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Morpho-anatomical affinities and evolutionary relationships of three paleoendemic podocarp genera based on seed cone traits

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- **Background and Aims:** The three relict genera *Pherosphaera*, *Microcachrys* and *Saxegothaea* in Podocarpaceae produce quite distinct seed cone types in comparison with other genera and do not form a clade along with *Acmopyle*. The detailed seed cone morpho-anatomy of these three relict genera and affinities with other podocarps are poorly known. This study aims to understand the seed cone morpho-anatomy and affinities among these three disjunct relict genera and with other podocarps.
- **Methods:** We comparatively analysed the seed cone morpho-anatomical traits of the three podocarps genera and used ancestral state reconstruction to understand the evolution of these traits.
- **Key Results:** We described the seed cone morpho-anatomical structures of the three relict genera in detail. The three genera produce aggregated multiovulate cones. Both *Microcachrys* and *Saxegothaea* have an asymmetrical free cup-like epimatium. Both species of *Pherosphaera* lack an epimatium. The ancestral state reconstruction implies that the presence of an epimatium is an ancestral trait in podocarps and is independently lost in *Pherosphaera* and *Phyllocladus*. The seed cones are fleshy in *Microcachrys* and non-fleshy in *Saxegothaea* and *Pherosphaera*. The seed cone macrofossils of both extinct and living podocarps also show the presence of an epimatium and fleshiness in podocarps.
- **Conclusions:** Altogether, the morpho-anatomy suggests that *Pherosphaera*, *Microcachrys* and *Saxegothaea* present affinities with each other and other podocarps, but the reconstruction of the ancestral seed cone in Podcarpaceae is quite complex due to multiple convergent evolutions of several structures. These structures (e.g. epimatium, aril and receptaculum) are of low taxonomic value but of great evolutionary and ecological significance, and are responsive adaptations to ever-changing environmental conditions.

Key words: Conifers, evolution, endemism, Gondwana, histology, seed dispersal, Podocarpaceae, reproductive biology.

INTRODUCTION

The Podocarpaceae today is one of the dominant conifer families, with 20 genera and >200 species, mostly in the Southern Hemisphere. Based on recent phylogenetic analyses, three major clades have been recognized – the Prumnopityoid clade, the Podocarpoid clade and the Dacrydioid clade. However, there is also a paraphyletic group of four genera outside these clades that comprise distinct lineages. These genera include three with compound seed cones – *Saxegothaea*, *Microcachrys* and *Pherosphaera*, and one with 1–2 ovules – *Acmopyle*, like other podocarps (Leslie *et al.*, 2017; Andruchow-Colombo *et al.*, 2019). The Podocarpoid and Dacrydioid clades contain most of the extant species and appear to have radiated during the Cenozoic, based on their current habitat preferences and the inferred molecular divergence dates. In contrast, members of the paraphyletic group of genera have restricted distribution and, along with some members of the Prumnopityoid clade, appear to reflect deeper divergences (Knopf *et al.*, 2012; Leslie *et al.*, 2017). These genera now have both restricted and disjunct distributions. *Saxegothaea* has one extant species in southern Chile and southern Argentina; *Microcachrys* contains one extant species in the alpine vegetation of central and western Tasmania, Australia; and *Pherosphaera* has two extant species, one in montane areas of Tasmania and the other on sandstone ledges of waterfalls in the Blue Mountains, New South Wales, Australia (Carpenter *et al.*, 2011).

Reproductive structures and traits are important for determining the evolutionary relationships of conifers and other groups, and they also provide insight into ecological interactions and adaptation to environmental changes (Yang *et al.*, 2015; Leslie *et al.*, 2018; Klaus and Matzke, 2020). Female reproductive structures are a critical component of any species and they are often strongly influenced by ecological interactions, including pollination, protection of the developing seeds and seed dispersal (Tomlinson *et al.*, 1991; Contreras *et al.*, 2017). In the conifers, seed cones have performed these multiple functions through time, and the adaptations at any one time can probably be co-opted or changed (within developmental limitations) to work as well as possible for the next required function (Leslie, 2011). Some conifer families have responded to changing environments through time by diversifying their seed cones (Yang *et al.*, 2015). For example, two of the currently dominant conifer families, Pinaceae and Podocarpaceae, have had distinctly different outcomes in seed cone evolution, i.e. hard scaly cones and fleshy cones, respectively (Contreras *et al.*, 2017).

These relict genera are considered to be surviving lineages from the Palaeozoic and Mesozoic eras (Doyle and Looby, 1939; Doyle, 1945; Axsmith *et al.*, 1998; Cantrill and Falcon-Lang, 2001). However, tracing the evolutionary history of these three extant genera is difficult because the morphological changes that have accumulated through time are represented by only a small number of extinct species in the fossil record (Looby and Doyle, 1939). These three relict Podocarpaceae genera have had a great deal of uncertainty in their relationship with other podocarp genera and in the interpretation of their origin. This is exemplified by the fact that they have demonstrated instability within various phylogenetic analyses. This morphological distinctiveness of these genera led some taxonomists to place them within their own families (e.g. Saxegothaeaceae, Pherospheraceae; Gaussen, 1974; Doweld and Reveal, 1999). The seed cone diversity in Podocarpaceae suggests several different evolutionary pathways to produce cones with different morpho-anatomical functional traits and structures (Tomlinson *et al.*, 1991; Tomlinson, 1992; Restemeyer, 2002). No other conifer family shows such diversity in seed cone functional traits and structures. There is also a remarkable difference in seed cone morphology between the Podocarpaceae and their nearest living relative, the Araucariaceae, and the fossil record provides no clear evidence for the early evolutionary history of seed cones in the Podocarpaceae. Although morphological aspects of conifers have been considered in general in previous studies (Tomlinson *et al.*, 1991; Tomlinson, 1992; Restemeyer, 2002; Contreras *et al.*, 2017; Leslie *et al.*, 2018; Herting *et al.*, 2020; Klaus and Matzke, 2020), there remains a lack of comprehensive studies on the detailed morpho-anatomy, functional structures and trait evolution of seed cones of the Podocarpaceae. The absence of such studies has resulted in some confusing interpretations and terminology for functional structures and traits. The focus of our research has been to investigate the detailed seed cone morpho-anatomy of functional structures and trait evolution of Podocarpaceae. This study is focused on evaluating the evolution and loss of seed cone functional structures in these three palaeoendemic genera (*Saxegothaea*, *Microcachrys* and *Pherosphaera*) to establish their relationship with other podocarps. We assessed the detailed seed cone morpho-anatomy for consistent affinities of functional structures and traits among these three disjunct relict genera and with other podocarps.

MATERIALS AND METHODS

General morphology and distribution

Pherosphaera has occasionally monoecious individuals, while both species are generally dioecious evergreen shrubs. Archer (1850) established the genus *Pherosphaera*, including two species (*P. hookeriana* and *P. fitzgeraldii*); they occur in mountainous areas with high rainfall and close to running water. *Pherosphaera hookeriana* W. Archer bis ex Hooker is the type species of the genus and is distributed in montane areas of Tasmania (Australia), where it reproduces asexually by root suckers as well as by sexual reproduction (Worth *et al.*, 2018). *Pherosphaera fitzgeraldii* (F. Mueller) F. Mueller ex Hooker f. is a critically endangered species distributed on sandstone ledges of waterfalls in the Blue Mountains, New South Wales (Australia). It is an evergreen shrub with spirally arranged, scaly leaves. The pollen grain of *Pherosphaera* ranges in size from 30 to 45 µm and has three smooth sacci (Elliott, 1948*b*).

Microcachrys tetragona is a monotypic paleoendemic species, restricted to alpine vegetation in central and western Tasmania, Australia (Carpenter *et al.*, 2011). It is a highly branched, low-growing shrub with leaves in opposite pairs. Hooker (1845) described this monotypic genus as having trisaccate pollen grains (Lawson, 1923*a*,*b*). Seed cones of *Microcachrys* are fleshy and bright red. *Saxegothaea conspicua* Lindley (Mañio) is a small- to medium-sized tree (Restemeyer, 2002), native to Southern Chile and Southern Argentina.

Seed cone collection

Seed cones at different developmental stages were collected from the Australian National Botanic Gardens, Canberra, Mount Lofty Botanical Garden, South Australia and The Tasmanian Arboretum, Devonport, Tasmania, Australia. For this study, we used living seed cones (ten seed cone replicates for each species) and we have stored four specimens of each species in the plant spirit collection of the University of Adelaide, Australia. The spirit collection voucher numbers are (1) UOA-SC219-22 for *Saxegothaea conspicua*, (2) UOA-SC223-226 for *Microcachrys tetragona*, (3) UOA-SC227-230 for *Pherosphaera fitzgeraldii* and (4) UOA-SC231-234 for *Pherosphaera hookeriana*. We also collected complete plant specimens which are lodged in the State Herbarium of South Australia (AD).

Taxon processing and sectioning

Whole seed cones, plus longitudinal and cross-sections of the seed cones were photographed with a Nikon-SMZ25 stereomicroscope. Specimens were fixed in 200 mL of FAA (100 mL of 95 % ethanol + 80 mL of dH_2O + 20 mL of 37 % formaldehyde solution) immediately after collection. For the histology, seed cones were fixed for 48–72 h and then processed for a 48 h cycle on a Sakura Tissue-Tek VIP6 Vacuum Infiltration Tissue Processor. They were then embedded in paraffin wax (Sakura Tissue Tek embedding centre). Longitudinal and cross-sections of 8–10 µm thickness were subsequently produced using a Leica RM 2235 Rotary Microtome and stained with H & E (DAKO Cover stainer) and Toluidine blue. The slides were observed under light microscopy and photographed at various magnifications.

Scanning electron microscopy

For scanning electron microscopy (SEM) the seed cones were fixed for 3 d in the fixative [4.0 % paraformaldehyde/1.25 % glutaraldehyde in phosphate-buffered saline (PBS) solution]. The samples were then washed twice for 5 min in PBS and 4 % sucrose. The samples were dehydrated twice in 70 % ethanol for 8 h each time, twice in 90 % ethanol for 8 h each time and twice in 100 % ethanol for 8 h each time. The dehydrated samples were placed in 100 % ethanol and hexamethyldisilazane (HMDS) (1:1) for 1 h. The HMDS was then pipetted out and the samples were air dried. The samples were mounted on an aluminium stub with sticky tabs and coated with platinum. These specimens were observed using a scanning electron microscope (Model Philip XL-30) installed at Adelaide Microscopy in the University of Adelaide.

Terminology used

Some of the important terms used are defined here and illustrated from taxa across the Podocarpaceae. Receptaculum/podocarpium: a fleshy structure formed when the peduncle or cone axis fuses with the bracts of the seed cone into a fleshy structure (Tomlinson *et al.*, 1991; Restemeyer, 2002). The receptacle can be formed by both fertile and sterile bracts. The receptacle is usually brightly coloured. In Fig. 1A–C, the receptaculum of *Podocarpus totara* is formed by fertile bracts. Aril: usually a fleshy or papery structure originating at the base of the ovule from an outgrowth of the funicle, not the testa or integument. *Phyllocladus hypophyllus* has two fleshy bracts; one is fertile and one is sterile to form a ring-like structure (Fig. 1D–F). The aril is free or in some cases fused with the seed (Dörken *et al.*, 2019). Epimatium: a fleshy or non-fleshy structure in Podocarpaceae considered as homologous to the ovuliferous scale (Figs 1G–L and 4E–H). The epimatium is usually brightly coloured (Dörken *et al.*, 2019). Ovuliferous/seed scale: a modified shoot that bears the ovule (Florin, 1954; Leslie and Losada, 2019). Herting *et al.* (2020) propose based on ontogenetic studies that seed scale is a modified funiculus. Fertile bract: a modified or specialized leaf that subtends the fertile scale/epimatium (Fig. 1G–H). Fleshy sterile bracts: bracts modified into a swollen and fleshy structure (Fig. 1J). Cone axis: axis of the cone. Micropyle: opening in the integument of an ovule (Figs 1B, D, H, K and 4A–F). Testa: outer layers of the seed (seed coat) protecting the embryo. The testa can sometimes be sub-divided into the exotesta, mesotesta and endotesta (Fig. 1C, F, I, L). Nucellus: the megasporangium of seed plants, often visible as the layer of cells in the central part of an ovule, surrounding the megagametophyte (Fig. 4H).

Measurements and character mapping

Different morpho-anatomical and embryological characters were recorded (Table 1). The anatomical layers were measured from mature seed cones. The measurements were taken using ImageJ 1.8.0_112 software. For ancestral reconstruction, we used the dated phylogeny of based on three chloroplast (*rbcL*, *matK*, *trnL–trnF*) and three nuclear genes (*NEEDLY*, *PHYP* and *ITS2*). The characters were mapped for their evolution using RASP 4.2-BayesTraits (Yu *et al.*, 2020) and Mesquite 3.6 (Maddison and Maddison, 2019) with the maximum likelihood (ML) and Markov chain Monte Carlo (MCMC) reconstruction method.

RESULTS

Pherosphaera *seed cone morpho-anatomy*

Pherosphaera hookeriana produces non-fleshy multiovulate cones occurring at the tip of the very short lateral shoots (Figs 2A–C and 4C, D). The seed cones are pink-reddish, ovoid-shaped, 2–4 mm long and 1–2.5 mm wide. The seed cones consist of 2–5 seeds, each with a fertile bract, and additionally the cone has 5–8 ovate concave sterile bracts. The seed size is about 1.2–1.8 × 0.4–0.8 mm. The ovules are erect.

The transverse section of the seed shows the following three major anatomical zones (Fig. 2D–F). (1) Testa: 8–14 compact layers of rounded and isodiametric cells forming the protective cover of the seed. The testa is differentiated into the exotesta made up of two layers of rounded cells forming the outer zone of the testa, the mesotesta which is a sclerified layer consisting of 3–5 layers of dense rounded cells forming the hard protective cover of the ovule, and the endotesta with 5–7 non-lignified layers of parenchymatous cells surrounding the nucellus. (2) Nucellus: 2–4 layers of small dense round cells. (3) Megagametophyte: 10–16 dense layers surrounding the straight embryo. The fertile bract that subtends the ovule has a single enlarged resin duct with a vascular bundle.

Pherosphaera fitzgeraldii produces non-fleshy multiovulate cones. There are usually 2–8 seeds per seed cone, each positioned on a fertile bract, similar to *P. hookeriana* (Fig. 4A–D). The seed size is 1–2.5 × 0.5–0.8 mm. The ovules are erect (Figs 2G–L and 4A, B). The *P. fitzgeraldii* mature seed cone consists of three anatomical zones, similar to *P. hookeriana* (Table 1).

The fertile bract attached to the ovule has a single enlarged resin duct with a vascular bundle in both species. Both aril and epimatium are absent in *Pherosphaera*. The outer layer of the exotesta is free at some points from the inner layer.

Microcachrys *seed cone morpho-anatomy*

Microcachrys tetragona produces bright red, fleshy, multiovulate cones at the tip of short branches (Fig. 3A). The cones are ovoid to globose, 2.5–8 mm long and 2–5 mm wide. Seeds are ovoid, 10–28 in number, each one surrounded by a fleshy epimatium and fertile bract (Fig. 3A–D).

The mature ovules are inverted and are positioned on a fleshy epimatium and bract (Fig. 3C–E). The fertile bract is at a right angle to the cone axis. The seed size is 1–2.5 × 1–2 mm. Four major anatomical regions/zones were observed in the mature seed cone (Fig. 3F). (1) Epimatium: 4–8 layers of small and large cells, covering half of the ovule initially and later covering the lower part of the mature seed (Figs 3C–E and 4E, F). (2) Testa: 4–6 compact layers of small and large cells. The testa is differentiated into the exotesta consisting of two layers of rounded cells forming the outer zone of the testa, the mesotesta which is a sclerified layer consisting of 2–3 layers of dense elongated cells forming the hard protective cover of the ovule, surrounding the nucellus and the endotesta made up of 2–3 layers of dense cells. The outer layer of the exotesta is free at some points from the inner layer similar to *Pherosphaera*.

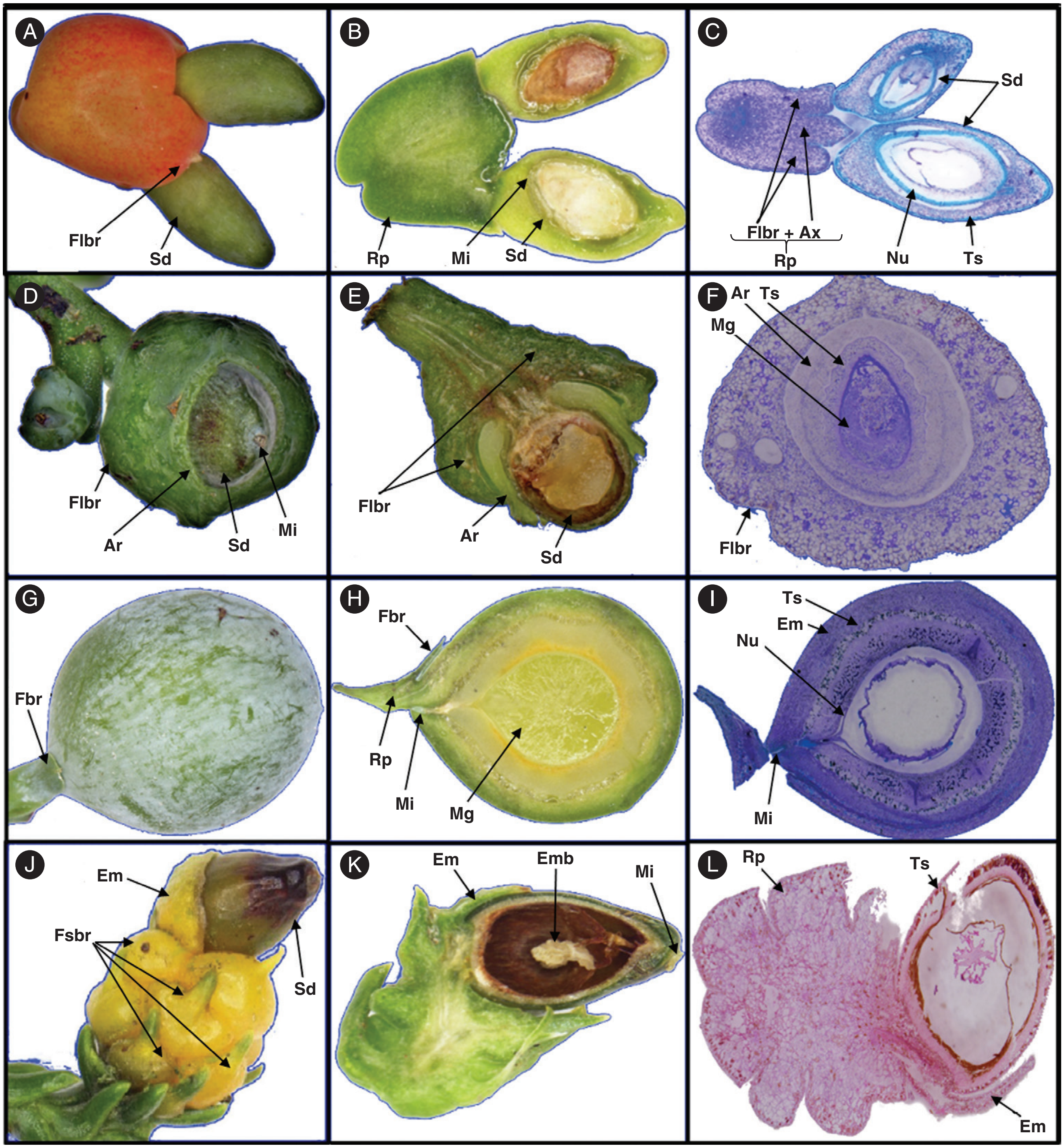

FIG. 1. Demonstrating different morpho-anatomical structures in Podocarpaceae. (A–C) *Podocarpus totara*, (D–F) *Phyllocladus hypophyllus*, (G–I) *Afrocarpus falcatus* and (J–L) *Dacrydium cupressinum*.

(3) Nucellus: a protective cover of the embryo consisting of 3–5 layers of dense, smaller cells. A double-layered megaspore membrane is also present. (4) Megagametophyte: 5–8 layers of large cells surrounding the straight embryo. The fertile fleshy bract anatomy shows an outer cuticle layer and a double-layered hypodermis of sclereid cells, an enlarged vascular bundle and two enlarged resin ducts.

Saxegothaea *seed cone morpho-anatomy*

Saxegothaea conspicua is another genus in the Podocarpaceae that produces non-fleshy multiovulate cones on the terminal branches. The seed cones are ovoid to globose in shape, 10–20 mm long and 4–8 mm wide, with 2–6 seeds per cone, each surrounded by a triangular fertile bract (Fig. 3G–J). Seeds are ovoid, 3–4 mm long and 2–3 mm wide.

TABLE 1. *Seed cone morpho-anatomical qualitative and quantitative characters of the three paleoendemic Podocarps*

Characters	*Pherosphaera hookeriana*	*Pherosphaera fitzgeraldi*	*Microcachrys tetragona*	*Saxegothaea conspicua*
Reproductive cycle	1 year	1 year	1 year	1 year
Cone shape	Ovoid	Ovoid	Ovoid–globose	Ovoid–globose
Cone size (mm)	2–4 × 1–2.5	2–4.5 × 1.5–2.5	2.5–8 × 2–5	10–20 × 4–8
Colour	Dry brownish	Dry brownish	Bright red	Dry brownish
Number of seeds per cone	2–5	2–8	10–28	2–6
Seed size (mm)	1.2–1.8 × 0.4–0.8	1–2.5 × 0.5–0.8	1–2.5 × 1–2	3–4 × 2–3
Seed shape	Ovoid	Ovoid	Ovoid	Ovoid
Seed surface	Rugose	Rugose	Smooth	Smooth
Seed colour	Shiny dark brown	Dark brown	Dark brown	Dark brown
Ovule	Orthotropous	Orthotropous	Anatropous	Anatropous
Free nuclei	16	16	40–64	16
Aril	Absent	Absent	Absent	Absent
Epidermal layers	1	1	1	1
Epidermal cell shape	round- isodiametric	round- isodiametric	round-rectangular	rectangular
Testa layers of cells	8–14	8–12	4–9	4–7
Nucellus	2–4	4–6	3–5	3–6
Embryo shape	Straight	Straight	Straight	Straight
Embryo size (mm)	0.3–0.5 × 0.1–0.2	0.2–0.6 × 0.1–0.3	0.3–0.5 × 0.1–0.2	0.2–0.4 × 0.1–0.2
Fertile bracts	2–5	2–8	10–28	2–6
Sterile bracts	5–8	4–8	12–30	15–20
Sclereid cells in testa	Present	Present	Present	Present
Sclereids in fertile bract	Present	Present	Present	Present
Resin canals in fertile bract	Present	Present	Absent	Present
Stomata on bracts	Present	Present	Present	Present
Fleshy bracts	Absent	Absent	Present	Present
Epimatium	Absent	Absent	Present	Present
Epimatium colour	–	–	Bright red	Yellowish
Dispersal	Barochory	Barochory	Zoochory	Barochory

The ovules are inverted, with each one positioned on a fertile bract. The seed size is 3–4 × 2–3 mm. Four major anatomical zones were observed in the mature cone (Fig. 3K, L). (1) Epimatium: 6–8 layers of round and rectangular cells, covering half the ovule (Figs 3K and 4G, H). (2) Testa: 3–4 layers of small rounded, elongated and rectangular cells. The testa is differentiated into the exotesta, a single or double layer of rounded cells forming the outer zone of the testa, and the endotesta, a sclerified layer consisting of 3–5 layers of dense elongated cells forming the hard protective cover of the ovule, surrounding the nucellus. (3) Nucellus: the protective cover of the embryo which consists of 3–6 layers of small cells. A double-layered megaspore membrane was also observed, similar to that in *Microcachrys*. (4) Megagametophyte: 4–6 layers of large cells surrounding the straight embryo. The ovule is attached directly to one fertile bract with three vascular bundles (two small on the upper side towards the ovule and one large on the lower side of the ovule) and one large resin duct.

Comparison of seed cone morpho-anatomical characters

All three genera produce multiovulate cones. However, the seed cones of *Pherosphaera* and *Saxegothaea* are non-fleshy, while *Microcachrys* has fleshy seed cones. An aril is absent in all three genera. An epimatium is present in both *Saxegothaea* and *Microcachrys*, but absent in *Pherosphaera*. The ovule is erect in both species of *Pherosphaera* and inverted in *Saxegothaea* and *Microcachrys*. The shape of the seed cone is ovoid in *Pherosphaera*, and ovoid to globose in *Microcachrys* and *Saxegothaea*. *Microcachrys* has 10–28 seeds per cone, *Pherosphaera* has 2–8 and *Saxegothaea* has 2–6. The seed surface is rugose in *Pherosphaera* and smooth in both *Saxegothaea* and *Microcachrys* (Fig. 4). The testa morphology and anatomy are similar in all three genera. Fleshy bracts are present in *Microcachrys* but absent in both *Saxegothaea* and *Pherosphaera*. Stomata were observed on the fertile bracts of all species. All genera have a straight embryo. The seed cones of *Pherosphaera* and *Microcachrys* are smaller than those of *Saxegothaea* (Fig. 5).

DISCUSSION

Seed cone reproductive and morpho-anatomical comparisons

Multiovulate cones. Pherosphaera, *Saxegothaea* and *Microcachrys* all produce aggregated multiovulate cones, and this distinguishes them from other podocarp genera. Non-aggregated multiovulate cones are also present in other genera of Podocarpaceae, e.g. *Phyllocladus*, *Lagarostrobos* and *Prumnopitys*. Production of multiovulate cones is also common in other conifers (Tomlinson, 1992, 1994). The seed cone and seed size in these aggregated seed cones are smaller in comparison with non-aggregated and solitary seed cones (Supplementary data Fig. S1). The ovules of *Saxegothaea* and *Microcachrys* are inverted, while *Pherosphaera* has erect ovules. Inverted ovules are common in other conifers (Stiles, 1912; Elliott, 1948*a*; Herting *et al.*, 2020). These relict genera have several fertile bracts, each with a single ovule arranged in a small compact strobilus, and this clearly distinguishes

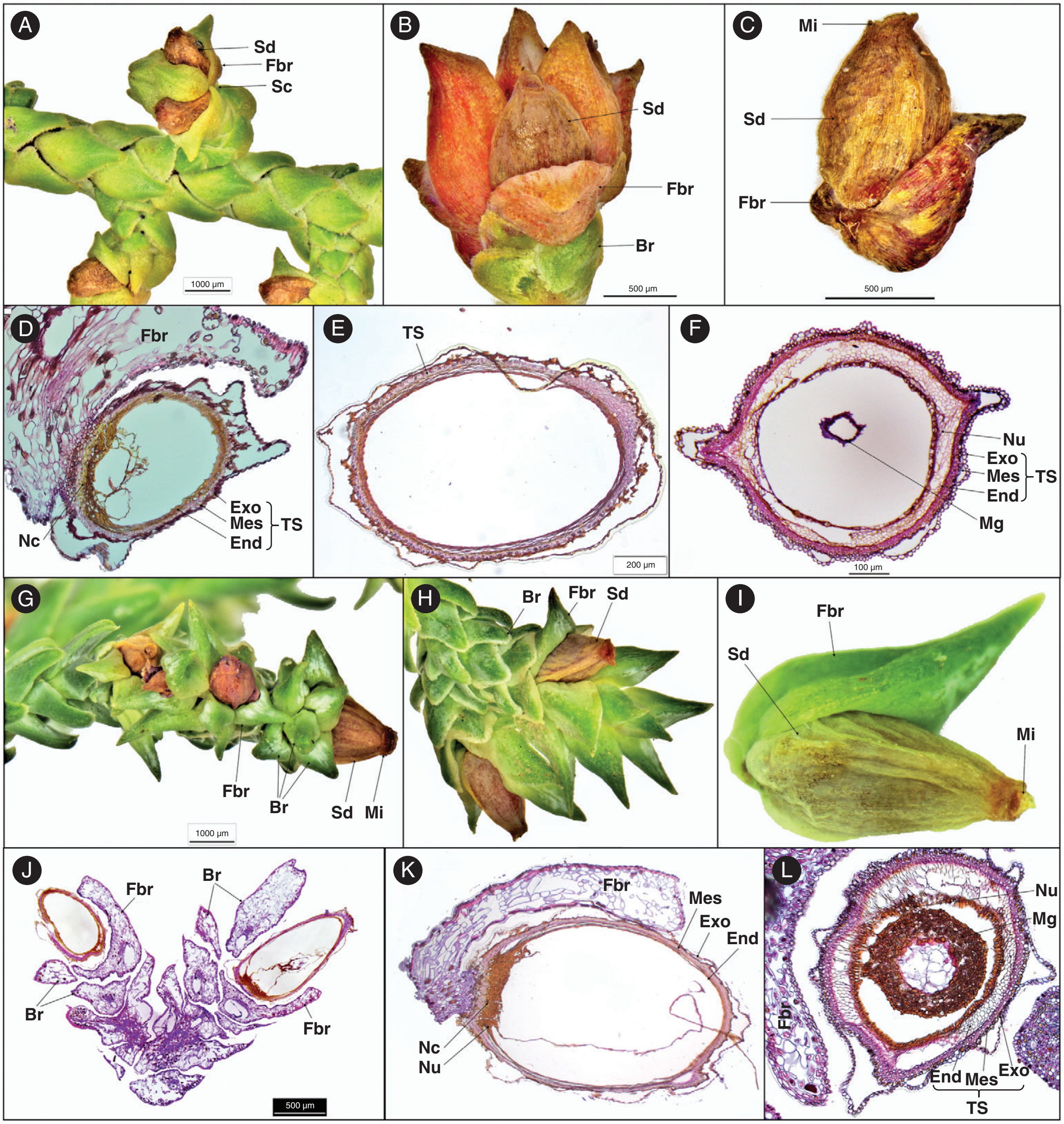

FIG. 2. Seed cone morpho-anatomical features of *Pherosphaera hookeriana* (A–F) and *P. fitzgeraldii* (G–L). (A) Seed cone branch with seed cones (Sc), seed (Sd) and fertile bract (Fbr); (B) seed cone with the seed, fertile bract and bract; (C) seed and fertile bract; (D) longitudinal section of a cone scale seed unit showing fertile bract, nucellar cap (Nc) and layers of testa (TS), i.e. exotesta (Exo), mesotesta (Mes) and endotesta (End); (E) longitudinal section of a single seed cone showing the testa; (F) seed cone cross-section showing layers of the testa, i.e. exotesta, mesotesta and endotesta; (G) seed cone branch showing seed, fertile bract, micropyle (Mi) and bract (Br); (H) seed cone showing seed (Sd), fertile bract and bract; (I) single seed cone showing seed, fertile bract and micropyle; (J) longitudinal section of seed cone branch cone showing seed, fertile bract and bract; (K) longitudinal section of a single seed cone showing fertile bract, nucellus (Nu), nucellar cap and layers of testa, i.e. exotesta, mesotesta and endotesta; and (L) seed cone cross-section showing fertile bract, nucellus, megagametophyte (Mg) and layers of testa, i.e. exotesta, mesotesta and endotesta.

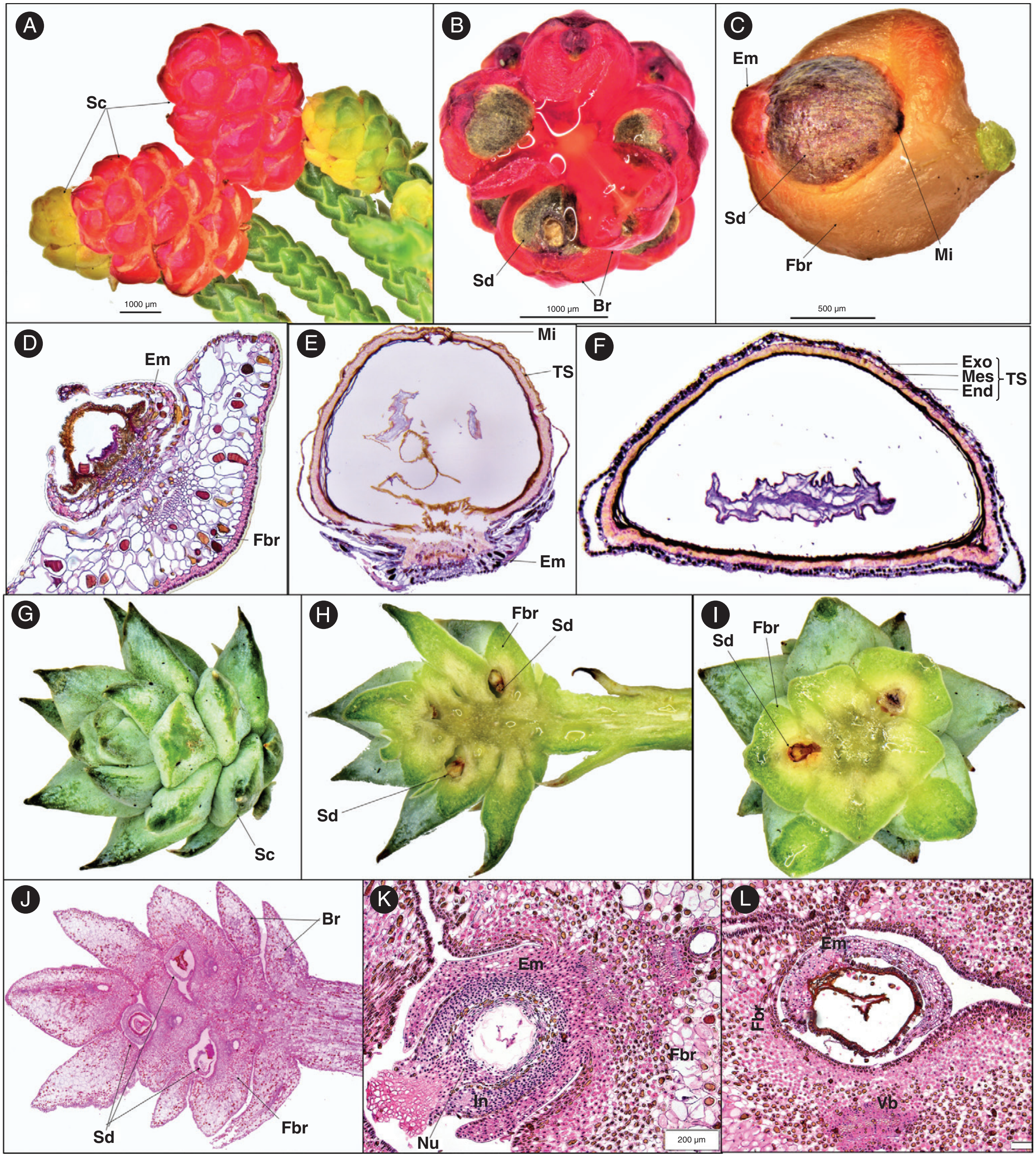

Fig. 3. Seed cone morpho-anatomical features of *Microcachrys* (A–F) and *Saxegothaea* (G–L) showing: (A) fleshy red and young yellowish seed cones; (B) seed cone cross-section view showing seed (Sd) and bract (Br); (C) seed cone with epimatium (Em), seed (Sd), fertile bract (Fbr) and micropyle (Mi); (D) young ovule development (post-pollination) shows fertile bract, epimatium and ovule (Ov); (E) longitudinal section of single seed cone showing testa (TS), micropyle (Mi) and epimatium; (F) seed cone cross-section showing the different layer of testa, i.e. exotesta (Exo), mesotesta (Mes) and endotesta (End); (G) seed cone (Sc); (H) seed cone longitudinal section showing fertile bract (Fbr) and seed (Sd); (I) cross-sections showing the seed cone with the fertile bract and seed; (J) longitudinal section of seed cone showing fertile bract, seed and bract; (K) longitudinal section of developing ovule (post-pollination) showing epimatium, nucellus (Nu), fertile bract and integument (In); and (L) cross-section of developing ovule (post-pollination) showing epimatium, fertile bract and vascular bundle.

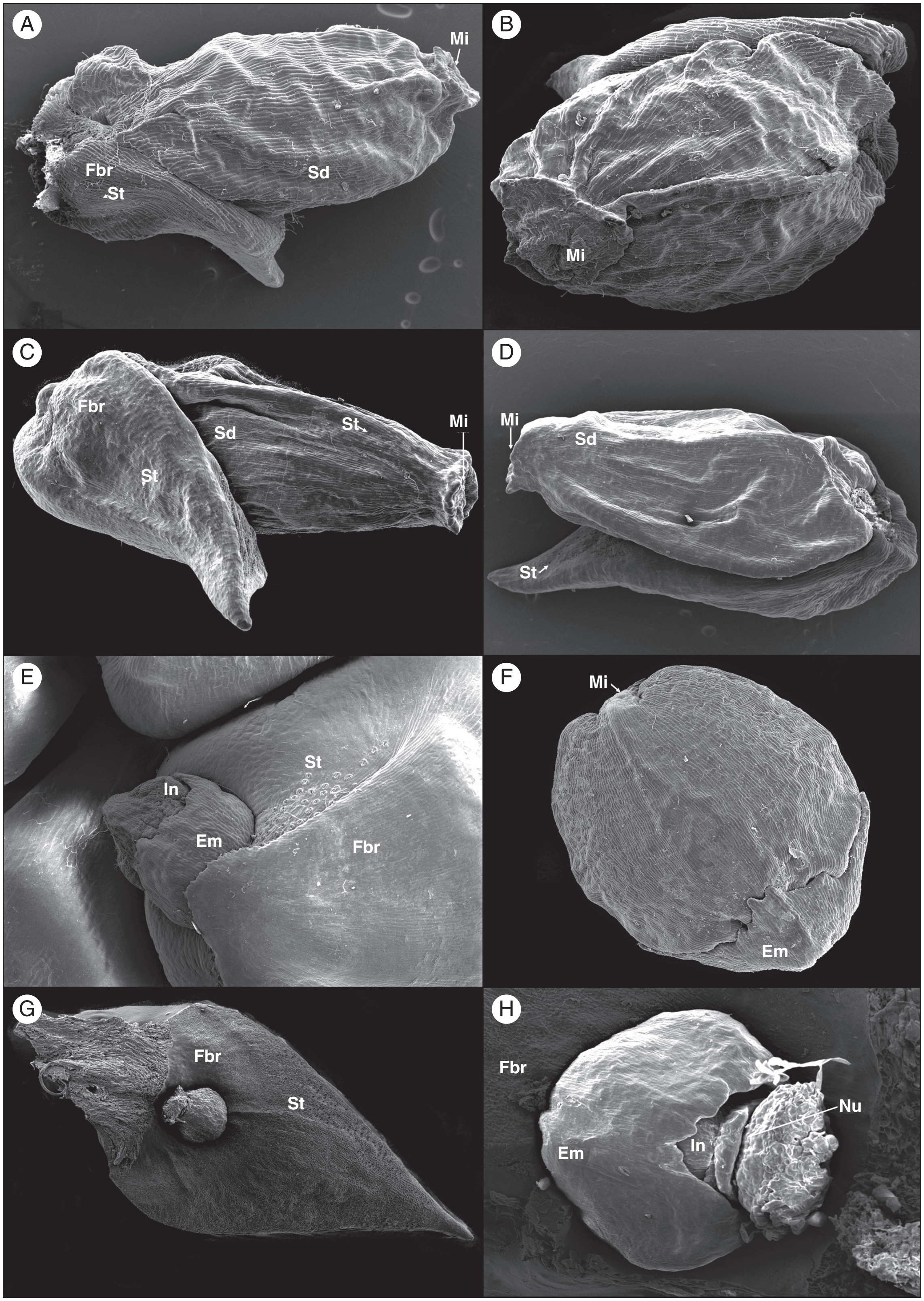

FIG. 4. SEM micrographs of *Pherosphaera fitzgeraldii* shows seeds with a fertile bract (A and B); *P. hookeriana* shows seeds with a fertile bract (C and D); *Microcachrys tetragona* shows the developing ovule (E) and seed cone with epimatium (F); and *Saxegothaea conspicua* showing developing ovule-post pollination with epimatium, nucellus and fertile bract (G and H).

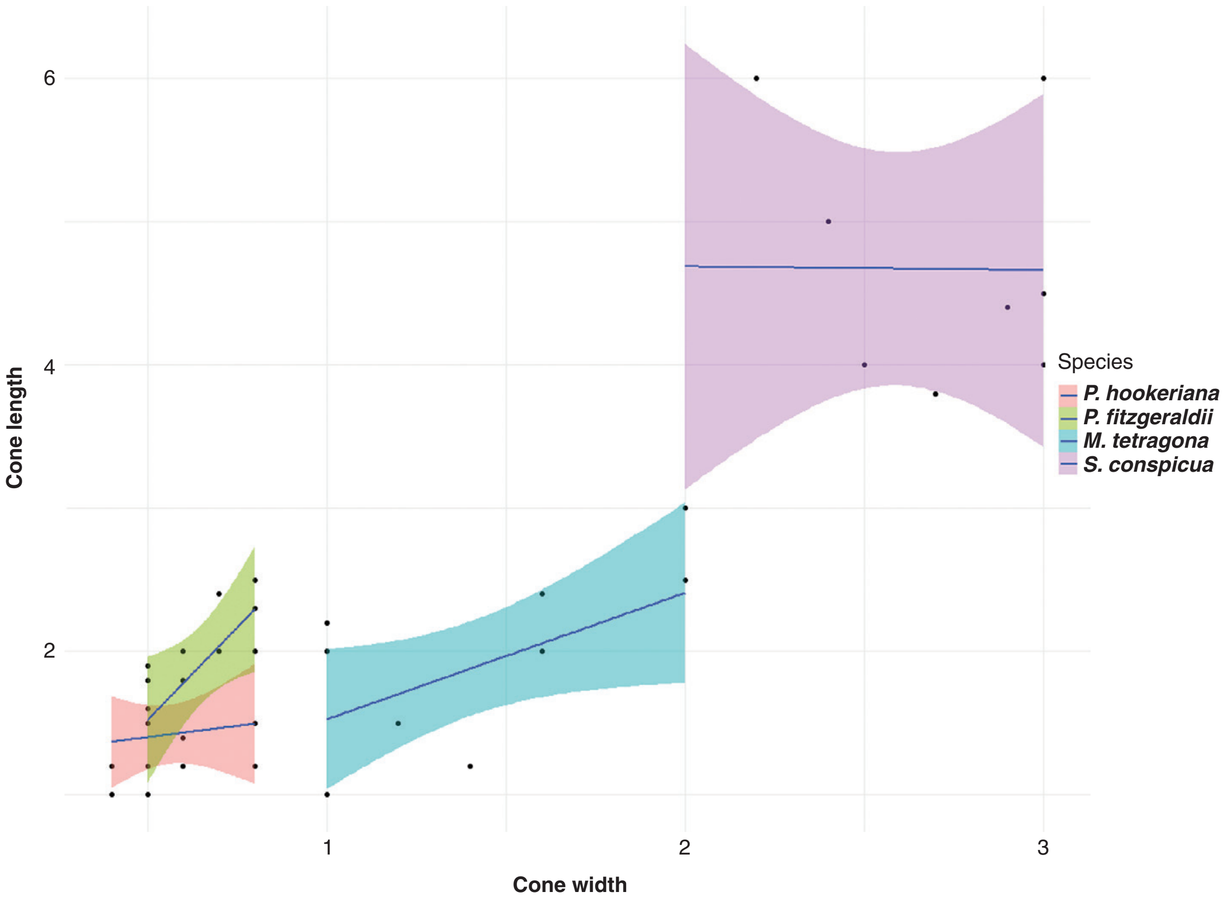

FIG. 5. Seed cone size (length × width, mm) based on ten replicas, showing individual cone size range and comparison with other species. *Pherosphaera hookeriana* and *P. fitzgeraldii* have smaller seed cone sizes as compared with *Microcachrys tetragona* and *Saxegothaea conspicua*.

them from all other genera. However, despite the wide variation among Podocarpaceae cone morphology, all have a single ovule per bract–scale complex (Tomlinson *et al.*, 1991; Contreras *et al.*, 2017).

Epimatium traits. A free asymmetrical cup-shaped epimatium is present in *Saxegothaea* and *Microcachrys* but absent in *Pherosphaera* (Supplementary data Fig. S2). The epimatium is fleshy in *Microcachrys* but not in *Saxegothaea* (Fig. 3). A similar well-developed epimatium with an asymmetrical morphology is present in other podocarps, e.g. *Lepidothamnus*, *Dacrydium*, *Falcatifolium*, *Manoao* and *Lagarostrobos*. The ancestral state reconstruction implies that the presence of epimatium is an ancestral trait in podocarps (Fig. 6). According to Elliott (1948*b*), the absence of an epimatium could be the result of an evolutionary reduction responsible for the production of erect ovules in *Pherosphaera*. The function of the epimatium has been hypothesized as allowing the production of an inverted ovule, which is vital for pollen scavenging (Tomlinson, 1992). An epimatium is considered equivalent to the ovuliferous scale in conifers (Tomlinson, 1992) and, for example, Herting and Stützel (2020) reported that the epimatium development in the Podocarpaceae resembles the development of the ovuliferous scale in the Araucariaceae. A general comparison of the ovule position, epimatium and attachment of the fertile bract in seed cones of *Saxegothaea*, *Microcachrys* and *Pherosphaera* with *Pinus* is shown in Fig. 7.

Megagametophyte and embryo traits. The number of archegonia present varies from two in *Pherosphaera* to three in *Saxegothaea*, and five or six in *Microcachrys*. Similar variation has been observed previously in the number of archegonia for different Podocarpaceae, e.g. *Phyllocladus* has one, *Podocarpus* has 11 and *Microcachrys* has 5–6 (Lawson, 1923*a*; Quinn, 1986). However, Wilson and Owens (1999) reported that *Podocarpus totara* usually has 4–6, but occasionally only two archegonia per ovule. Twenty archegonia per ovule are reported in both *Podocarpus nivalis* and *Afrocarpus falcatus* (Boyle and Doyle, 1953; Osborn, 1960). A gametophyte with a few large archegonia is an ancestral state that is absent from *Pherosphaera*, and the archegonial complex in *Microcachrys* is regarded as a derived condition (Lawson, 1923*a*, *b*; Taylor *et al*., 2005). In *Saxegothaea*, archegonia are enclosed by thick layers of female gametophyte with a small depression over the neck (Looby and Doyle, 1939), and this condition was also observed in *Pherosphaera hookeriana* by Elliott (1948*a*). Similarly, the presence of several relict nuclei in the *Pherosphaera* proembryo was reported by Elliott (1948*b*), and the current study endorses those findings. Similarly, we observed two-celled and hence binucleate embryos in *Pherosphaera* and *Saxegothaea*, and Doyle and Looby (1939) reported two-celled

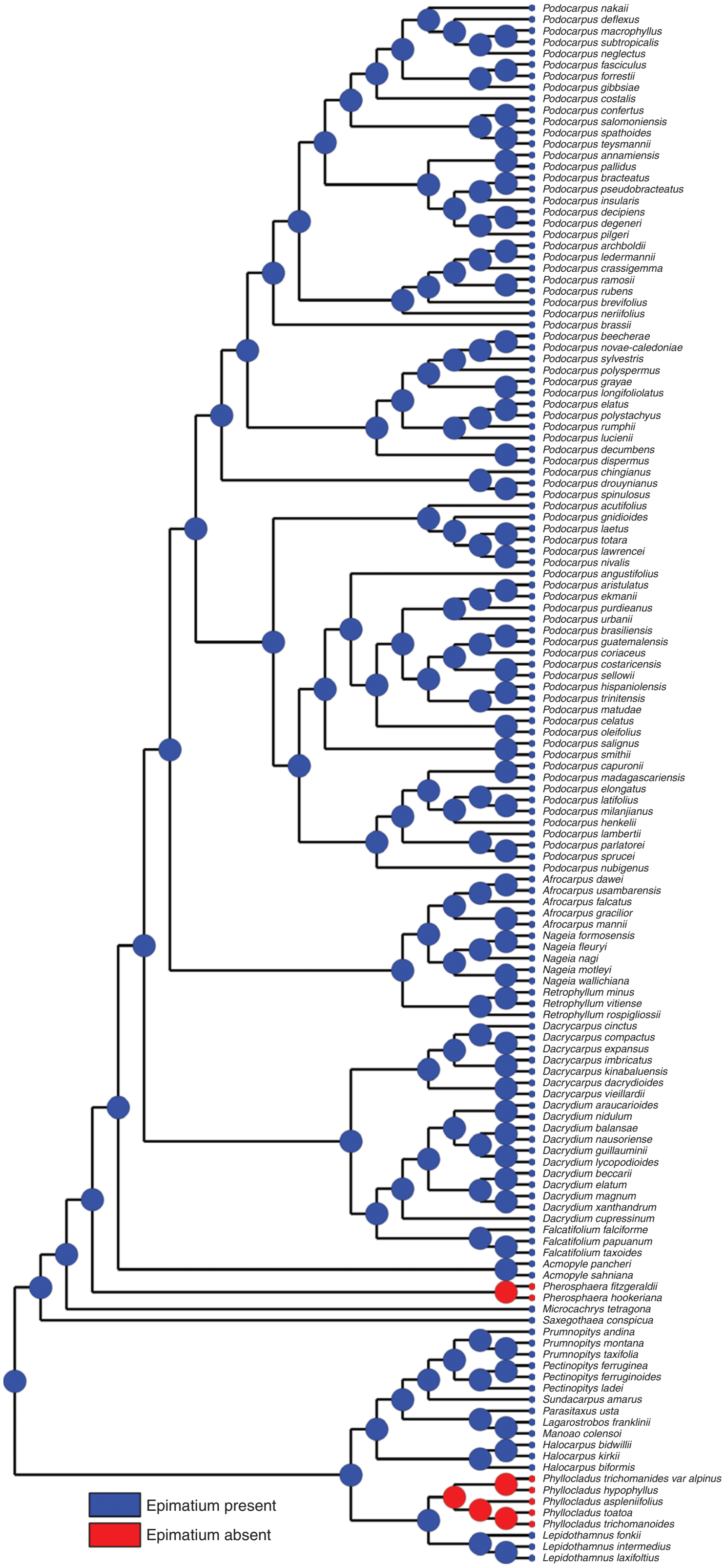

FIG. 6. Character mapping of the presence of an epimatium in different genera of Podocarpaceae using maximum likelihood.

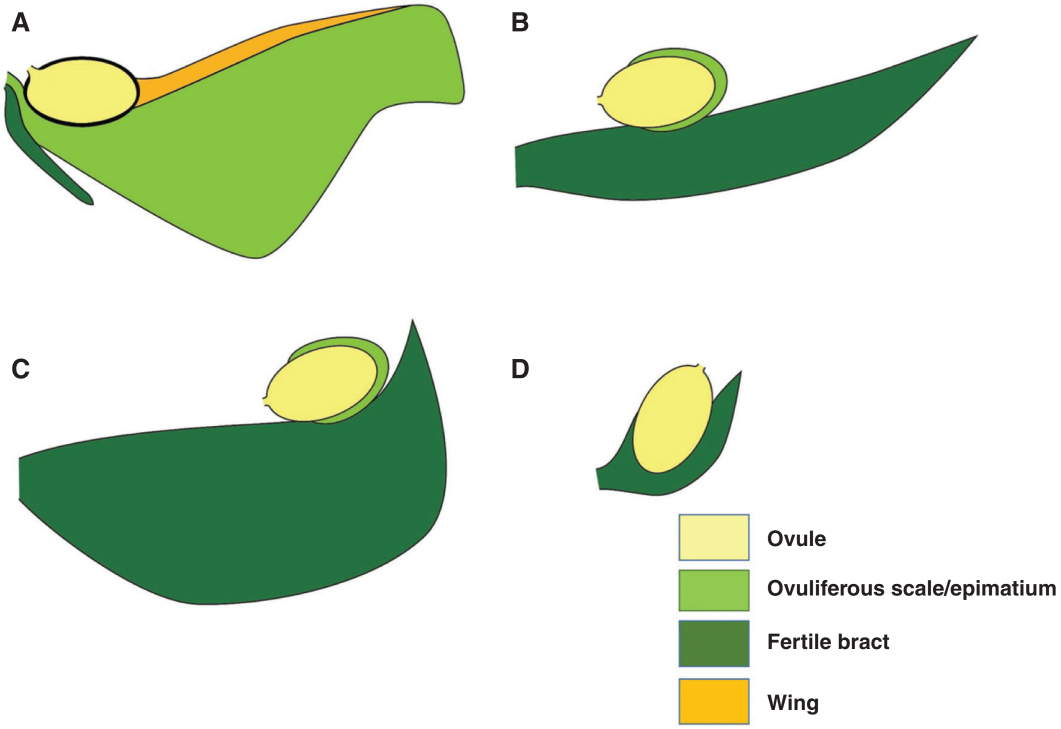

Fig. 7. Diagrammatic representation of a single seed cone scale from *Pinus* (A), *Pherosphaera* (B), *Saxegothaea* (C) and *Microcachrys* (D). *Pinus* is used to represent a typical coniferous cone scale arrangement. The ovule with micropyle is shown in yellow, the bract scale is in dark green and the ovuliferous scale and epimatium is in light green. In (B) (*Saxegothaea*) and (C) (*Microcachrys*), the epimatium is interpreted as a homologous structure to ovuliferous scale in other conifers. In (D) (*Pherosphaera*), the epimatium has disappeared completely.

units in *Saxegothaea* as a result of the direct division of the proembryo original cell nucleus with subsequent wall formation. The Podocarpaceae typically has a binucleate, single-celled embryonic condition (Elliott, 1948*a*). Lawson (1923*b*) suggested that two-celled units are derived in Podocarpaceae; however, given that such units occur in all other conifers (Elliot, 1948*a*), this is probably the ancestral condition, and the single-celled state in Podocarpaceae is derived.

Testa. In the three relict genera, the testa is papery and can be differentiated into an exotesta, mesotesta and endotesta (Figs 2 and 3). The outer testa is fleshy in some podocarp genera (e.g. *Retrophyllum*, *Afrocarpus*, *Prumnopitys* and *Pectinopitys*) and papery in other genera (e.g. *Dacrydium*, *Dacrycarpus* and *Falcatifolium*). The podocarp genera and species with fleshy outer testa have hard or woody endotesta.

Bract traits. Similarities were observed in the anatomy of the fertile and sterile bracts of the seed cone of *Pherosphaera*, *Saxegothaea* and *Microcachrys*, showing that the fleshy or swollen bracts of the latter two genera result simply from the accumulation of water, rather than more complex anatomical modifications. Restemeyer (2002) also reported that in *Microcachrys* the desiccated leaves change into fleshy cone bracts. Several resin ducts were observed in the seed cone bracts of fleshy and swollen bracts. Thomson (1909) also reported similarities in the bract–scale complexes of *Saxegothaea* and *Microcachrys*.

Fleshiness and ancestral seed cones

The ancestral state reconstruction indicates that different functional structures and traits evolved multiple times in Podocarpaceae. The ancestral state reconstruction of fleshiness in podocarps suggests that it is an ancestral trait, and *Saxegothaea* and *Pherosphaera* have evolved non-fleshy seed cones independently (Fig. 8). Based on statistical approaches, Klaus and Matzke (2020) suggested that the ancestral seed cones in Podocarpaceae were non-fleshy, and the earliest transition from non-fleshy into fleshy seed cones (e.g. at the base of the clade that includes *Podocarpus*, *Afrocarpus*, *Nageia*, *Retrophyllum*, *Dacrydium*, *Falcatifolium* and *Dacrycarpus*) occurred in the Late Cretaceous (approx. 82.4 million years ago), and these taxa are mainly bird dispersed. The ancestral state reconstruction of Herting *et al.* (2020) also implies that the ancestral seed cones in Podocarpaceae were fleshy. However, several fleshy structures occur across the clades and genera. These fleshy and non-fleshy structures shift multiple times, and more extensive studies are required in the Prumnopityoid, Podocarpoid and Dacrydioid clades.

Comparisons with other taxa

Stiles (1912) considered *Pherosphaera* to be closely related to *Phyllocladus* (which has a simple fleshy bract and aril) as both have erect ovules, and Doyle and Looby (1939) considered *Pherosphaera* to be an advanced derivative of *Phyllocladus*.

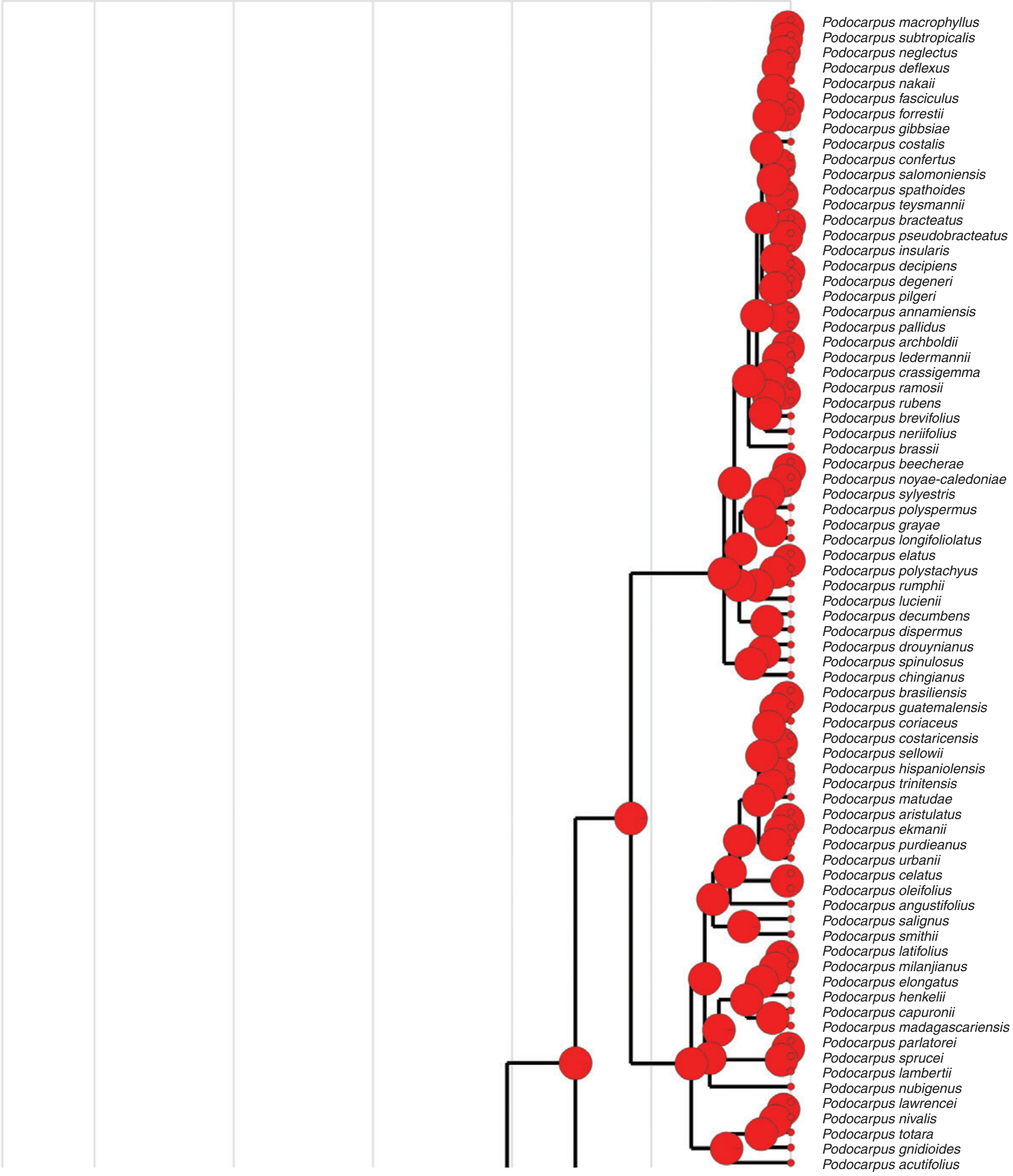

FIG. 8. Character mapping of seed cone morphology in different genera of Podocarpaceae using maximum likelihood.

However, embryology, morpho-anatomy and phylogenetics do not support a close relationship between *Pherosphaera* and *Phyllocladus* (e.g. polyembryony is distinctly different in these two genera, the presence of an aril, independent loss of the epimatium and *Phyllocladus* clade formation with other podocarps, namely the Prumnopityoid clade) (Elliott, 1948*b*; Leslie *et al.*, 2018).

The megaspore membrane is well developed in *Pherosphaera*, *Saxegothaea* and *Microcachrys* (Lawson, 1923*a*, *b*). The presence of a megaspore membrane is reported in all conifers, but its appearance varies among different groups. For instance, in the Taxaceae, it is thin and inconspicuous, while in the Podocarpaceae it is thick and obvious (Fiordi *et al.*, 1996). The similarity in the megaspore membrane between *Pherosphaera* and *Microcachrys* led Doyle (1945) to suggest that these genera are closely related. Elliott (1948*b*) reported that *Pherosphaera* is consistent with the Podocarpaceae and especially with *Dacrydium* in its gamete development, except that the nucellus is free from the integument in *Dacrydium*. One other peculiar feature of *Saxegothaea* is the absence of a pollination drop as the nucellus protrudes from the micropyle and forms a papillous stigma-like surface as a receptive structure, and gamete delivery to the ovule is achieved by the growth of a pollen tube (Tomlinson *et al.*, 1991). *Saxegothaea* also has non-saccate pollen, similar to Araucariaceae, *Larix*, *Pseudotsuga* and *Tsuga* (Tomlinson, 1994). *Microcachrys* has a unique arrangement of seed cone bracts among podocarps, with the bracts or bract–scale complexes arranged in alternate whorls of four. This character resembles *Diselma archeri* (Cupressaceae) more than its closer suggested relative *P. hookeriana*, which has spirally arranged leaves. Seed cones of *Saxegothaea* show a gradual transition from foliage to sterile and fertile bracts,

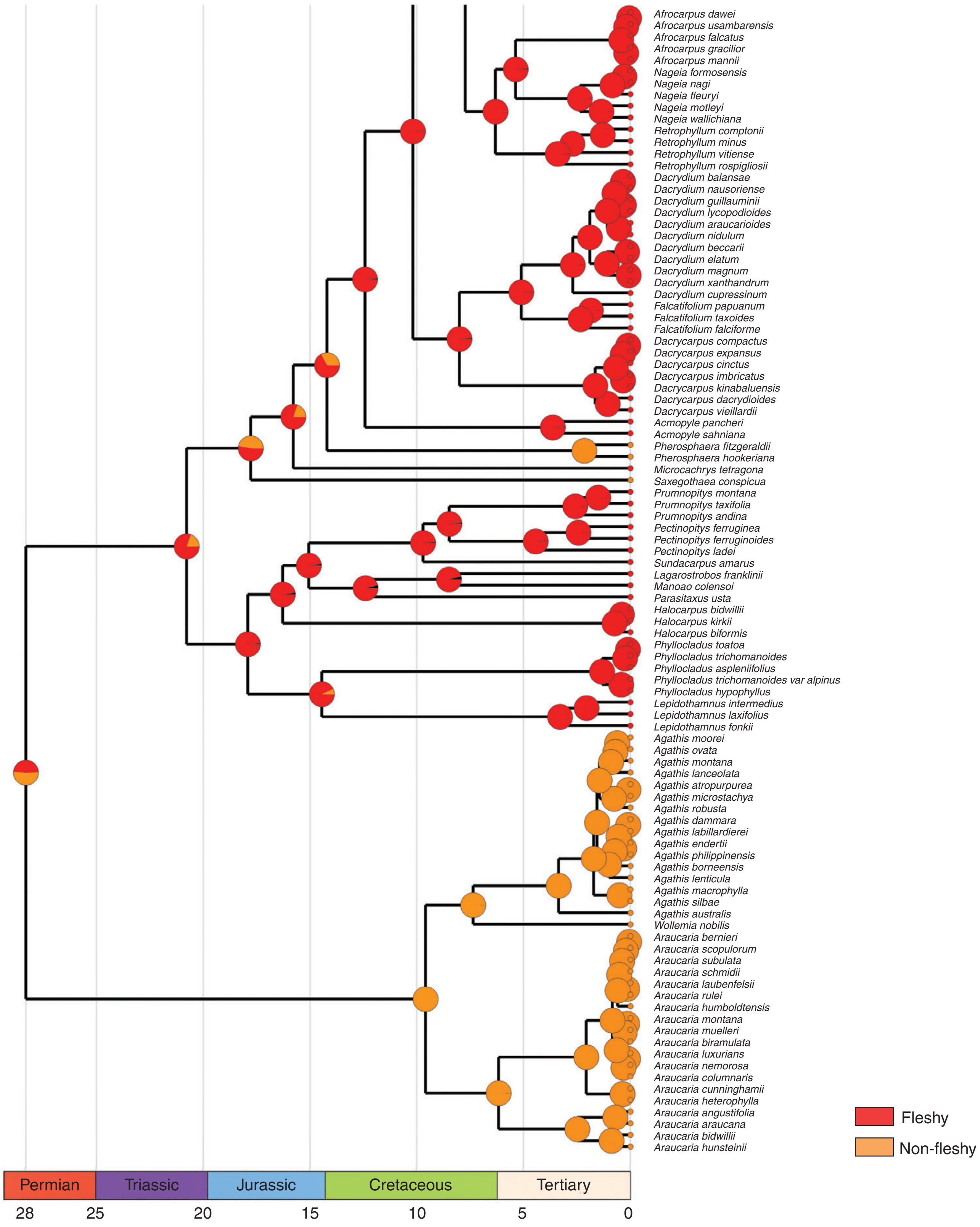

FIG. 8. Continued

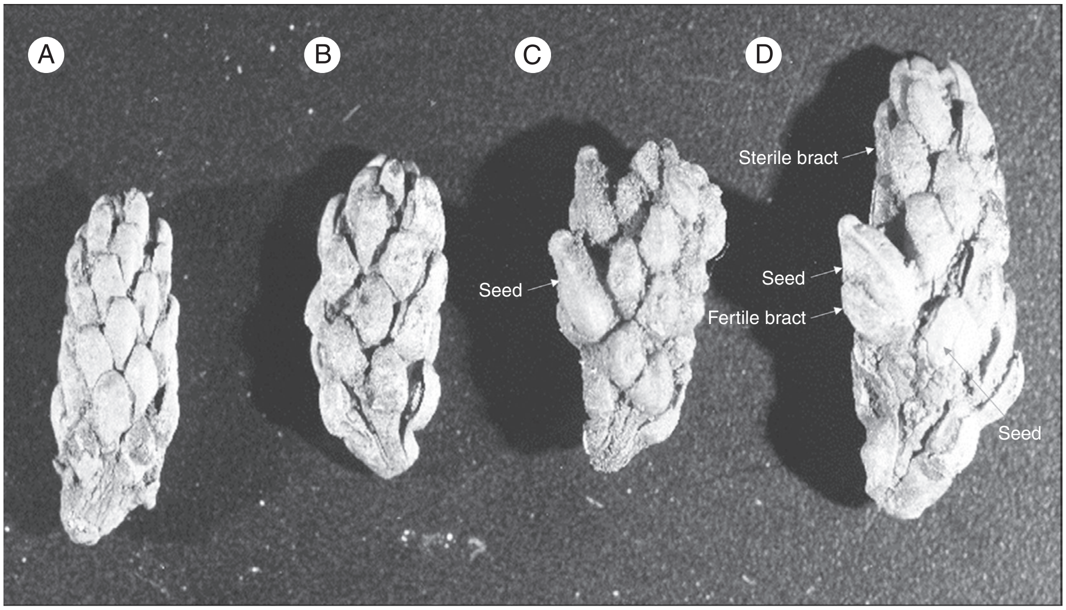

FIG. 9. The seed cones (A–D at different levels of maturity) of an undescribed *Fecundistrobus* species from Winton, Queensland show morphological similarities with *Pherosphaera* seed cones as having non-fleshy bracts. The photo was taken by Dr M. D. Peters and is from the collection held in the David T. Blackburn Palaeobotany Collection at the University of Adelaide. Abbreviations: Ar, aril; Ax, cone axis; Br, bract; Edo, endotesta; Em, epimatium; Ep, epidermis; Exo, exotesta; Emb, embryo; Fbr, fertile bract; Fs, fertile scale; Fsbr, fleshy sterile bract; Flbr, fleshy bract; Gm, gynophore membrane; In, integument; Mes, mesotesta; Mg, megagametophyte; Mi, micropyle; Nu, Nnucellus; Nc, nucellar cap; Ov, ovule; Rc, resin canal; Rd, resin duct; Pc, pollen cone; Po, pollen; Rp, receptaculum/ podocarpium; Sc, seed cone; Sd, seed; Sl, sclereid; Slsc, fleshy sarcotesta-like seed coat; Ts, testa; Vb, vascular bundle.

which is similar to some Araucariaceae species, e.g. *Araucaria rulei* and *A. columnaris* (Young, 1910). Similarly, *Saxegothaea* resembles *Araucaria* and *Microcachrys* in the arrangement of vascular bundles (enlargement of the vascular bundle and resin ducts) in the fertile bracts of the seed cone (Stiles, 1908). Gaussen (1974) and Woltz (1985) established *Saxegothaea* as a separate family, Saxegothaeaceae, due to its resemblance to Araucariaceae.

Wilde (1944) proposed that the podocarps follow two lines of evolution; one is the reduction of the fertile branch system while retaining large leaves, and the second is the retention of numerous fertile branches while the leaves are reduced (imbricate). *Pherosphaera* and *Microcachrys* fall in the second category. Retention of numerous fertile branches and reduced leaves is common to all three genera studied here. Despite these genera looking morphologically distinct from other podocarps, they share a similar seed cone reproductive development, functional structures and morpho-anatomy with each other and with other podocarps. Similarly, the ancestral state reconstructions also provide an insight on the evolution and loss of structures and traits (Contreras *et al.*, 2017; Leslie *et al.*, 2017).

What the fossil record tells us about the initial seed cones in Podocarpaceae

The fossil record also provides an insight into the relictual distribution of *Saxegothaea*, *Microcachrys* and *Pherosphaera* in the Podocarpaceae. An undescribed extinct seed cone, informally named *Fecundistrobus*, from the middle Cretaceous of Winton, Queensland (Peters, 1985) shows morphological similarities to *Pherosphaera* and *Saxegothaea* as the fleshy fertile scale and receptacle are absent (Fig. 9). *Fecundistrobus* also has sterile bracts below the fertile bracts, similar to *Saxegothaea*, *Microcachrys* and *Pherosphaera*, with an even distribution of ovules. Erect ovules are present in both *Fecundistrobus* and *Pherosphaera* (Peters, 1985). The fossilized seed cone of an extinct species (*Friisia lusitanica*) with an inverted seed and epimatium from the Cretaceous of western Portugal resembles *Microcachrys* (Mendes and Kvaček, 2020). Other seed cones from the Jurassic of Yorkshire (*Scarburgia hallii*) and the Middle Jurassic of Poland (*Harrisiocarpus gucikii* and *H. cracoviensis*) also resemble *Microcachrys* (Reymanówna, 1987). *Mehtaia rajmahalensis* (from the Jurassic of India) is another extinct genus showing similarities to *Fecundistrobus* and *Pherosphaera* (Vishnu-Mitre, 1958). Rao (1972) reported the fossil seed cone of the extinct species *Nipaniostrobus sahnii* from the Cretaceous Rajmahal Hills in India as showing morphological resemblance to *Saxegothaea*, *Microcachrys* and *Pherosphaera*. It can be inferred that podocarps once had an Indo-Australian distribution. The sterile foliage of the extinct species *Microstrobos sommervillae* (*Pherosphaera sommervillae*) from the Early Eocene Buckland sediments in south-eastern Tasmania) also supports *Pherosphaera* as a paleoendemic genus (Townrow, 1965).

Conclusions

This study provides insights into the seed cone morphology and trait evolutions in three relict genera, *Pherosphaera*, *Microcachrys* and *Saxegothaea*, with discrete and widely separated distributions. Despite the probable extinction of closely related sister species and genera, and the evolution of morphological structures as adaptations to changing environmental conditions, they still share basic reproductive traits with other podocarps. The detailed seed cone morpho-anatomy and ancestral state reconstruction imply that *Pherosphaera*, *Microcachrys* and *Saxegothaea* have evolved and lost traits. These three relict genera produce aggregate multiovulate cones. Some fossil podocarp taxa and sister family Araucariaceae also have multiovulate cones. The seed cone morpho-anatomy and reproductive biology unequivocally place these genera in the family Podocarpaceae. Evaluation of the fossils and these extant paleoendemic genera suggests that the ancestral seed cones were fleshy and non-fleshy seed cones (*Saxegothaea* and *Pherosphaera*) twice independently. However, podocarps demonstrate complex multiple convergent evolutions and are also characterized by substantial variation in seed cone structure and basic architecture, and this is reflected in the relict lineages studied here. Given their deep branches, these lineages suggest that such variation has always been characteristic of the group.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: comparison of seed cone size and seed size of Podocarpaceae. Figure S2: epimatium morphology mapping in different genera of Podocarpaceae using maximum likelihood. Figure S3: seed size shows that *Saxegothaea* has a comparatively larger seed size, followed by *Microcachrys* and *Pherosphaera*. Figure S4: *Pherosphaera hookeriana* and *Pherosphaera fitzgeraldii*.

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