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THE STOMATAL COMPLEX OF *PODOCARPUS* OBSERVED IN CROSS-SECTION USING CRYOFRACTURE: A PRELIMINARY STUDY

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Cryofracture of living material and fracture at room temperature of herbarium material were used to obtain cross-sections of the stomatal complexes of four species of *Podocarpus* (Podocarpaceae) for scanning electron microscopy. Cross-sections of the stomata of one species in *Podocarpus* subgenus *Foliolatus* section *Foliolatus* (*Podocarpus rubens*), one in *Podocarpus* section *Globulus* (*Podocarpus beecherae*), one in *Podocarpus* subgenus *Foliolatus* section *Longifoliolatus* (*Podocarpus insularis*) and one in subgenus *Podocarpus* section *Australis* (*Podocarpus nivalis*) were studied. The architecture of the stomatal complex, including the wax plug, is described. It was found that the wax plug sits high in the stomatal antechamber in *Podocarpus rubens*, *P. beecherae* and *Podocarpus decipiens* and about halfway up the chamber in *P. nivalis*. A ridge, which appears to correspond to the crease where the guard cells meet, exists on the underside of the wax plug in *Podocarpus beecherae*, *P. decipiens* and *P. rubens*; its presence in *P. nivalis* requires confirmation. In addition, ridges within the stomatal antechamber were observed when viewing the cross-sections of *Podocarpus decipiens* and *P. rubens*, the internal surface of the cuticle of *P. decipiens*, *Podocarpus teysmannii*, *P. insularis* and *Podocarpus milanjianus*, and the external surface of the cuticle of *Podocarpus chinensis*, *Podocarpus macrophyllus* and *Podocarpus pilgeri.* These ridges may consist of wax and be a result of epitaxis.

Keywords. Cryofracture, epistomatal chamber, Podocarpaceae, *Podocarpus*, stomata, stomatal antechamber, wax plugs.

Introduction

Podocarpus L'Hér. ex Pers. is the largest genus in the family Podocarpaceae, with about 100 species (Farjon, 2010). *Podocarpus* has two subgenera, *Podocarpus* subgenus *Podocarpus* and *Podocarpus* subgenus *Foliolatus* de Laub. (de Laubenfels, 1985). In species within *Podocarpus* subgenus *Podocarpus*, the stomata possess a distinct raised rim around their openings, a structure known as a Florin ring, a term coined by Buchholz & Gray (1948: 52) and named after the morphologist and specialist on gymnosperms Rudolf Florin, who first discovered them in conifers (Florin, 1931). In contrast, species within *Podocarpus* subgenus *Foliolatus* are characterised by the

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absence of Florin rings (de Laubenfels, 1985). The Florin ring may function in reducing wettability of the leaf surface, as suggested by Pariyar *et al.* (2017) for *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. In addition, Mohammadian *et al*. (2009) found that in *Agathis robusta* (C.Moore ex F.Muell.) F.M.Bailey the Florin rings had a noticeable, although not quantifiable, effect in deflecting fungal hyphae away from the stomata and so protecting them from fungal invasion. Investigation of this in *Podocarpus* was outside the scope of this study.

Species of *Podocarpus* subgenus *Podocarpus* are found in habitats ranging from forest to alpine heath in Southeast Australia, Tasmania, New Zealand and Chile, as well as in the tropical highlands of Africa and America (including the Caribbean Islands), and rarely in the tropical lowlands of those areas (de Laubenfels, 1985). In contrast, species of *Podocarpus* subgenus *Foliolatus* are especially abundant in the Asian and Pacific tropics (lowlands and highlands), in subtropical areas of eastern Asia and in Australia, and are absent from Africa and the Americas (de Laubenfels, 1985). The two subgenera overlap only in two areas: the Pacific tropics of north-eastern Australia and on the island of New Caledonia (de Laubenfels, 1985).

The cross-section of the stomatal complex of *Podocarpus* has not previously been studied using either light microscopy or scanning electron microscopy (SEM). Indeed, there have been very few such studies of the stomatal complex of any gymnosperm. However, SEM can provide further detail and information on the architecture of the stomatal complex and the wax plug and how they function as a unit.

Two early studies were those by Hanover & Reicosky (1971) and Jeffree *et al.* (1971). Hanover & Reicosky's paper concerned surface wax deposits on the foliage of various conifers including *Picea pungens* Engelm., while that by Jeffree *et al.* studied epicuticular wax in what they and numerous other workers have termed the *stomatal antechamber* (e.g. Jeffree *et al.*, 1971; Appleby & Davies, 1983; Morvan, 1987, *Podocarpus* [various organs, but not stomata in cross-section]; Karabourniotis *et al.*, 2001). The stomatal antechamber is equivalent to the space in the stomatal complex that is exterior to the subsidiary cells and below the surface of the leaf. This cavity was also termed the *epistomatal chamber* by Hanover & Reicosky (1971), and that term has likewise been used by numerous later workers (e.g. Yoshie & Sara, 1985; Prior *et al.*, 1997; Vovides & Galicia, 2016). Mill & Whiting (2012) used 'peristomatal chamber' for the same cavity in the stomatal complex of *Podocarpus orarius* R.R.Mill & Whiting, but that expression has been used by no other authors and is rejected here. Instead, following Jeffree *et al.* (1971), Morvan (1987) and subsequent authors, we use 'stomatal antechamber'.

Materials and Methods

Cross-sectioning

Leaf samples were taken from either herbarium or living specimens of four species of *Podocarpus* conserved at the Royal Botanic Garden Edinburgh (Table 1).

TABLE 1. Voucher and collection details for material of species of *Podocarpus* examined

Subgenus	Section	Species	Type of specimen	Origin of specimen	Herbarium specimen no.	Voucher of living specimen	Accession number and qualifier, if any	Year of collection of herbarium specimen or voucher (V)	Images provided	Leaf length × width (mm)[a]
Podocarpus	*Australis* de Laub.	*Podocarpus nivalis* Hook.	Living	New Zealand	*Lancaster* 1305	*Whiting* 9	19850452A	2009 (V)	LCS, TCS	3–20 × 2–2.5
	Podocarpus	*Podocarpus milanjianus* Rendle[b]	Herbarium	Zimbabwe	*Taylor* 1766			1956	ISC with wax ridges	50–170 (less when stunted) × 5–16
Foliolatus de Laub.	*Foliolatus* de Laub.	*Podocarpus rubens* de Laub.	Living	Sulawesi, Indonesia	*Smith & Galloway* 205	*Whiting* 13	20000597A4	2009 (V)	LCS, TCS	30–60 × 5–10 (adult; juvenile larger)
		Podocarpus insularis de Laub.	Herbarium	New Guinea	*Croft & Katik* 14966			1973	ISC with wax ridges	55–150 × 7–18
	Longifoliolatus de Laub.	*Podocarpus decipiens* N.E.Gray[c]	Herbarium	Fiji	*McKee* 2842			1955	LCS, TCS and ISC with wax ridges	63–130 × 7–16
	Polystachyus de Laub.	*Podocarpus macrophyllus* (Thunb.) Sweet	Living	Japan	*Page* 9868	*Whiting* 8	19763612A2	1976, 2009 (V)	ESC with wax ridges	Normally 40–100 × 4–10
		Podocarpus chinensis (Roxb.) Wall. ex Forbes[d]	Herbarium	Tobago		*Broadway* 2934		1909	ESC with wax ridges	Usually 35–70 × 4–7
	Globulus de Laub.	*Podocarpus teysmannii* Miq.[e]	Herbarium	Burma (Myanmar)	*Keenan* et al. 3081			1961	ISC with wax ridges	Mostly 100–145 × 15–21
		Podocarpus beecherae de Laub.[f]	Living	New Caledonia	*de Laubenfels* s.n. (*Spicer* s.n.)	*Whiting* 16	20030919A	2002, 2009 (V)	TCS	30–60 × 3–4.5

TABLE 1. (*Continued*)

Subgenus	Section	Species	Type of specimen	Origin of specimen	Herbarium specimen no.	Voucher of living specimen	Accession number and qualifier, if any	Year of collection of herbarium specimen or voucher (V)	Images provided	Leaf length × width (mm)[a]
	Gracilis	*Podocarpus pilgeri* Foxw.	Living	Vietnam	*Northern Vietnam First Darwin Expedition* 170	*Whiting* 18	20022521	2002, 2009 (V)	ESC with wax ridges	Mostly 25–45 × 5–7

ECS, external surface of the cuticle; ICS, internal surface of the cuticle; LCS, longitudinal cross-section; TCS, transverse cross-section.

[a] Adapted from Farjon (2010) with extreme values omitted, except: *Podocarpus beecherae* from de Laubenfels (2003), *P. decipiens* from Gray (1955) and *P. teysmannii* from measurements of the holotype specimen of its synonym *P. epiphyticus* at E.

[b] Often synonymised with the South African *Podocarpus latifolius* (Thunb.) R.Br. ex Mirb. (e.g. de Laubenfels, 1985; White *et al.*, 2001), but retained as a separate species by Farjon (2010), the view followed here.

[c] *Podocarpus decipiens* was regarded as a synonym of the widespread *P. neriifolius* D.Don by de Laubenfels (1985) and Farjon (2010) but kept separate by Smith (1979). Cuticle micromorphologies of the two taxa show differences (Whiting, 2009), and in her protologue Gray (1955) pointed out anatomical differences. Smith (1979) retained *Podocarpus decipiens* as a distinct species, and the same view was recently accepted by de Laubenfels (2015), who classified it in *Podocarpus* section *Longifoliolatus*.

[d] Treated as *Podocarpus macrophyllus* var. *maki* Siebold & Zucc. by Farjon (2010).

[e] The studied material was the holotype of *Podocarpus epiphyticus* de Laub. & Silba. This name was regarded as a synonym of *Podocarpus neriifolius* by Farjon (2010), but cuticle micromorphology shows distinct differences from that species (Whiting, 2009). Most recently, *Podocarpus epiphyticus* was synonymised with *P. teysmannii* by de Laubenfels (2015).

[f] A recently described species (de Laubenfels, 2003) that has been synonymised with *Podocarpus novae-caledoniae* Vieill. by Farjon (2010). Cuticle micromorphologies of the two taxa are markedly different, however (Whiting, 2009), so *Podocarpus beecherae* is retained here. This is also in agreement with the most recent treatment of these two species by de Laubenfels (2015), who transferred *Podocarpus beecherae* to *Podocarpus* section *Globulus*.

Cross-sections of living material (*Podocarpus nivalis* Hook., *P. rubens* de Laub. and *P. beecherae* de Laub.) were obtained using cryofracture. A section of the leaf was cut into slices of c.5 × 7 mm and placed on to a stub that had a layer of colloidal graphite C303, allowing the segments to be placed with the longitudinal or transverse edge up. The specimen was rapidly frozen using liquid nitrogen subcooled to about −210°C and transferred to the cold stage of a Gatan Alto 2500 cryopreparation system, where the leaf segments were fractured using a blade at a temperature of about –175°C. Cross-sections of herbarium material (*Podocarpus decipiens* N.E.Gray) were obtained by fracture of the dried leaf, using tweezers at room temperature. These slices were mounted with the longitudinal or transverse edge up on a specimen stub with a carbon adhesive disc and Electrodag 915 high-conductivity paint.

Cuticle preparation

The cuticle of six other taxa of *Podocarpus* (*P. chinensis* (Roxb.) Wall. ex Forbes,[1] *P. insularis* de Laub., *P. macrophyllus* (Thunb.) Sweet, *P. milanjianus* Rendle, *P. pilgeri* Foxw., *P. teysmannii* Miq.[2]: Table 1) was also examined, although cross-sections of these species were not undertaken. To observe the internal surface of the cuticle (hereafter abbreviated ISC), the cuticle was isolated following the method of Alvin & Boulter (1974), with changes made to suit Podocarpaceae based on work by Kershaw (1997), Stockey *et al*. (1998) and Stark Schilling & Mill (2011). Six leaf slices of around 0.8 cm^2 were placed in glass vials with 6 mL of 20/120% (16.66%) solution of dihydroxidodioxidochromium (H_2CrO_4, 'chromic acid'). The vials were sealed and kept at room temperature in a fume hood for 96 h. Isolated cuticles were removed from the chromic acid solution, washed with distilled water and left to dry on filter paper for at least 4 h. Cuticles were checked for homogeneity under the light microscope. Two cuticle slices were mounted on an aluminium stub using carbon adhesive discs. The external surface of the cuticle (ESC) was observed using untreated leaf slices. These samples were mounted on to stubs as above. The leaf segments were sputter coated with 60%:40% gold:palladium for 2 min at 12 mA in an Emscope SC500 sputter coater.

Scanning electron microscopy

Examination of all samples by SEM, whether they were cryosectioned, fractured or not, was done using a Hitachi S-4700 II field emission SEM at the University of Edinburgh. Accelerating voltage was 5 kV. Working distance varied from 11.9 to 15.2 mm for the cross-sections, and from 13.0 to 16.6 mm for the ISC and ESC. It was found that optimal images of leaf cuticles were obtained by setting LensMode to Analysis, although images of *Podocarpus beecherae*, *P. decipiens* and *P. nivalis* in

[1] Also known as *Podocarpus macrophyllus* var. *maki* Siebold (see Table 1).

[2] As noted in Table 1, the material here regarded as *Podocarpus teysmannii* (following de Laubenfels, 2015), was the holotype of *Podocarpus epiphyticus* de Laub. & Silba.

cross-section were taken with LensMode set to Normal. Magnifications ranged from approximately 1.3 to 6 k [i.e. × 1300–6000], as given exactly in the SEM data imprinted at the bottom of each figure.

Results

Cross-sections of the stomatal apparatus of *Podocarpus rubens* (Fig. 1), *P. beecherae* (Fig. 2) and *P. decipiens* (Fig. 3) revealed the inside of the stomatal antechamber with wax plug intact. The wax plug sat high in the stomatal antechamber, in contact with the subsidiary cells and with a relatively wide gap between the wax plug and the guard cells. The wax plug of *Podocarpus nivalis* (Fig. 4) was missing for the transverse cross-section (TCS: Fig. 4A) and appeared damaged in the longitudinal cross-section (LCS: Fig. 4B).

Transverse cross-section

The TCS of the stomatal apparatus is illustrated for *Podocarpus rubens* (Fig. 1A,B), *P. beecherae* (Fig. 2A–D) and *P. nivalis* (Fig. 4A). This view shows a cut through the two lateral subsidiary cells and through the two guard cells. All transverse sections revealed closed guard cells, a narrow stomatal antechamber and a wax plug with a central ridge on the underside (apart from in *Podocarpus nivalis*, in which the wax plug was not present). The wax plug of *Podocarpus beecherae* appeared very smooth on the underside compared with a rough upper surface consisting of protruding wax rods (Fig. 2A–C). The guard cells in *Podocarpus beecherae* were usually almost round in cross-section (Fig. 2A–C), although they were elliptical in the sample used for Fig. 2D; whereas those of *P. rubens* (Fig. 1A,B) and *P. nivalis* (Fig. 4A) were very elongated and elliptical, although this may vary. The lateral subsidiary cells tended to arch over the guard cells, coming to approximately halfway, where a distinct triangle of cell wall material (probably including cuticle and wax) sat on top of the guard cell (seen in all three species for which transverse sections were obtained: Figs 1B tcwc, 2A–C, 4A). The most notable difference between the species was the presence in *Podocarpus nivalis* of a Florin ring created by the lateral subsidiary cells and that appears to be composed of cell wall material and cuticle (Fig. 4A frcw, 4B).

Longitudinal cross-section

The LCS of the stomatal apparatus is illustrated for *Podocarpus rubens* (Fig. 1C–F), *P. decipiens* (Fig. 3C–F) and *P. nivalis* (Fig. 4B). This view shows a section through the two polar subsidiary cells and one mostly complete guard cell. No ridge could be seen on the underside of the wax plug when seen in this view (the ridge appearing to correspond with the area where the two guard cells meet). In this view, wax crystals could clearly be seen on the guard cells and on the stomatal antechamber walls, where wax ridges occasionally occurred in *Podocarpus rubens* (Fig. 1C,D,F) and *P. decipiens* (Fig. 3F). In *Podocarpus nivalis*, a layer of wax covered the stomatal complex and a wax

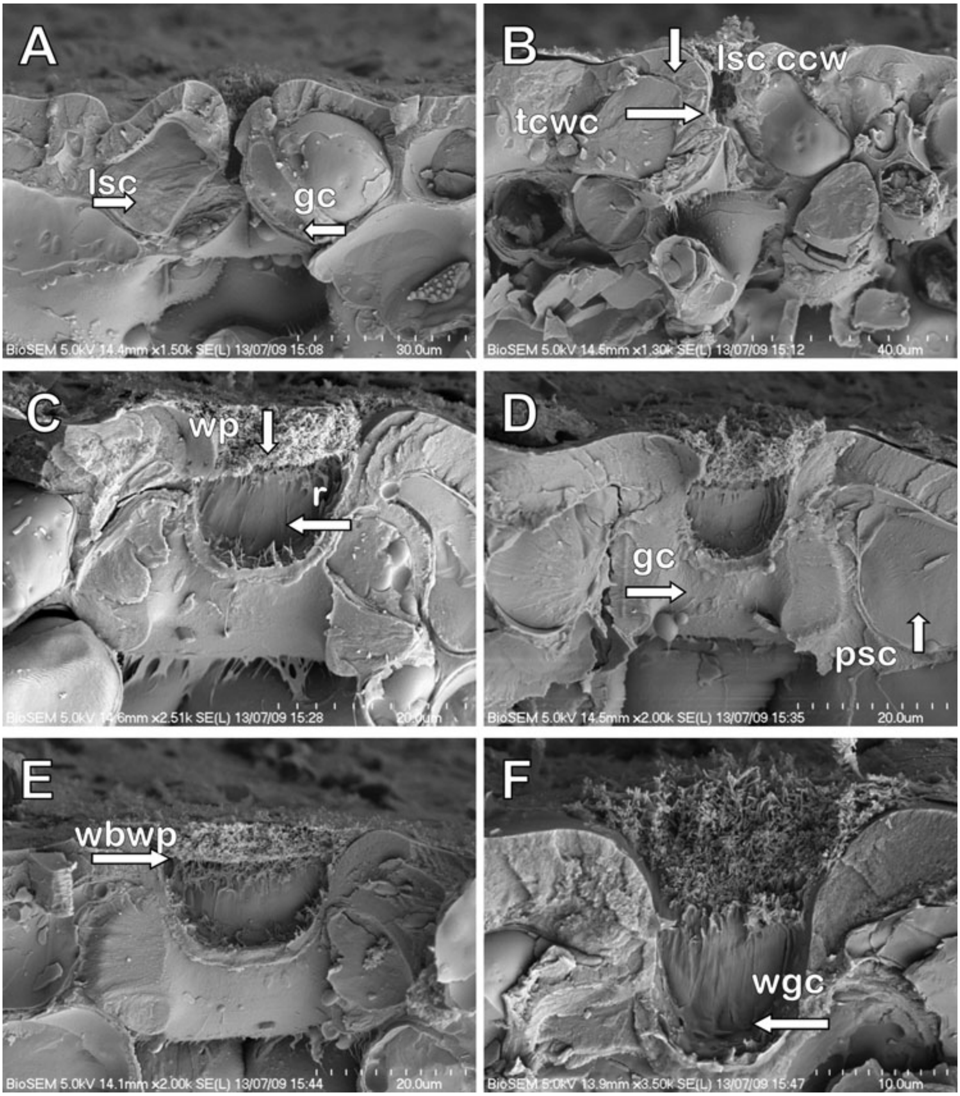

FIG. 1. *Podocarpus rubens*: cross-sections of stomatal complexes under scanning electron microscopy. A, Transverse cross-section (TCS) showing lateral subsidiary cell (lsc) and guard cell (gc). B, TCS showing cuticle and cell wall of lateral subsidiary cell (lsc ccw) and triangular area of cell wall and cuticle (tcwc). C, Longitudinal cross-section (LCS) showing wax plug (wp) and ridges (r). D, LCS showing polar subsidiary cell (psc) and guard cell (gc). E, LCS showing wax below the wax plug (wbwp). F, LCS showing wax on guard cell surface (wgc). Scale bar and magnification shown in each image.

plug appeared to sit halfway up the stomatal antechamber (Fig. 4B). In *Podocarpus rubens* (Fig. 1D) and *P. decipiens* (Fig. 3D), a portion of the polar subsidiary cells, mostly cell wall material, arched almost completely over the polar ends of the guard cells; the same was observed to a greater extent in *P. nivalis* (Fig. 4B). The presence

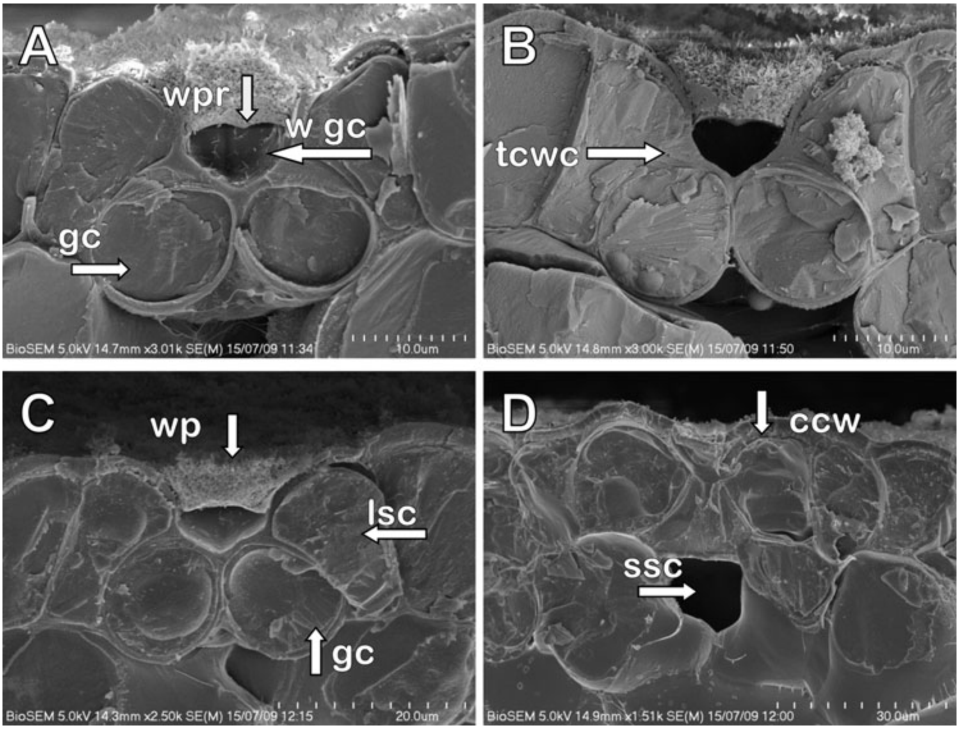

FIG. 2. *Podocarpus beecherae*: cross-sections of stomatal complexes under scanning electron microscopy. A, Transverse cross-section (TCS) showing wax plug ridge (wpr), wax on the guard cell surface (w gc) and guard cell (gc). B, TCS showing triangular area of cell wall and cuticle (tcwc). C, TCS showing wax plug (wp), lateral subsidiary cell (lsc) and guard cell (gc). D, TCS showing substomatal cavity (ssc) and cuticle and cell wall (ccw). Note the elliptical guard cells in this sample. Scale bar and magnification shown in each image.

of the Florin ring in *Podocarpus nivalis* was less obvious than in TCS, and the Florin ring appeared to consist of cell wall material. The polar ends of the guard cells tended to be oblong to reniform in LCS in all three species. An internal view of the stomatal complex of *Podocarpus decipiens* was seen, resulting from fracturing of the leaf during cross-sectioning. Wax was seen on the inside area of the stomata and a groove on the underside of the guard cells (Fig. 3A).

Ridges within the stomatal antechamber

Ridges within the stomatal antechamber were observed in the cross-sections of *Podocarpus rubens* and *P. decipiens*, and also when viewing the ISC of *P. decipiens* (Fig. 5A–C), *P. teysmannii* (Fig. 5D,E), *P. insularis* (Fig. 5F) and *P. milanjianus* (Fig. 6A), as well as the ESC of *P. pilgeri* (Fig. 6B), *P. chinensis* (Fig. 6C,D), and *P. macrophyllus* (Fig. 6E,F). The ridges appeared to be particularly prominent and

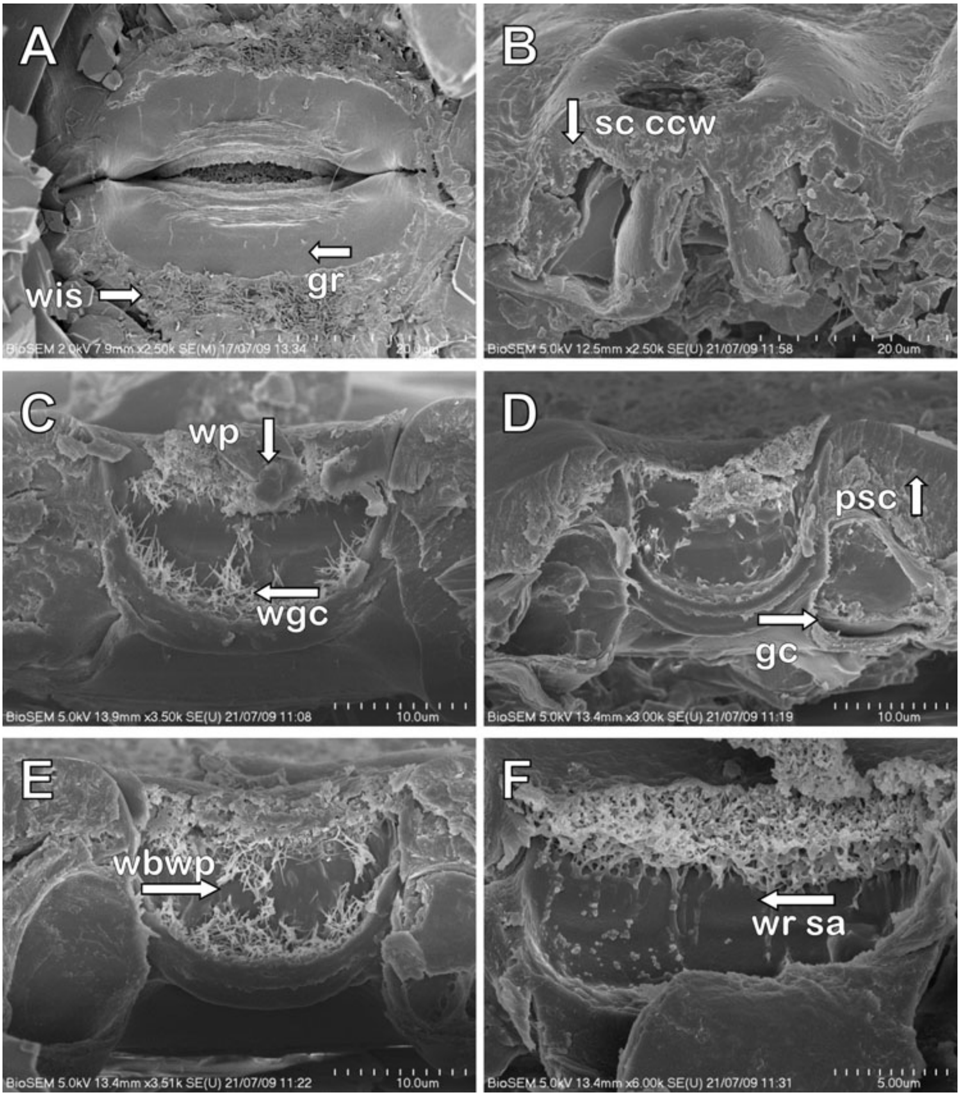

FIG. 3. *Podocarpus decipiens*: stomatal complexes under scanning electron microscopy. A, Inner surface of stomatal complex, showing wax inside stomata (wis) and groove (gr). B, Outer view of stomatal complex, showing cuticle and cell wall of subsidiary cells (sc ccw). C, Longitudinal cross-section (LCS) showing wax plug (wp) and wax on guard cells (wgc). D, LCS showing polar subsidiary cell (psc) and guard cell (gc). E, LCS showing wax below wax plug (wbwp). F, LCS showing wax ridges in the stomatal antechamber (wr sa). Scale bar and magnification shown in each image.

regularly spaced in *Podocarpus decipiens*, *P. teysmannii* and *P. macrophyllus*. In *Podocarpus decipiens* (Fig. 5B) and *P. teysmannii* (Fig. 5E), a cracked wax plug, which would have been positioned at the top of the stomatal antechamber, could also be

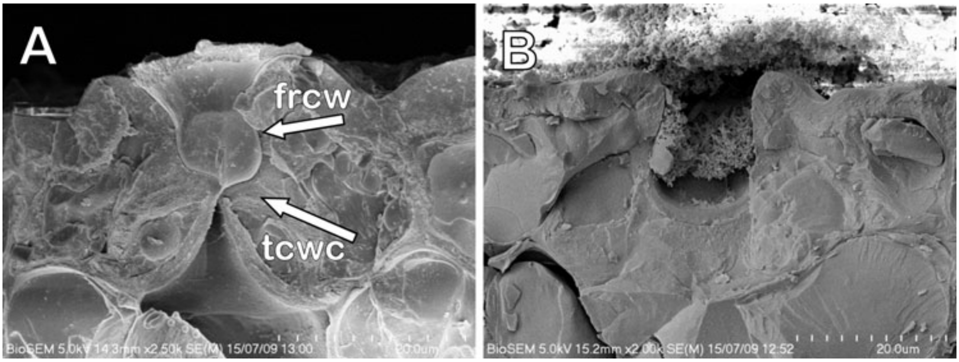

FIG. 4. *Podocarpus nivalis*: cross-sections of stomatal complexes under scanning electron microscopy. A, Transverse cross-section showing Florin ring made up of cell wall material and cuticle (frcw) and triangular area of cell wall and cuticle (tcwc). B, Longitudinal cross-section. Scale bar and magnification shown in each image.

seen. The ridges were less prominent and less regularly spaced in *Podocarpus insularis, P. pilgeri, P. chinensis* and *P. milanjianus.*

Wax plugs

The wax plug of *Podocarpus rubens* seen in LCS and TCS appeared at the very top of the stomatal antechamber, in line with the top of the subsidiary cells (Fig. 1A–F). In *Podocarpus decipiens*, the wax plug sat slightly below the level of the subsidiary cells (Fig. 3B–E), whereas in *P. nivalis* it was situated halfway up the stomatal antechamber (Fig. 4B), although this may have been the result of damage. The wax plug of *Podocarpus beecherae* seen in TCS often had the outer edges lower than the subsidiary cell edges, although the central area or the edges of the wax plug bulged up to the height of the subsidiary cells (Fig. 2A–C). Overall, the wax plug occurred as a flat layer when viewed in LCS and was more irregular when viewed in TCS, often with a ridge on the lower surface and bulges on the upper surface.

Dimensions

In total, measurements were taken from seven different stomata in LCS for *Podocarpus decipiens*, four in LCS for *P. rubens*, one in LCS for *P. nivalis*, three in TCS for *P. beecherae* and one in TCS for *P. rubens* (Table 2). *Podocarpus rubens* was the only species with images of undamaged stomata available in both LCS and TCS, with the main difference being the shape of the wax plug (Fig. 1B,C).

Table 2 shows that in *Podocarpus* subgenus *Foliolatus*, on average the stomatal antechamber was deeper in *P. rubens* (17.33 μm in LCS and 16 μm in TCS) than in any other species examined, while it was shallowest in *P. beecherae* (10.77 μm in TCS). The deepest stomatal antechamber, although by less than 1 μm, was that of *Podocarpus*

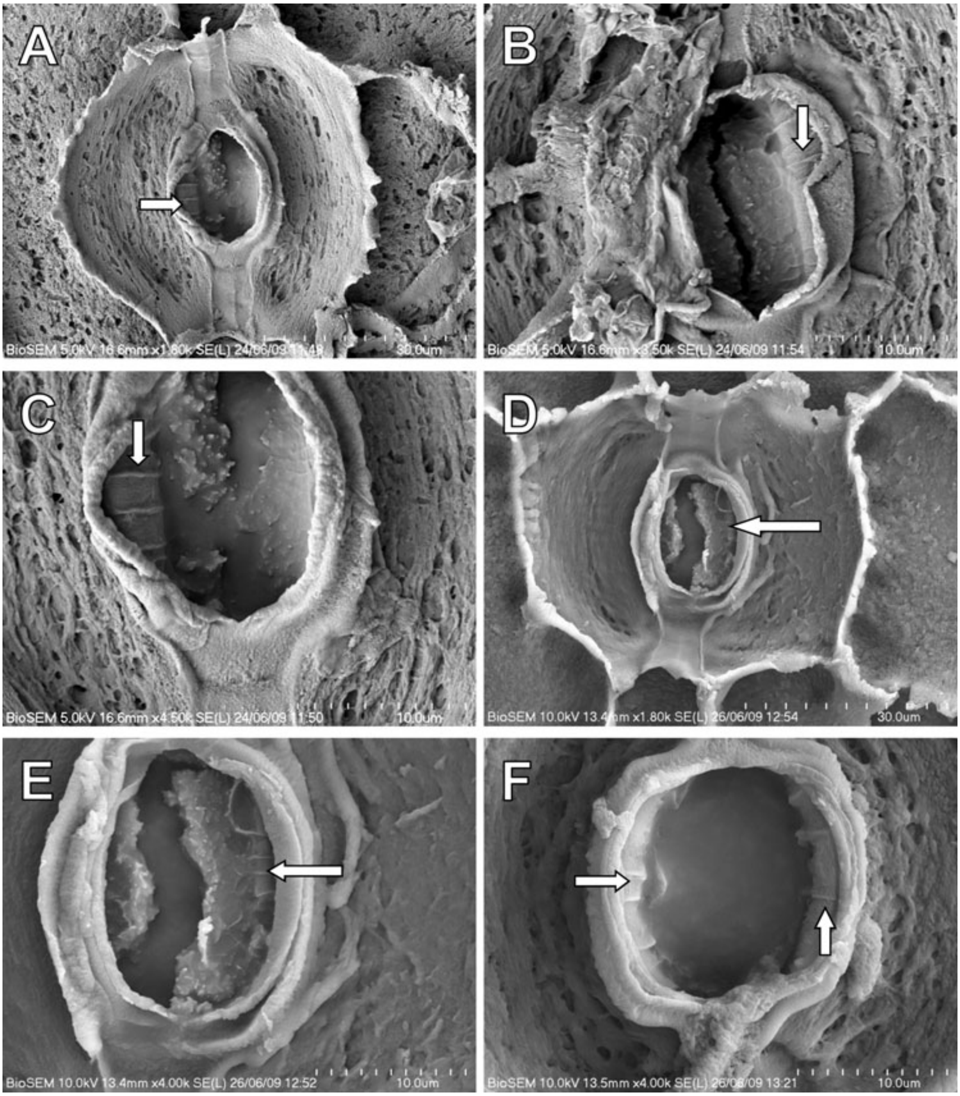

Fig. 5. Internal surfaces of cuticle of *Podocarpus* species, showing ridges on the cuticle that appear to be in the stomatal antechamber: A–C, *Podocarpus decipiens*; D and E, *P. teysmannii*; F, *P. insularis*. Scale bar and magnification shown in each image.

nivalis (18 µm). The wax plug was similar in thickness for *Podocarpus rubens* (6.5 µm in both LCS and TCS), *P. nivalis* (6 µm) and *P. beecherae* (6.17 µm), although it was markedly thinner in *P. decipiens* (3.76 µm). The gap between the base of the wax plug and the upper surface of the guard cells was fairly similar between the studied species, varying only by up to 3 µm, being greatest in *Podocarpus rubens* (10.83 µm in LCS) and shallowest in *P. beecherae* (7.9 µm in TCS). The mean percentage of the

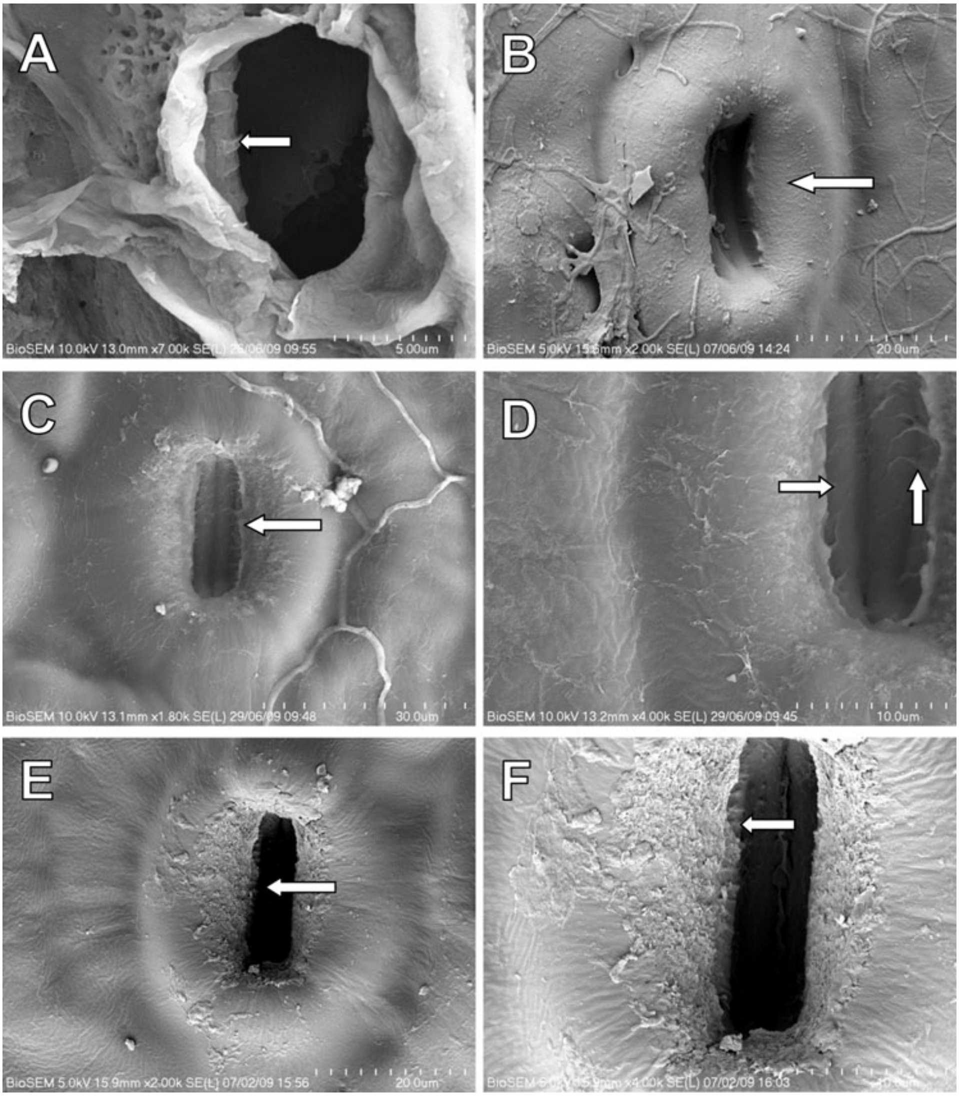

FIG. 6. Internal (A) and external (B–F) surfaces of cuticle of *Podocarpus* species, showing ridges on the cuticle that appear to be in the stomatal antechamber. A, *Podocarpus milanjianus*; B, *P. pilgeri*; C and D, *P. chinensis*; E and F, *P. macrophyllus*. Scale bar and magnification shown in each image.

stomatal antechamber taken up by the gap between the lower surface of the wax plug and the upper surface of the guard cells was greatest in *Podocarpus decipiens* (68%), if measuring depth from the top of the wax plug, or in *P. nivalis* (67%), if measuring to the top of the subsidiary cells, whereas it was least in *P. beecherae* (46% to top of wax plug, or 43% to top of subsidiary cells).

Table 2. Measurements taken from longitudinal cross-sections of *Podocarpus decipiens*, *P. rubens* and *P. nivalis*, and transverse cross-sections of *P. rubens* and *P. beecherae*

Species	No. of stomata measured	Mean depth of stomatal antechamber from top of wax plug to base of chamber (μm)	Mean depth of stomatal antechamber from level with top of subsidiary cells to base of chamber (μm)	Mean thickness of wax plug (μm)	Mean depth of gap (G) between base of wax plug and upper surface of guard cells (μm)	Stomatal antechamber (measured from base of chamber to top of wax plug, W, or top level of subsidiary cells, S) taken up by wax plug (%)	Stomatal antechamber taken up by G (%)	Placement of wax plug	Type of cross-section
Podocarpus decipiens	7	11.87	15.47	3.76	8.11	W: 32 S: 27	W: 68 S: 55	Slightly below level of subsidiary cells	Longitudinal
Podocarpus rubens	4	17.33	17.33	6.50	10.83	W: 37 S: 37	W: 63 S: 63	At very top of chamber, level with top of subsidiary cells	Longitudinal
Podocarpus rubens	1	16.00	16.00	6.50	9.50	W: 41 S: 41	W: 59 S: 59	Wax plug sits at very top of chamber and bulges in centre	Transverse

TABLE 2. (*Continued*)

Species	No. of stomata measured	Mean depth of stomatal antechamber from top of wax plug to base of chamber (μm)	Mean depth of stomatal antechamber from level with top of subsidiary cells to base of chamber (μm)	Mean thickness of wax plug (μm)	Mean depth of gap (G) between base of wax plug and upper surface of guard cells (μm)	Stomatal antechamber (measured from base of chamber to top of wax plug, W, or top level of subsidiary cells, S) taken up by wax plug (%)	Stomatal antechamber taken up by G (%)	Placement of wax plug	Type of cross-section
Podocarpus beecherae	3	10.77	11.43	6.17	7.90	W: 54 S: 57	W: 46 S: 43	Wax plug edges often lower in chamber, although central area or edges bulge up to level of subsidiary cells	Transverse
Podocarpus nivalis	1	–	18.00	6.00	–	S: 33	S: 67	Halfway up chamber (although possibly damaged)	Longitudinal

Discussion

We studied cross-sections of three species of *Podocarpus* subgenus *Foliolatus* (*Podocarpus beecherae*, *P. decipiens* and *P. rubens*) and one of *Podocarpus* subgenus *Podocarpus* (*P. nivalis*). Cross-sections of the stomatal complex provide a basis for understanding the architecture of the stomatal complex with regard to the guard cells, subsidiary cell shape, Florin ring (where present) and wax plug. Very few examples of studies on cross-sections of gymnosperm stomata seem to exist. One such study is that by Johnson & Riding (1981), who used light microscopy and SEM to study the structure and ontogeny of two species of *Pinus* L. (Pinaceae). In addition, a study by Mohammadian *et al.* (2009) used cryofracture to examine the cross-section of the stomatal complex of *Agathis robusta*. Their study also found that the wax plug occurred as a layer of wax that sat high in the stomatal antechamber (although no images were provided to make comparisons with the material that we studied). Our study represents the first of its kind for *Podocarpus*, and is also one of the first studies to use either cryofracture techniques or fracture at room temperature to examine the stomata of any gymnosperm and apply the results in a taxonomic context. Within Podocarpaceae, the same techniques have been used to study the cross-section of the stomata of the monospecific genus *Sundacarpus* (J.T.Buchholz & N.E.Gray) C.N.Page (Whiting, 2009).

More images are needed, in particular for *Podocarpus* subgenus *Podocarpus*, before generalisations can be drawn, although the images provided here do give some insight into the organisation and functioning of the stomatal complex in *Podocarpus*. In all species of *Podocarpus* subgenus *Foliolatus* examined in cross-section, the wax plug sat high in the stomatal antechamber and was in contact with the subsidiary cell walls, but with a relatively large gap between the wax plug and the guard cells. The underside of the wax plug had a central ridge that appeared to correspond with the crease where the guard cells meet (seen in *Podocarpus beecherae* and *P. rubens*). This is most clearly seen in *Podocarpus beecherae*, in which the shape of the upper surface of the guard cells and the shape of the lower surface of the wax plug (including the ridge) are similar although do not quite match. Material of both *Podocarpus beecherae* and *P. rubens* originated from young trees kept in a glasshouse (Table 1). Both these factors could have had an effect on the wax plug; for example, the ridge may disappear over time and the production of wax may vary. Had more samples, from leaves of different ages, been studied, these questions could perhaps have been addressed, but doing so was outside the scope of this study. Wax plug shape and thickness and placement within the stomatal antechamber may also vary in nature over time or depending on the seasons, as was found for *Podocarpus macrophyllus* by Morvan (1987). A cycle of wax plug extrusion, solidification and degradation was found to occur in cultivated *Agathis robusta* (Mohammadian *et al.*, 2009).

The wax plug of *Podocarpus nivalis* appeared to sit halfway up the stomatal antechamber, although it may have been damaged during cryofracture. A view of the external surface of the cuticle in this species revealed wax plugs with cracks, which could indicate that the wax plugs of this species are easily damaged during cryofracture or drying of specimens (Whiting, 2009) or that the wax plug may have been damaged because of the ageing of the leaf or changing seasons. Alternatively, it could indicate the presence, as opposed to absence, of specific fungi in the stomata, as was found in *Pinus strobus* L. by Deckert *et al.* (2001). In cultivated *Agathis robusta*, it was found that wax plugs were degraded in winter and that leaves over 2 years old had incomplete, irregularly shaped wax plugs (Mohammadian *et al*., 2009). There does appear to be more wax overall present in the stomatal antechamber of this species, which could indicate that it has a much thicker wax plug than *Podocarpus decipiens, P. beecherae* and *P. rubens.* The stomatal antechamber in *Podocarpus* subgenus *Podocarpus* potentially has more depth/height because the Florin ring, providing the possibility of having a thicker wax plug and still leaving a fairly large gap between the guard cells and wax plug. Wax plugs were found to block fungal hyphae entering the stomata in *Agathis robusta* (Mohammadian *et al*., 2009), although Deckert *et al.* (2001) found that in *Pinus strobus* the epistomatal chamber acts as a microhabitat for specific, presumably beneficial, 'stomatal fungi' that are protected from desiccation and the effects of ultraviolet light by the wax plug; the hyphae of these fungi never protruded into the stomatal pore or occupied the substomatal space. A thicker wax plug may also last longer and be more resistant to the seasons, which would provide protection against harmful fungi for a greater period of time. Studies of more species of *Podocarpus* subgenus *Podocarpus* are needed to test this hypothesis.

The ridges within the stomatal antechamber observed in the cross-sections of *Podocarpus rubens* and *P. decipiens* and when viewing the ISC of *P. decipiens*, *P. teysmannii*, *P. insularis* and *P. milanjianus* and the ESC of *P. pilgeri*, *P. chinensis* and *P. macrophyllus* were a novel discovery. They may be present in a greater number of species (in *Podocarpus* and in other genera), although difficulty can be encountered in observing them. In the case of the ISC, the ridges are hidden when the guard cells are closed and can be seen only when the stomatal antechamber is collapsed slightly inwards. In addition, these ridges may be hidden from view by the wax plug when observing the ESC. The process of removing the wax may also remove the ridges if they consist of wax only. These ridges were observed mainly in species of *Podocarpus* subgenus *Foliolatus*, although a similar pattern was observed on the internal cuticle of *P. milanjianus* from *Podocarpus* subgenus *Podocarpus*, but not in other members of that subgenus examined (Whiting, 2009).

Examination of young leaves of another, recently described species of *Podocarpus* subgenus *Foliolatus*, *Podocarpus orarius* R.R.Mill & Whiting, found that in the developing stomata the wax plug appears to lie on top of the guard cells within the stomatal antechamber (Mill & Whiting, 2012, 'peristomatal chamber'). In this species, the developing lateral subsidiary cells form narrow ridges on either side of the guard cells. It may be that as the leaf expands, the subsidiary cells push the guard cells downwards. If this is so, it may cause the cuticle to be stretched in this region of the stomatal antechamber. This could cause the polymers of the cuticle to align, which in turn could make the wax crystals orient in the direction of the polymers, thereby creating ridges by a process of epitaxis. It is possible that these ridges are a result of wax crystals being produced by the guard cells and then travelling up the epistomatal wall to become incorporated into the wax plug. The cross-sections of *Podocarpus decipiens* and *P. rubens* appear to show that wax crystals are produced by the guard cells and then climb the epistomatal wall to the wax plug. It was noted that a triangular area of cell wall material (from the guard cell and the subsidiary cell) and cuticle sits on top of the guard cell. This area, as well as the lateral subsidiary cells, may become increasingly curved inwards during development of the stomatal complex and stomatal antechamber; ridges may then occur in this region's inner area. The subsidiary cells have a specific and complex shape in comparison with the surrounding epidermal cells. The subsidiary cells are likely to have a number of functions, which may include creating the Florin ring and surrounding groove (in subgenus *Podocarpus*), positioning of guard cells, wax production, raising the wax plug from the guard cells, and creating a greater depth for a thicker wax plug to occur (in subgenus *Podocarpus*). The subsidiary cells may therefore have an important role in the development of the stomatal complex as well as in the functioning of the stomata (as found by Johnson & Riding, 1981).

Few images were available from which to take measurements, in particular for *Podocarpus nivalis*, the only species of subgenus *Podocarpus* that was studied. Ideally, more cross-sections would need to be done, with more species and more specimens per species to obtain more accurate dimensions. However, the measurements taken give a general idea as to the proportions of the stomatal antechamber taken up by the wax plug and the gap between the wax plug and the guard cells. In *Podocarpus beecherae*, the younger specimen (a sapling 40 cm tall when sampled) was noticeable in having the greatest proportion of the stomatal antechamber taken up by the wax plug and therefore the least proportion taken up by the gap between the guard cells and wax plug. Possibly in a mature tree (and mature leaf) this gap may have increased in depth and/or the wax plug be lifted higher. However, images of *Podocarpus beecherae* were all in TCS, which could have affected the measurements, because in *P. rubens* the proportion of the stomatal antechamber taken up by the wax plug was slightly higher in TCS than in LCS. Interestingly, the wax plug mean thickness was fairly constant in *Podocarpus rubens* (TCS and LCS), *P. beecherae* and *P. nivalis*, although it was on average thinner in *P. decipiens*. The sample of *Podocarpus decipiens* was the only one that was taken from a herbarium specimen (Table 1). More work would need to be done to see if, and in what way, the wax plug changes in morphology during the process of making a herbarium specimen, which includes drying, as well as over time in the herbarium. The data, however, suggest that either such changes do take place, or that (given the similarities between the wax plug thicknesses of *Podocarpus rubens*, *P. beecherae* and *P. nivalis*) differences in thickness do occur between some species but these differences are not correlated with the subgeneric placement of the species.

In the LCS of *Podocarpus rubens*, the wax plug appears as a rectangular layer mostly flush with the upper level of the lateral subsidiary cells, although it appears slightly sunken in *P. decipiens*. This sunken wax plug may also be a consequence of the sample coming from a herbarium specimen (the wax plug shrinking as it dries out). More work is necessary to determine the true placement and shape of the wax plug in *Podocarpus nivalis*.

Conclusions

Although the results presented here must be regarded as a preliminary study, they show the potential for cross-sections of stomata obtained by cryofracture and fracture at room temperature to be used in taxonomic studies. Very little is known about the variation in stomatal cross-sectional morphology in gymnosperms, or indeed any group of plants, and it is hoped that this study will stimulate further research in this field. As well as further SEM of a wider range of leaf ages than was possible to be studied here, light microscopy of samples histochemically stained with Sudan III and IV (to show waxes and cuticles) and floroglucinol/HCl (to illustrate lignin in the stomatal complexes) would be highly desirable.

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