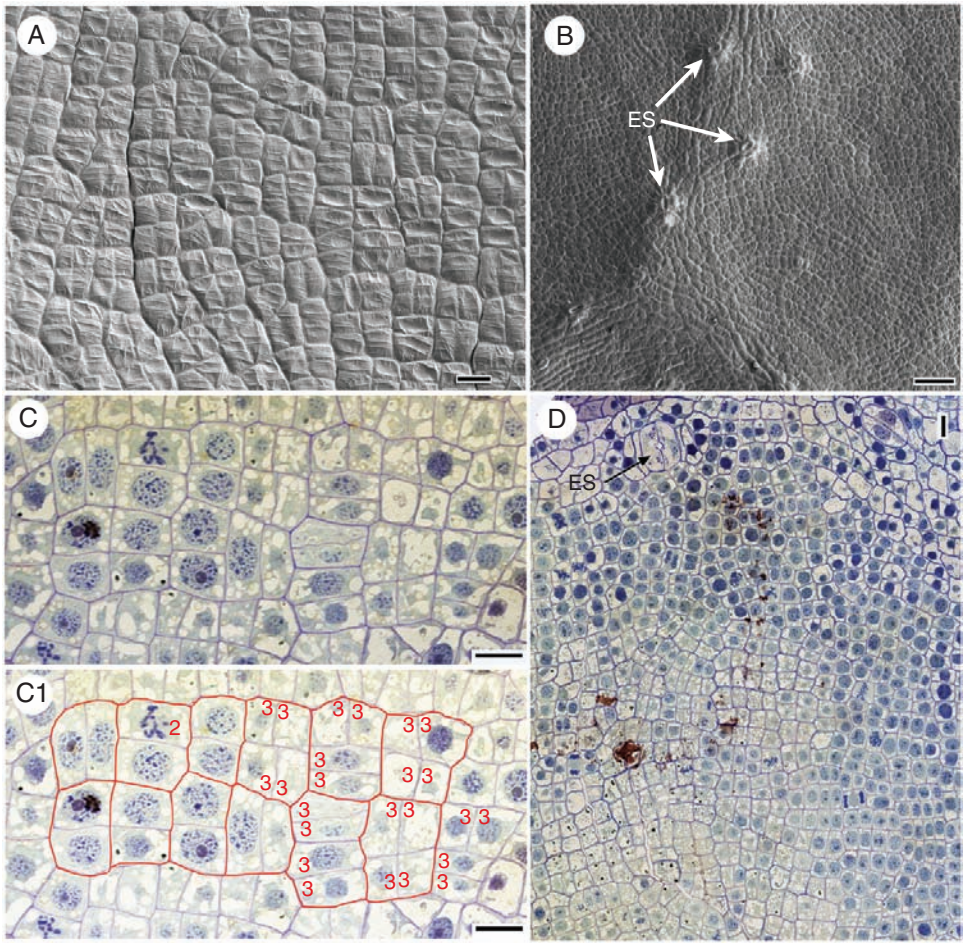

FIG. 6. *Gnetum gnemon*: abaxial surfaces of very young leaves with protodermal cells. (A, B) SEM; (C, D) LM. (A) Detail of intercostal surface showing quartet prepatterning, with protodermal cells arranged in groups of four in a 'squared' arrangement. (B) Early-formed stomata (ES) located above veins. (C, C1) Detail of intercostal region at slightly older stage than (A), in which protodermal cells have undergone at least one round of symmetrical divisions to form further quartets. (C1) Same image as (C), with cell quartets outlined (2 indicates cell undergoing a further symmetrical mitosis; 3 indicates cells that have already undergone a further symmetrical mitosis). (D) View showing both costal and intercostal regions of protodermal cells. ES indicates an early-formed stoma located over a vein or close to it. Scale bars: (A, C, D) = 10 µm; (B) = 50 µm.

parallel with the axis to form a pair of GCs. The outer two cells form mesogene LSCs. Polar neighbour cells adjacent to the GCs undergo further divisions perpendicular to the axis. As the epidermis thickens anticlinally, the GCs become sunken and the LSCs enlarge until they overarch the GCs.

DISCUSSION

Our observations reveal profound differences between the three genera of Gnetales (Table 2, Fig. 10), not only in mature structure and development of stomata, but also in epidermal prepatterning during early leaf development. These differences, though clearly heritable, can be partly explained by a range of factors. For example, the greater GC length in *Welwitschia* relative to its sister genus *Gnetum* is consistent with a whole-genome duplication in the *Welwitschia* lineage (Li *et al.*, 2015; Wan *et al.*, 2018). Similarly, the contrasting GC lengths that we observed among different *Ephedra* species are consistent with a range of genome sizes documented in this genus, which is highly unusual among gymnosperms in possessing a high proportion of polyploid species (Leitch and Leitch, 2013). Other factors that potentially contribute to observed differences are outlined below.

Differences in plant surface morphology in Gnetales are correlated with their contrasting ecological preferences

Contrasting morphological traits in the three gnetalean genera, though they are clearly heritable, can be at least partly correlated with their different ecological preferences. *Gnetum* grows in constantly mesic and shady primary tropical rainforests and is not ecophysiologically well adapted to the better-lit canopy, whereas *Ephedra* and *Welwitschia* are confined to extremely arid environments that are subject to high irradiation and temperatures and hence support few competing species (Eller *et al.*, 1983; Henschel and Seely, 2004; Feild and Balun, 2008; Krüger *et al.*, 2017). Such ecological traits include wood structure and hydraulics (Carlquist, 2012) and pollen structure

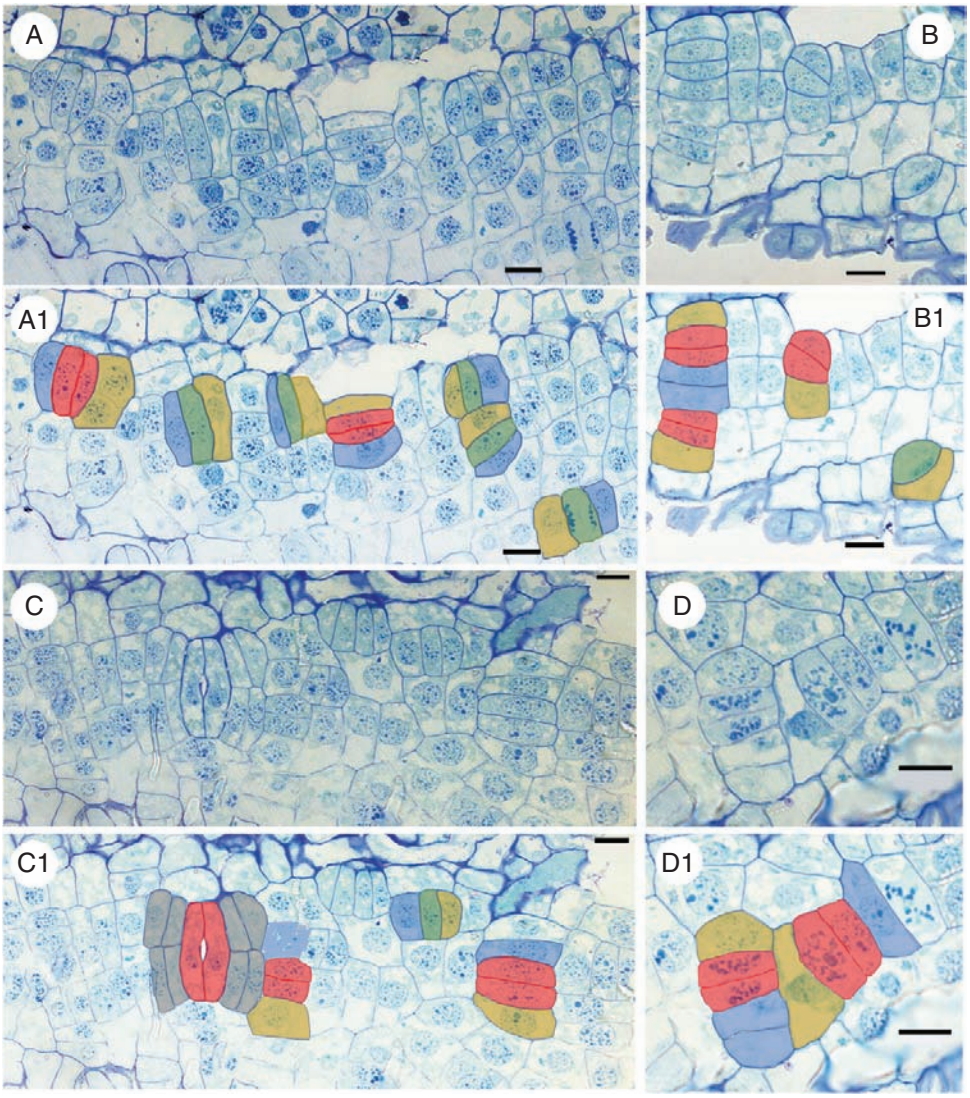

FIG. 7. *Gnetum gnemon*: developing stomata on abaxial surfaces. Each image is paired with a version in which some cells are artificially coloured. (A, A1) Region with two stomata formed and five guard mother cells (GMCs), the right-hand GMC undergoing symmetrical division. (B, B1) Region in which one recently formed stoma and a GMC have resulted from asymmetrical divisions at an acute angle. (C, C1) Region with a large early-formed stoma surrounded by several neighbour cells that have already undergone a series of mitoses. (D, D1) Region with two recently formed stomata. Colours: green, GMC; red, guard cell; yellow and blue, mesogene lateral subsidiary cells (LCSs); grey, LSCs that have undergone further mitoses. Scale bars = 10 µm.

(Osborn, 2000; Bolinder *et al.*, 2015). Pollen of *Ephedra* and *Welwitschia* is ellipsoidal and polyplicate with characteristic longitudinal ribs, a feature that facilitates dehydration and subsequent rehydration during pollen dispersal, whereas pollen of the mesic genus *Gnetum* is spherical and spiny. Indeed, Carlquist (2012) suggested that the failure of Gnetales to effectively compete with angiosperms in a wide range of ecological niches is better explained by other factors, such as the constraints of their relatively slow reproduction, rather than by any structural or hydraulic advantages of angiosperms. This inference, though speculative, is reinforced by the presence of a massive female gametophyte with a coenocytic growth phase in most seed plants, including Gnetales but excluding angiosperms (Rudall and Bateman, 2019*a*).

The stomatal differences observed here (Table 2) can also be partly explained by ecophysiology. In the relatively mesic genus *Gnetum*, the GCs are flush with the surface or even slightly raised. In contrast, in both *Ephedra* and *Welwitschia*, the stomatal GCs are so deeply sunken below the leaf surface that they are not visible in surface view; in optical and paradermal sections they appear surrounded by mesophyll cells and are adjacent to the neighbour cells only on their outer surfaces, thus limiting transpiration and maximizing water economy. The epidermis of the desert plant *Welwitschia* is protected by a remarkable crystalline cuticle that extends over the LSCs into the stomatal pores, but not into the walls of the GCs (Fig. 8D). Krüger *et al.* (2017) used energy-dispersive spectrometry to determine that these highly unusual crystals [which were earlier reported by Takeda (1913*a*)] are composed of calcium oxalate. Their hypothesis that the crystalline layer helps to both moderate the extreme temperatures and reflect excessive light in the desert environment is potentially corroborated by their absence

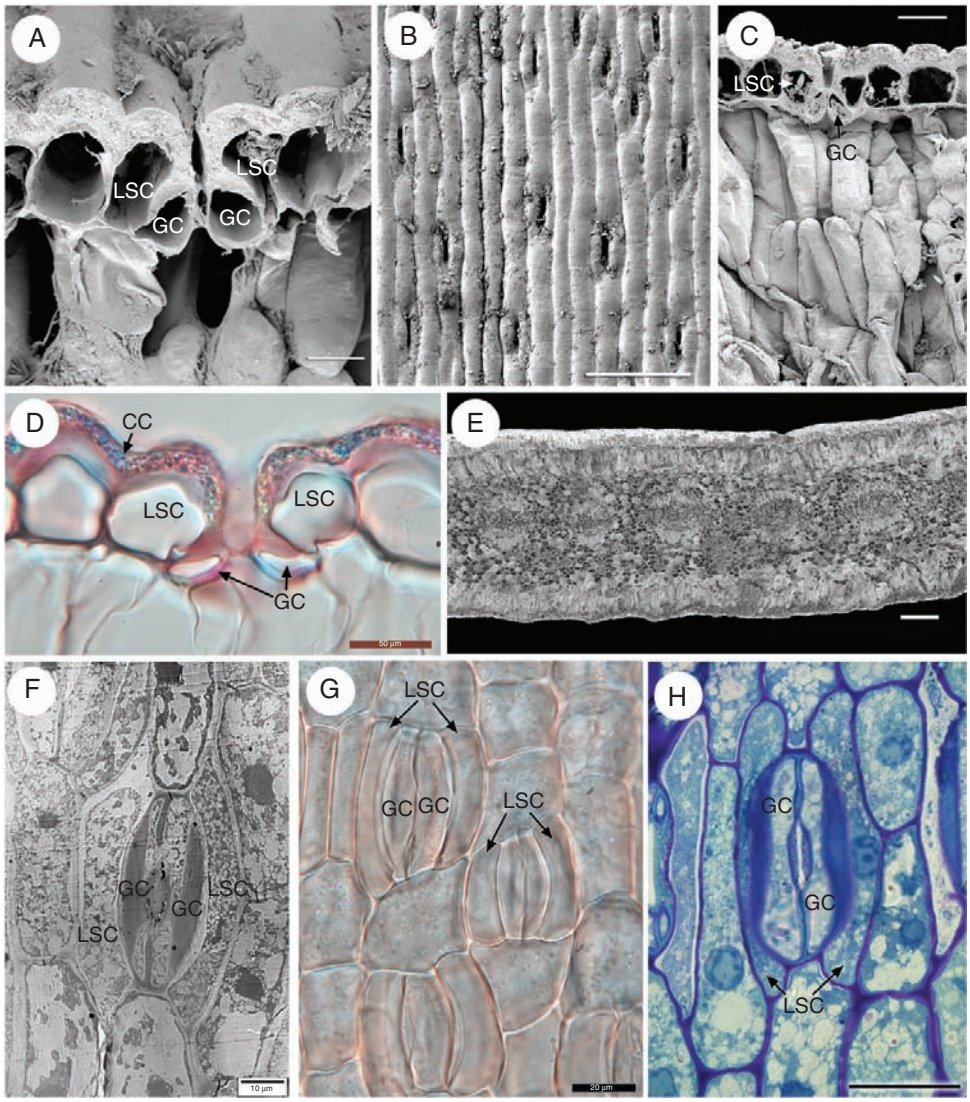

FIG. 8. *Welwitschia mirabilis*. (A) Transverse section of an adaxial stomatal pore showing sunken guard cells (SEM). (B) Abaxial epidermis showing stomatal pores oriented parallel to each other (SEM). (C) Transverse section of epidermis through stoma (SEM). (D) Transverse section of an adaxial stomatal pore showing sunken guard cells and thick cuticle embedded with crystals (LM). (E) Transverse section of leaf (SEM). (F) Paradermal section of young stoma (TEM). (G) Optical paradermal section of young stomata (DIC). (H) Paradermal section of young stoma (LM). Scale bars: (A, F) = 10 µm; (B) = 100 µm; (C, E, G, H) = 20 µm; (D) = 50 µm. CC, crystalline cuticle; GC, guard cell; LSC, lateral subsidiary cell.

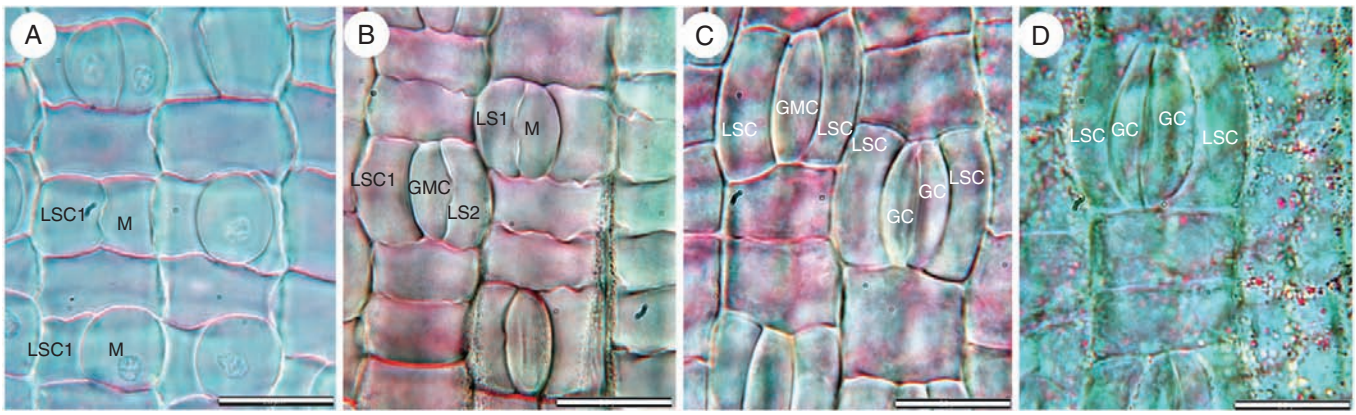

FIG. 9. *Welwitschia mirabilis*. (A–D) Optical paradermal sections of young stomata (DIC), showing successive developmental stages. Scale bars = 20 µm. GC, guard cell; GMC, guard mother cell; LSC, lateral subsidiary cell (numbered in sequence of formation); M, meristemoid.

TABLE 2. *Comparison of stomatal traits in genera of Gnetales (see also Fig. 10)*

	Ephedra	*Gnetum*	*Welwitschia*
Leaves	Reduced, scale-like	Petiolate; elliptical lamina with a central midrib and reticulate venation	Long-lived, linear, strap-like with parallel venation
Stomatal location	In linear cell files on stem; also sparsely present on leaves	On abaxial leaf surface	On both leaf surfaces
Stomatal origin	(On stems) stomata derived from intercalary meristem above each node	Most stomata derived from expanding intercostal regions throughout lamina	Stomata derived from intercalary meristem at leaf base
Epidermal prepatterning	Linear	Quartet	Linear
Guard mother cell (GMC) formation	GMCs formed directly from meristemoids	Each GMC formed from central cell of a triad formed by two successive mitoses of a meristemoid	Each GMC formed from central cell of a triad formed by two successive mitoses of a meristemoid
Guard cell (GC) orientation	Non-random; long axes of GCs parallel with organ axis	Initially non-random; stomata oriented in regular pattern parallel or perpendicular; later disrupted	Non-random; long axes of GCs parallel with leaf axis
Mature stomata	GCs deeply sunken, overarched by LSCs; GC length ~25–40 µm	GCs flush with surface or slightly raised; GC length ~20 µm	GCs deeply sunken, overarched by LSCs; GC length ~40 µm
Lateral neighbour cells/ lateral subsidiary cells (LSCs)	Perigene; derived from oblique divisions in lateral neighbour cells in adjacent cell files	Mesogene; GMC and initial pair of LSCs together form a triad	Mesogene; GMC and pair of LSCs together form a triad

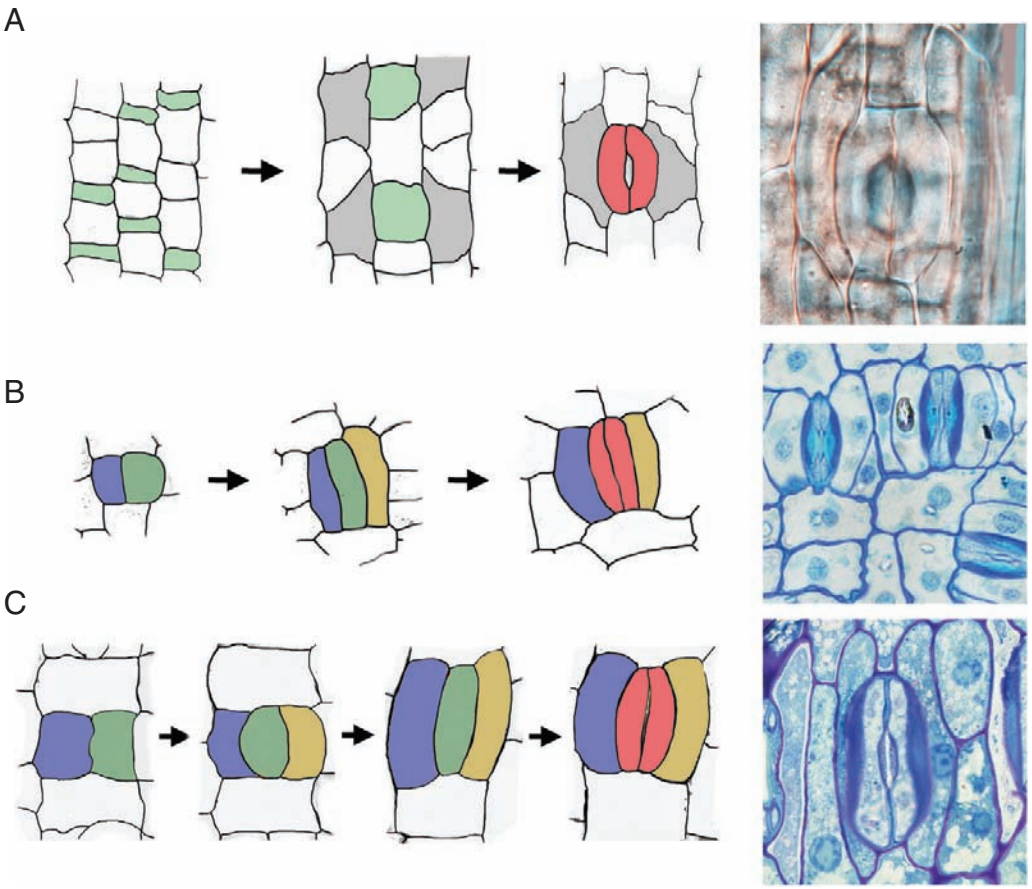

FIG. 10. Summary diagrams of stomatal development in (A) *Ephedra*, (B) *Gnetum* and (C) *Welwitschia*, with photomicrographs in the right-hand column. In the diagrams cells are coloured/textured as follows: green, meristemoid or guard mother cell; dark grey, perigene lateral subsidiary cell (LSC); blue, first mesogene LSC; yellow, second mesogene LSC; red, guard cell. In (A) both LSCs are perigene. In (B) and (C) the stomatal meristemoid divides twice and passes through a distinct triad stage before achieving the full complement of four cells in the stomatal complex. In *Gnetum* (B) the LSCs themselves often undergo secondary mitosis, but further LSC division does not occur in *Welwitschia* (C).

from the GCs, which are sunken and subject to a different microenvironment within the deep pore.

Contrasting patterns of stomatal development among genera of Gnetales

Conversely, the profound differences in stomatal development between *Ephedra* on the one hand and *Gnetum* and *Welwitschia* on the other (Table 2, Fig. 10) are not readily explained by ecophysiology. These differences, also observed in previous studies of stomata of Gnetales (e.g. Florin, 1931), support other data indicating that *Gnetum* and *Welwitschia* are a sister pair, each themselves representing ancient and divergent lineages, and *Ephedra* is even more distantly related. This phylogenetic relationship is inferred from cladistic analyses, both molecular and morphological (e.g. Doyle and Donoghue, 1986; Doyle, 1996, 2006; Nixon *et al.*, 1994; Rothwell and Serbet, 1994; Hilton and Bateman, 2006; Graham and Iles, 2009; Ickert-Bond *et al.*, 2009; Coiro *et al.*, 2018; Ran *et al.*, 2018). It is supported by morphological studies of several characters, including cone structure and nodal anatomy (e.g. Eames, 1952; Ickert-Bond and Renner, 2016).

Stomatal structure and development in *Ephedra* more closely resemble those of some other extant gymnosperms (especially conifers) than *Gnetum* and *Welwitschia.* Lateral neighbour cells in *Ephedra* are modified (albeit weakly) relative to other pavement epidermal cells and possess more granular contents, suggesting that they could have a physiological role, at least at some stage in their life history. Similarly, in many conifers the lateral neighbour cells appear crescent-shaped in surface view, though they are rarely described as LSCs (Florin, 1931). These cells are derived from cell lineages adjacent to the GMC and undergo oblique divisions in the region of the GMC. They form crescent-shaped cells that ultimately overarch the GCs. Thus, we here categorize them as perigene LSCs. In *Pinus* (sister to the remaining extant conifers), early stomatal development resembles that of *Ephedra* (Johnson and Riding, 1981). Stomata are traditionally reported as anomocytic or haplocheilic (i.e. lacking LSCs) in *Ephedra*, despite illustrations depicting modified lateral neighbour cells with oblique end walls (e.g. Florin, 1931; Pant and Mehra, 1964*a*). However, Kunzmann *et al.* (2011) described stomata in different *Ephedra* species as anomocytic to occasionally tetracytic, thus acknowledging the presence of weakly modified subsidiary cells.

Similarities between stomatal development in Gnetales and other extant gymnosperm lineages remain to be examined in more detail (reviewed by Rudall *et al.*, 2013; Rudall and Bateman, 2019*b*), with particular focus on comparisons with *Ephedra*, which is relatively plesiomorphic in Gnetales. Among other extant gymnosperms, in most cycads the guard cells are deeply sunken and the stomatal apparatus is surrounded by a distinct ring of cells (e.g. Florin, 1931; Coiro and Pott, 2017); the precise developmental origin of the encircling cells requires further exploration, though they are reportedly perigene, at least in *Cycas* (Pant and Mehra, 1964*b*). Epidermal and stomatal development in *Ginkgo* is relatively chaotic and some amplifying divisions occur on the expanding leaf blades (Rudall *et al.*, 2012).

In contrast with the perigene LSCs of *Ephedra*, our study shows that the relatively distinct LSCs of *Gnetum* and *Welwitschia* are mesogene cells. Indeed, examination of mature leaves of *Welwitschia* would lead to prediction of mesogene LSCs because these cells are clearly arranged in the same cell file as the GCs. Admittedly, this inference is not always reliable; close integration of the LSCs with the GCs as a single functional unit is also achievable with perigene LSCs, as is the case in grasses (Payne, 1979; Rudall *et al.*, 2017). Many studies have described the stomata of *Gnetum* and *Welwitschia* as paracytic (e.g. Kausik, 1974; Kunzmann *et al.*, 2011), with clearly modified LSCs, but few have examined development. Takeda's (1913*a*) and Florin's (1934) studies of *Welwitschia mirabilis*, made primarily using hand sections and illustrated only by line drawings, remain the only publications prior to the present one that describe early stomatal development in this remarkable genus. Even Rodin's (1958*a*, *b*) detailed studies of leaf development in *Welwitschia* included descriptions of fully formed stomata but failed to record early stomatal development, which occurs in a restricted region close to the point of leaf insertion on the stem. Our results confirm earlier observations (Takeda, 1913*a*; Florin, 1934) that the LSCs and GCs in *Welwitschia* are indeed formed from the same initial meristemoid, which divides twice to form a group of three cells, termed a triad by Rudall *et al.* (2013).

In *Gnetum,* our study shows similar developmental triads with mesogene LSCs, as also reported for *G. gnemon* by Takeda (1913*b*) and confirmed by most later authors (Florin, 1931; Kausik, 1974; Nautiyal *et al.*, 1976), though some contradictory accounts exist that failed to find mesogene LSCs (Maheshwari and Vasil, 1961; Inamdar and Bhatt, 1972). The triad pattern of development is apparently rare; current evidence suggests that among extant taxa it otherwise occurs only in the non-seed-plant *Equisetum* (Cullen and Rudall, 2016) and in a few angiosperms belonging to the magnoliid clade (reviewed by Rudall and Bateman, 2019*b*).

Stomatal patterning is partly related to organ growth

Among Gnetales, the primary photosynthetic organs – the stems in *Ephedra* and the leaves in *Gnetum* and *Welwitschia* – possess strikingly contrasting morphologies; they also achieve growth and expansion by different means. Stems of *Ephedra* that already possess mature epidermal cells in the upper part of the internode continue to add cells from an intercalary meristem located a short distance above the subadjacent node (Fig. 1B), in a 'diaphragm' region that can later form a dehiscence layer (Widmoyer, 1950, 1954; Cresson and Evert, 1993). The two persistent leaves of *Welwitschia* (Fig. 1C, D) continue to add cells throughout the life of the plant from an intercalary meristem at their base (Rodin, 1958*a*). Development of the relatively angiosperm-like leaves of *Gnetum* (Fig. 1E–G) proceeds initially by basal expansion, then by means of a marginal meristem accompanied by plate meristem activity in the areoles between the veins, where the stomata are formed (Rodin, 1967; Tomlinson and Fisher, 2005). Thus, leaf development in *Gnetum* most closely resembles that of angiosperms, which grow by a combination of different types of stem-cell activity, including intercalary, marginal and plate meristems.

Much remains to be understood about the genetic bases for contrasting patterns of organ development. Recent research convincingly demonstrates a role for genes of the WUSCHEL (WUS) clade of the WOX (WUSCHEL-related homeobox) gene family (Nardmann and Werr, 2013), but detailed comparative gene expression studies of different types of stem-cell activity are currently lacking. Interestingly, two *WUS* genes found in *Gnetum gnemon* (*GgWOXX* and *GgWOXY*) apparently lack orthologues in other seed-plant genomes, indicating that they represent ancestral sequences that were lost from other seed-plant lineages (Nardmann and Werr, 2013; Wan *et al.*, 2018). Expression of *WOX3* genes marks marginal or plate meristems during leaf expansion in a similar manner in *Arabidopsis* and *Gnetum*.

Our study demonstrates that differences in cell patterning in the mature leaf epidermis of *Gnetum* and *Welwitschia* relate more to their contrasting modes of early leaf expansion rather than to later development. Epidermal prepatterning in the expanding leaf blades of *Gnetum* undergoes a 'quartet' developmental phase, in contrast with the exclusively linear development of *Welwitschia*. Quartet prepatterning (*sensu* Bünning and Sagromsky, 1948; Barlow and Lück, 2009; Rudall *et al.*, 2019*b*) results in stomata being formed almost simultaneously in a single cluster and oriented perpendicular to each other rather than exclusively in the same direction. A similar quartet type of prepatterning is widespread in angiosperms, including *Amborella*, the sister taxon to all other extant angiosperms (Rudall and Knowles, 2013). This close similarity between early leaf expansion in *Gnetum* and angiosperms partly explains other lamina similarities. For example, *Gnetum* displays higher vein length per unit area compared with other gymnosperms (Sack and Scoffoni, 2013), though it is lower than that of many angiosperms; in this respect *Gnetum* is most closely comparable with shade-adapted angiosperms from lowland tropical rainforests (Feild and Balun, 2008).

Conversely, there is a divergence in subsequent stomatal development between *Gnetum* and early-divergent angiosperms. In *Amborella*, stomatal meristemoids are formed by an asymmetrical mitosis of a protodermal cell that results in random orientation of the stomatal pores. In contrast, in *Gnetum* the protodermal mitosis is approximately symmetrical and parallel with the long axis of the cell. Furthermore, within a single protodermal quartet, two guard-cell lineages often form perpendicularly to each other. Despite occasional reports of random stomatal orientation in this genus, our results show that this perpendicular orientation is partly maintained in older leaves in *Gnetum*, resulting in relatively non-random orientation relative to the nearest veins, especially at younger stages.

Leaves of *Gnetum* are often compared with those of eudicots (e.g. *Arabidopsis*) because both groups possess a petiole and a broad lamina with reticulate venation, albeit with lower vein density in *Gnetum* (Feild and Balun, 2008). However, later epidermal expansion in *Gnetum* clearly differs from that of reticulate-veined eudicots. The iterative mitoses of epidermal ground cells that occur in *Gnetum* are generally symmetrical and result in linear chains of cells rather than the asymmetrical (and hence inwardly spiralling) amplifying divisions that characterize many eudicots. In *Arabidopsis*, asymmetrical amplifying divisions are at least partly controlled by the bHLH protein *SPEECHLESS* (*SPCH*) (Bergmann and Sack, 2007; MacAlister and Bergmann, 2011). Amplifying divisions are rare or absent from monocots and some early-divergent (ANA-grade) angiosperms such as *Amborella* and Nymphaeaceae (Carpenter, 2005; Rudall and Knowles, 2013; Rudall *et al.*, 2017). It remains to be evaluated whether the symmetrical expansion divisions of *Gnetum* are controlled by genetic factors similar to those that govern amplifying divisions in eudicots.

Comparison with fossil Gnetales

The fossil record of Gnetales extends back to the early Cretaceous and indicates that this lineage was once far more species-rich than today. The three surviving relictual genera of Gnetales represent tantalizing clues to this past diversity. Several Cretaceous macrofossils that include well-preserved vegetative structures with stomata have recently been assigned to Gnetales (Rydin *et al.*, 2003; Kunzmann *et al.*, 2009, 2011; Yang *et al.*, 2015; Ickert-Bond and Renner, 2016). Yang *et al.* (2015) described a Chinese fossil from the Early Cretaceous Yixian Formation as a new species of *Ephedra*; it possessed paired strap-shaped leaves, but the stomata were not described. Three new genera that resembled Gnetales (*Cariria*, *Cearania*, *Cratonia*) have been described from the Early Cretaceous Crato formation of Brazil (Rydin *et al.*, 2003; Kunzmann *et al.*, 2009, 2011). The genus *Cariria* possessed entire oval, thick leaves with parallel venation and stomata on both surfaces (Kunzmann *et al.*, 2011). The stomata of *Cariria* were predominantly slightly sunken and axially or obliquely oriented, with an undetermined number of subsidiary cells. In a detailed discussion and comparison with both living and putative fossil Gnetales, Kunzmann *et al.* (2011) did not assign *Cariria* to this lineage, commenting that it may represent a closely related extinct lineage and noting similarities with not only Gnetales but also Bennettitales or Erdtmanithecales. The fossil genus *Cratonia* resembled cotyledons of *Welwitschia* in possessing similar venation and epidermal structure, together with axially oriented paracytic stomata (Rydin *et al.*, 2003). In contrast, *Cearania* had highly branched shoots with overlapping leaves; it more closely resembled *Ephedra*. However, its well-preserved stomata were apparently highly unusual and rather variable, with predominantly transverse or oblique orientation combined with axially elongated epidermal cells, perhaps indicating that the ephedroid lineage was once more diverse in stomatal structure.

Conclusions

Our comparative investigation of epidermal development in the three extant genera of Gnetales not only clarifies the homologies of the specialized lateral cells immediately adjacent to the stomata but also uncovers differences in early stomatal prepatterning. Perhaps not surprisingly, given the vast period that has elapsed since their phylogenetic divergence, the three extant genera display strong differences in both their mode of development and their mature structure. Contrasting developmental trajectories in the epidermis are genetically determined. They can be correlated partly with the contrasting ecological preferences of the three genera and partly with distinctions

in organ growth. Although *Ephedra* shares linear and intercalary growth patterns with *Welwitschia*, stomatal development in *Ephedra* differs significantly from that in both *Gnetum* and *Welwitschia*, more closely resembling that in other extant gymnosperm groups.

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