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The evolution of the stomatal apparatus: intercellular spaces and sporophyte water relations in bryophytes—two ignored dimensions

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Cryo-scanning electron microscopy shows that nascent intercellular spaces (ICSs) in bryophytes are liquid-filled, whereas these are gas-filled from the outset in tracheophytes except in the gametophytes of Lycopodiales. ICSs are absent in moss gametophytes and remain liquid-filled in hornwort gametophytes and in both generations in liverworts. Liquid is replaced by gas following stomatal opening in hornworts and is ubiquitous in moss sporophytes even in astomate taxa. New data on moss water relations and sporophyte weights indicate that the latter are homiohydric while X-ray microanalysis reveals an absence of potassium pumps in the stomatal apparatus. The distribution of ICSs in bryophytes is strongly indicative of very ancient multiple origins. Inherent in this scenario is either the dual or triple evolution of stomata. The absence, in mosses, of any relationship between increases in sporophyte biomass and stomata numbers and absences, suggests that CO_2 entry through the stomata, possible only after fluid replacement by gas in the ICSs, makes but a minor contribution to sporophyte nutrition. Save for a single claim of active regulation of aperture dimensions in mosses, all other functional and structural data point to the sporophyte desiccation, leading to spore discharge, as the primeval role of the stomatal apparatus.

This article is part of a discussion meeting issue 'The Rhynie cherts: our earliest terrestrial ecosystem revisited'.

1. Introduction

While recent structural, developmental, physiological and molecular data have raised lively polarized debates and vexed questions about the evolution and functioning of stomata [1–12], not to mention the unresolved issue of unitary versus multiple origins [13–21], these have focused almost exclusively on the guard cells and a veritable army of genes that might affect aperture changes in the same. A growing multitude of genes are thought to be allied to stomatal biology, particularly in the context of the roles of abscisic acid (ABA); many of these are also found in bryophytes, including the liverworts—the only extant land plant clade that completely lacks stomata [22,23]. However, hard experimental data on putatively active aperture movements *viz. reversible changes* in response to environmental cues, in non-seed plants are restricted to a handful of incisive studies on ferns [1,9,10,24]. Only small *unidirectional changes* (but never closure) have been recorded in the lycophyte *Selaginella uncinata* [11], the hornwort *Anthoceros punctatus* [25] and the two mosses [3] *Physcomitrella patens* and *Funaria hygrometrica*, both of which have unusual stomata with just a single guard cell [26–29]. In an earlier and much cited work, Garner & Paolillo [30] state that *Funaria* stomata respond to exogenous cues but do not provide any aperture measurements. It is interesting to note that the largest aperture changes in *Physcomitrella* and *Funaria* were in response to fusicoccin. This fungal toxin activates the H^+ATPase pump that initiates turgor-driven stomatal opening in vascular plants. However, close scrutiny suggests the possibility of serious

flaws in the Chater *et al*. data [3]. These include: (i) sampling size (three replicates of 40)—this is small for each experiment, particularly for *Funaria* where stomatal numbers often exceed 200 [7]; (ii) an absence of precise information on the maturational state of the sporophytes; and (iii) differences between the controls in the different experiments often exceeding those for the various treatments. For example, in *Funaria* the aperture areas in the ABA control exceed those following fusiccocin treatment.

Guard cells are just one key component of the stomatal apparatus: as highlighted by Corner [31], their subjacent schizogenous intercellular spaces (ICSs), developing by separation of contiguous primary walls through the middle lamella, are equally integral to the functioning of stomata in regulation of gaseous exchange. Since gas-filled ICSs rank alongside conducting cells, cuticles and matrotrophy [19,20,32] as one of the key innovations intrinsic to the body plans of land plants, it is all the more surprising that they have been almost completely overlooked in functional studies and their unitary origin assumed without question. Scrutiny of published electron micrographs [33] provides no evidence for their possible occurrence in the land plants' zygnematalean ancestors [34]. On top of this major omission we are not aware of any recent studies that have actually documented accurately the distribution of ICSs across bryophytes and pteridophytes let alone evaluated their possible evolutionary trajectories.

Probably the most informative account is by Sifton [35]. This draws attention to old disagreements (e.g. [36,37]) about possible differences in ontogeny between the several layers of chambers in the thalli of *Reboulia* from the single layer opening by air pores in *Marchantia*. It goes on to say that little has been written about air space tissues in liverworts outside the Marchantiales (today's Marchantiophyta, table 1) and that the ICSs in both generations in hornworts are similar to those in typical parenchyma in vascular plants. Moss gametophytes are said to lack schizogenous ICSs with any cavities therein (e.g. in *Campylopus polytrichoides*, now *C. pilifer*) being lysigenous with the remains of dead cells in their cavities. Like those in hornworts, the ICSs in moss sporophytes are said to resemble those in vascular plants. A subsequent review [42] makes but a brief mention of bryophytes while Ishizaki's [22] account of extracellular signalling in the regulation of cell separation focuses almost entirely on the air chambers in *Marchantia*. Most recent papers address cell separation either in the context of abscission or enzymatic activities associated with wall breakdown (e.g. [43–45]) and make no mention of bryophytes.

A further missing piece in our understanding of stomatal evolution is any investigation in bryophytes into a possible mechanism that might drive aperture changes akin to the potassium ion fluxes affecting guard cell turgor found in angiosperms [46]. Highly pertinent, in the only such study to date, is the absence of potassium fluxes in the pseudostomata in *Sphagnum* [47]. So-called because they lack pores and subjacent ICSs, these structures are thought to facilitate sporophyte desiccation thus promoting capsule dehiscence and spore discharge [18,47]. The same has also been proposed as the principle function of stomata in hornworts [21] and bryopsid mosses on the basis of (i) developmental changes in wall chemistry that render the guard cells inelastic [48,49]; (ii) delayed dehiscence in stomata-less mutants in *Physcomitrella* [2]; and (iii) field observations of moss sporophytes throughout their development [50]. These revealed that, once open, moss stomata never close regardless of the weather conditions. However, the potassium content of the guard cells and adjacent epidermal cells remains to be investigated.

The background to the present focus on ICSs and guard cell potassium was the discovery, by the use of cryo-scanning electron microscopy (CSEM), that nascent ICSs in hornwort and *Funaria* sporophytes are initially liquid-filled [15,51,52]. Whereas in tracheophytes the foliar ICSs are gas-filled from the outset, in hornwort and *Funaria* sporophytes replacement of liquid by gas in the ICSs occurs only after stomatal opening and long before stomatal maturation [51]. At odds with Sifton's [35] assumption of ICS homology, the demonstration that the origins of ICSs in hornwort and moss sporophytes might be fundamentally different from vascular plants raised a major question regarding possible multiple origins across land plants. This echoes past debates on the multiple origins of water-conducting elements in mosses and liverworts [32,53,54]. In addition, this chance finding has far reaching implications for both the functioning and origins of bryophyte stomata. Thus, we investigated the occurrence, contents and fates of the ICSs not only in moss sporophytes but also across bryophytes in both generations. Questions of particular interest were: Are ICSs absent in moss gametophytes as indicated by previous anatomical studies? How far might they occur in liverwort sporophytes following an illustration of ICSs but without comment in the setae of the basal liverwort *Haplomitrium* [41]? In complex thalloid liverworts are all the chambers gas-filled, even those not connected to air pores? Since filmy ferns are poikilohydric and lack stomata [55–58], how far might be the absence of ICSs related to poikilohydry? As ICSs are integral to stomatal regulation of gaseous exchange, what new light do the data on ICSs shed on the unitary or multiple origin of stomata?

A recurrent observation in the CSEM was that gametophytes of liverworts, hornworts and mosses, plus filmy fern leaves, exhibited cytorrhysis after minimal drying out over a few minutes during specimen preparation as illustrated by previous authors [59–61], whereas hornwort and moss sporophytes were unchanged. Similarly, in nature, moss sporophytes largely appear unchanged with the stomata remaining open throughout the life of the sporophytes even after days or even weeks without rain have left the subjacent gametophytes completely desiccated [50] (figure 1). In the context of water relations, this suggests that moss (and hornwort) sporophytes may be homiohydric and that ICS and stomata are attributes of homiohydry.

Poikilohydry is one of the key features separating bryophytes from tracheophytes [62,63]; among extant plants homiohydry is restricted to tracheophyte sporophytes. Remarkably, however, all the vital information on the physiology and cell biology of vegetative desiccation tolerance, i.e. how bryophytes are able to lose water and then recover upon rewetting, derives almost exclusively from the gametophytes [64,65]. For example, in a recent authoritative review sporophytes are not even mentioned [66]. We simply do not know how far sporophyte water relations might mirror or differ from those of the gametophytes. The only data that we are aware of that show rates of water loss from moss sporophytes [47] reveal these are much more in line with those in vascular plants, with waterproofing by the cuticle and waxes on moss sporophyte surfaces [26,67–69], plus stomata with varying apertures supporting the premise of homiohydry. Thus, to test this assumption, we present comparative data on rates of water loss from both generations in a range of mosses and hornworts.

Table 1. The occurrence of fluid-filled schizolytic internal intercellular spaces in liverworts and hornworts. Classifications follow Crandall-Stotler *et al.* [38] and Villarreal *et al.* [39] for liverworts and Stotler & Crandall-Stotler [40] for hornworts. C, present at cell corners only; G, fluid replaced by gas; M, large and surrounded by several cells.

class	order family	taxa examined	air chambers	gametophyte	sporophyte	absent (−) or air pores (AP)
Liverworts						
Haplomitriopsida	Treubiales	*Treubia lacunosa*	−	+M	+C	−
	Calobryales	*Haplomitrium gibbsiae*	−	−	+C	−
		Haplomitrium hookeri	−	−	+C[c]	−
Marchantiopsida	Blasiales	*Blasia pusilla*	−	+M[a]	+C	−
	Neohodgsoniales	*Neohodgsonia mirabilis*	multilayered	+M	?	AP
	Sphaerocarpales	*Sphaerocarpos texanus*	−	−	−	−
	Riellales	*Riella affinis*	−	−	−	−
	Lunulariales	*Lunularia cruciata*	one-layered	−	?	AP
	Exormothecaceae	*Exormotheca pustulosa*	one-layered	−	?	AP
	Marchantiales					
	Aytoniaceae	*Asterella australis*	multilayered	+M	?	AP
		Asterella tenera	multilayered	+M	?	AP
		Cryptomitrium oreoides	multilayered	+M	?	AP
		Mannia californica	multilayered	+M	?	AP
		Plagiochasma rupestre	multilayered	+M	?	AP
		Reboulia hemispherica	multilayered	+M	?	AP
	Marchantiaceae	*Marchantia foliacea*	one-layered	−	+C	AP
		Preissia quadrata	one-layered	−	?	AP
	Cleveaceae	*Athalamia hyalina*	multilayered	+M	?	AP
		Peltolepis quadrata	multilayered	+M	?	AP
		Sauteria alpina	multilayered	+M	?	AP
	Conocephalaceae	*Conocephalum conicum*	one-layered	−	?	AP
	Corsiniaceae	*Corsinia coriandra*	one-layered	−	?	AP
	Cyathodiaceae	*Cyathodium foetidissimum*	one-layered	−	−	AP
	Dumortieriaceae	*Dumortiera hirsuta*	rudimentary	+M[b]	+C	−
	Monocleaceae	*Monoclea forsteri*	−	−	+C	−
	Monoseleniaceae	*Monoselenium tenerum*	−	−	−	−
	Oxymitraceae	*Oxymitra cristata*	one-layered	−	−	AP
	Ricciaceae	*Riccia huebeneriana*	multilayered	+M	−	AP
		Riccia crystallina	multilayered	+M	−	AP

[a]*Nostoc* colonies only.
[b]Carpocephala stalks only.
[c]Illustrated in Schuster [41].

One final rather neglected aspect of sporophyte biology in mosses is the possible contribution of CO_2 uptake through the stomata to sporophyte biomass. Experimental studies have revealed that the relative contributions of the gametophytes via the placenta [70–73] to the growth of the sporophytes ranges from 90% and 80% in *Pleuridium acuminatum* and *Mnium hornum*, respectively, down to 50% in *Funaria hygrometrica* [62,74]. Food reserves of gametophytic origin, acquired by the young sporophytes in Polytrichales, are thought to make a significant contribution to biomass of the expanding capsules [75]. Although none of these studies addressed the possible contribution to sporophyte biomass overall via CO_2 acquisition through the stomata rather than directly through the sporophyte surface, it is interesting that the gametophytic contributions are inversely related to stomatal numbers in *P. acuminatum* (3–4) [26], *M. hornum* (21–38) and *F. hygrometrica* (160–220) [7]. However, this may simply be due to chance; whereas stomatal densities and numbers in vascular plants make sense in terms of a regulatory role for CO_2 and water exchange, in mosses numbers (and absences) differ enormously even between closely related genera with very similar ecologies [7,76]. We, therefore, recorded the weights of the sporophytes in a range of mosses from stomatal opening (i.e. before possible gaseous exchange through the apertures) to capsule dehiscence.

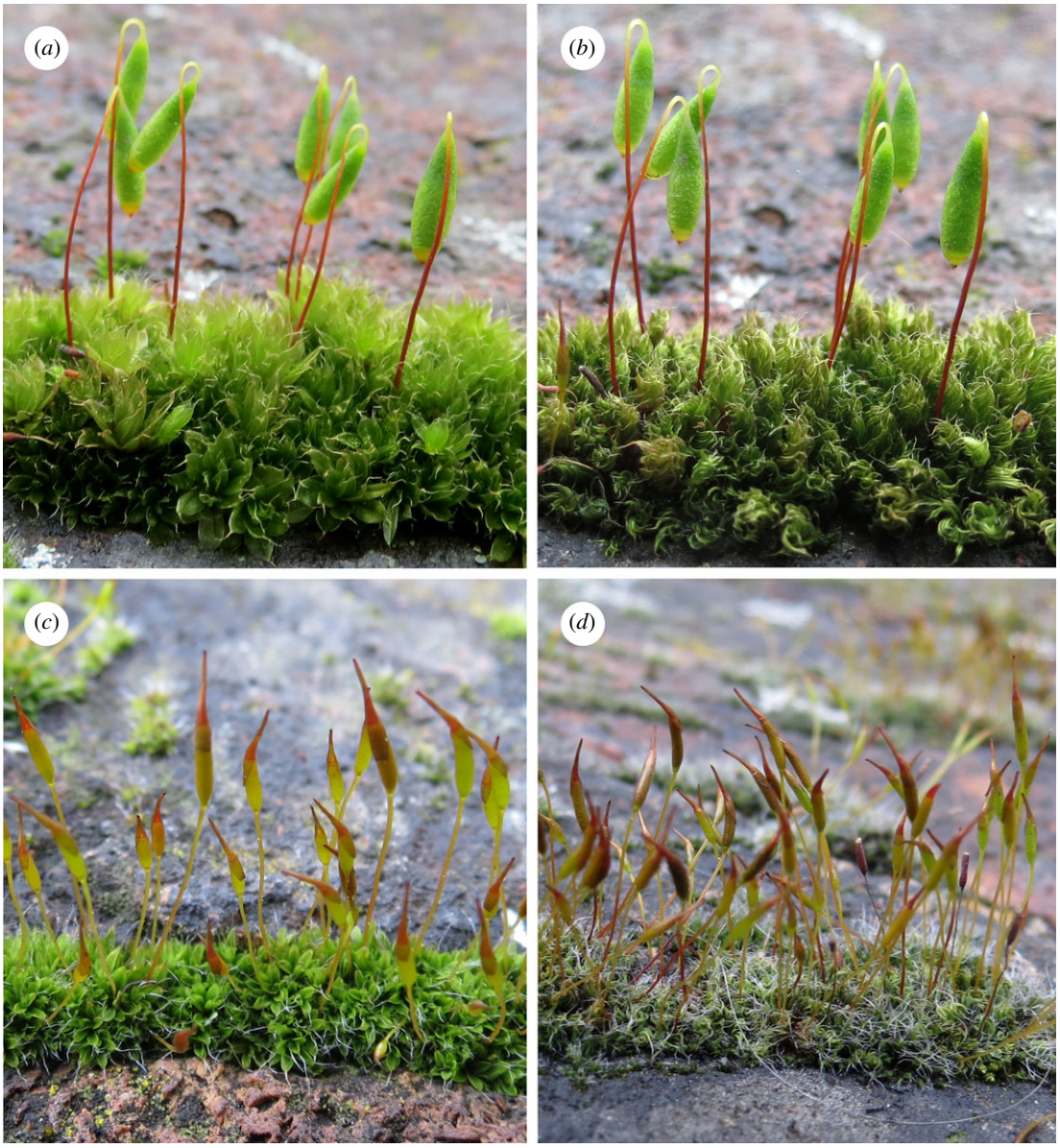

Figure 1. Homiohydric sporophytes and poikilohydric gametophytes. Colonies of *Bryum capillare* (*a*,*b*) and *Tortula muralis* (*c*,*d*) with green fully expanded capsules. Whereas the sporophytes remain unchanged after two weeks without rain the subjacent gametophytes become completely desiccated within 2–4 h (*b*,*d*).

2. Material and methods

(a) Microscopy

A wide selection of bryophytes and filmy ferns were collected from the wild and either observed immediately or maintained in growth cabinets. Full protocols for the light, standard SEM and CSEM images used in this account can be found in Duckett *et al*. [47] and Pressel *et al*. [51]. Nomenclature follows the legitimate names in Tropicos, www.tropicos.org.

(b) Potassium content of guard cells

Percentage weights of potassium were calculated from elemental X-ray spectra for both guard cells of at least four stomata plus adjacent epidermal cells of fully expanded green capsules of the mosses *Polytrichum juniperinum*, *Funaria hygrometrica*, *Physcomitrella patens*, *Tortula muralis*, *Bryum radiculosum* and *Philonotis fontata*. Prior to freezing, the mosses were maintained with fully hydrated gametophytes in a lighted growth chamber with irradiance 50 μmol m^{-2} s^{-1}, at 14–16°C, relative humidity 90%, i.e. under conditions likely to be optimal for stomatal opening. Readings taken from the sporophyte surfaces served as controls. Elemental spectra were obtained from stomata from at least four sporophytes of each species, by focused ion beam milling in a FEI quanta 3D FEG dual beam microscope (FEI Company, OR, USA) following the method by Duckett *et al*. [47].

(c) Rates of water loss

Rates of water loss (% fresh weight, FW) were calculated for a range of plants (mosses, a hornwort and vascular plants) listed in table 4. Plant materials were allowed to dry naturally in the laboratory atmosphere (70% RH, 20–22°C). Weight losses were measured at regular intervals from at least 20 moss and hornwort sporophytes (green, fully expanded in mosses and undehisced in hornworts) and vascular plants leaves, respectively, all freshly excised. Weights were recorded at regular intervals over a period of 72 h.

(d) Maturational weight increases

Dry weights were obtained by drying freshly collected sporophytes, at least 10 per species for each maturational stage (*viz*. immediately prior to stomatal opening, immediately following opening, open for two weeks and containing mature spores), overnight in an oven at 95°C. Percentage weight increases were calculated against the dry weights of the sporophytes immediately before stomatal opening.

3. Observations

(a) Intercellular spaces in lower land plants

The occurrence and nature of schizolytic internal ICSs based on our own CSEM observations in bryophytes and pteridophytes is summarized in tables 1 and 2.

(b) Gametophytes

Liverworts (figure 2*a–c*). At the base of the liverwort tree liquid-filled ICSs are associated with the fungal endophyte within the thalli of *Treubia* [79]. Unlike the liquid in other bryophyte ICSs, this highly mucilaginous material is exuded in large quantities from splits in the lower epidermis of the thalli and resembles that coating the naked subterranean fungus-containing axes in *Haplomitrium* [80]. Biochemical analyses are now needed to determine the composition of this material to ascertain whether it is the same as that found in other bryophyte ICSs. This would seem unlikely as its production is triggered by the fungal endophytes [81], whereas other ICS liquids are constitutive. It is also noteworthy that the contents of the lacunae in *Treubia* appear to be a product of the endoplasmic reticulum [79] while the mucilage produced by mucilage papillae in bryophytes is derived from the Golgi [82–85].

The Sphaerocarpales and Blasiales, the two orders sister to the rest of the Marchantiophyta [38], both completely lack internal gametophytic ICSs. The *Nostoc* colonies in Blasiales and hornworts are liquid-filled and open to the surface via pores from their inception [86]. Their development is triggered by the cyanobacteria, and cell expansion and division rather than schizolysis appears to be responsible for their increase in size. Until the composition of the matrix around the cyanobacteria and its origin, from either host or endophyte, have been determined we cannot judge how far these lacunae might be the same as other bryophytic ICSs.

Although schizolytic processes are involved in setting the initial framework in marchantialean air chambers [22,87], these almost exclusively involve surface cells. The cavernous lumina subsequently produced are almost exclusively the result of cell overgrowth and not cell separation: from the outset the chambers are open to the exterior of the thalli and are gas-filled throughout. However, examination of taxa with several strata of chambers within their thalli in addition to those opening onto the upper surface of the thalli via the pores (*Neohodgsonia mirabilis*, *Athalamia hyalina*, *Riccia huebeneriana*) reveals that some of the lower chambers are liquid-filled, thereby indicating a different and schizolytic origin (figure 2*a–c*). The occurrence of these large fluid-filled lacunae only in the Neohodgsoniaceae, Cleaveaceae and Ricciaceae, all later divergent lineages than the basal Sphaerocarpales and Blasiales [38], is perhaps indicative of separate evolution.

The only other place where we have seen fluid-filled ICSs in liverwort gametophytes are at the cell corners in the fleshy carpocephala stalks in *Dumortiera hirsuta* (figure 2*d*). We did not find them in the carpocephala stalks in *Asterella*, *Conocephalum*, *Lunularia* and *Marchantia* nor anywhere in the gametophytes of the Jungermanniopsida (simple thalloid and leafy liverworts).

Hornworts (figure 3*a–c*). The presence or absence of central sealed mucilage-filled chambers is a key character delimiting different hornwort genera [88] (figure 3*a*,*b*), except in *Notothylas* where these may be present or absent depending on the species [89]. As in *Blasia*, the ubiquitous *Nostoc* colonies (figure 3*c*) are open to the exterior from their inception and it is not clear how closely they are related to the internal mucilage chambers or how closely they match the *Nostoc* chambers in *Blasia*. In the former, the host filaments traversing the colonies are transfer cells, whereas in hornworts they are highly vacuolated [86]. The hornwort fungal endophytes are often associated with the *Nostoc* colonies but not the large mucilage chambers, and fungi are absent in *Blasia* [90].

Mosses. We did not encounter a single example of intercellular spaces in moss gametophytes from the Takakiopsida, Sphagnopsida and Andreaeopsida to the Hypnales in our SEM studies to date embracing 21 orders, 54 genera and over 100 species. Thus, this study confirms Sifton's [35] earlier observations.

Pteridophytes. The only gametophytic ICSs in pteridophytes are associated with the endophytic fungi in the Lycopodiales [91–93]. They also appear to be absent in the gametophytes of rhyniophytes [94–96], thus suggesting that they might not have been homiohydric despite the presence of a cuticle and stomata.

(c) Sporophytes

Liverworts (figure 2*d–l*). Our studies confirm the presence of ICSs, previously illustrated by Schuster [41], in the seta of *Haplomitrium* and demonstrate, by CSEM, that these are liquid-filled (figure 2*i*). Similar ICSs were found across all the major liverwort groups (figure 2*d*,*g*,*j–l*), but it should be noted that we targeted taxa with large fleshy setae and we do not know if this feature is also present in those with much simpler setae: there is no indication of their presence from the line drawings in Schuster [41]. We also discovered that the elongated setae in several genera including members of the Haplomitriopsida (*Treubia*), Marchantiopsida (*Monoclea*), Fossombroniales (*Allisonia*) and Jungermanniales (*Wettsteinia*) (figure 2*e*,*f*,*h*) contain central fluid-filled lysigenous cavities with the remains of broken cells around their periphery (figure 2*f*).

Hornworts (figure 3*d–i*). In hornwort sporophytes, there is an absolute correlation between the presence of stomata and initially liquid-filled ICSs in the photosynthetic cortical tissues [51] (figure 3*d*,*e*,*g*). However, this is not true for the columella, where ICSs are present not only in all the stomate genera (figure 3*f*) (*Anthoceros*, *Folioceros*, *Leiosporoceros Paraphymatoceros*, *Phaeomegaceros*) but also in the astomate *Megaceros* and *Nothoceros*. *Dendroceros* and *Notothylas* have yet to be examined but clearly this feature must be absent in species lacking a columella [89]. Astomate taxa consistently lack ICSs (figure 3*h*,*i*). Following sporophyte dehiscence, the initially fluid-filled columella ICSs become gas-filled down to deep within the involucres at a level where the substomatal ones are always liquid-filled.

Mosses (figures 4 and 5). From absent in the three basal lineages Takakiopsida, Sphagnopsida and Andreaeopsida, sporophytic ICSs are ubiquitous in the remainder of the mosses whether (figure 4*a–f*) or not (figure 5*d–f*) taxa have stomata. Gas gradually replaces their initially liquid-filled content over periods of one to four weeks following stomatal opening on green, fully expanded capsules (e.g. figure 4*e*,*f*). However, in some Polytrichales, where fully formed stomata may remain closed for up to four months, we frequently found gas-filled ICSs in capsules with unopened stomata (figure 5*a*). The same process of liquid replacement also occurs in fully expanded capsules in astomate taxa (figure 5*d–f*) within similar time periods, e.g. two to four

Table 2. The occurrence of schizolytic internal intercellular spaces in mosses and pteridophytes. L, liquid-filled throughout; G, gas-filled throughout; LG, liquid replaced by gas. Classifications follow Goffinet *et al*. [77] for mosses, Smith *et al*. [78] for ferns.

class	order	genera examined	gametophyte	sporophyte	stomata
Mosses					
Takakiopsida	Takakiales	*Takakia*	−	−	−
Sphagnopsida	Sphagnales	*Sphagnum*	−	−	−
Andreaeopsida	Andreaeales	*Andreaea*	−	−	−
Oedipodiopsida	Oedipodiales	*Oedipodium*	−	+LG	+
Polytrichopsida	Polytrichales	*Dawsonia*	−	+LG	+
		Dendoligotrichum	−	+LG	+
		Oligotrichum	−	+LG	+
		Polytrichum	−	+LG	+
		Polytrichastrum	−	+LG	+
		Atrichum	−	+LG	−
		Pogonatum	−	+LG	−
Tetraphidopsida	Tetraphidales	*Tetradontium*	−	+LG	+
		Tetraphis	−	+LG	−
Bryopsida	Buxbaumiales	*Buxbaumia*	−	+LG	+
	Diphysciales	*Diphyscium*	−	+LG	+
	Encalyptales	*Encalypta*	−	+LG	+
	Funariales	*Discelium*	−	+LG	+
		Ephemerella	−	+LG	+
		Entosthodon	−	+LG	+
		Funaria	−	+LG	+
		Physcomitrella	−	+LG	+
	Scouleriales	*Scouleria*	−	+LG	−
	Grimmiales	*Grimmia*	−	+LG	+
		Ptychomitrium	−	+LG	+
		Racomitrium	−	+LG	+
		Schistidium crassipilum	−	+LG	−
		Seligeria carniolica	−	+LG	−
		Seligeria calycina	−	+LG	+
	Archidiales	*Archidium*	−	+LG	−
	Dicranales	*Fissidens*	−	+LG	+
		Ceratodon	−	+LG	+
		Dicranum	−	+LG	+
		Pseudephemerum	−	+LG	+
		Campylopus	−	+LG	−
		Dicranella heteromalla	−	+LG	−
		Dicranella varia	−	+LG	+
	Pottiales	*Didymodon*	−	+LG	+
		Ephemerum	−	+LG	+
		Tortula	−	+LG	+
		Weissia	−	+LG	+
		Cinclidotus fontinaloides	−	+LG	−
		Micromitrium	−	+LG	−
	Splachnales	*Splachnum*	−	+LG	+
Tetraplodon		−	+LG	+	

(*Continued*.)

Table 2. (*Continued.*)

class	order	genera examined	gametophyte	sporophyte	stomata
	Bryales	*Bryum*	−	+LG	+
		Mnium	−	+LG	+
		Plagiomnium	−	+LG	+
	Bartramiales	*Bartramia*	−	+LG	+
		Conostomum	−	+LG	+
		Philonotis	−	+LG	+
	Orthotrichales	*Macromitrium*	−	+LG	+
		Orthotrichum	−	+LG	+
		Ulota	−	+LG	+
	Hedwigiales	*Hedwigia*	−	+LG	+
	Hypnodendrales	*Hypnodendron*	−	+LG	+
	Hookeriales	*Calyptrochaeta*	−	+LG	+
		Hookeria	−	+LG	+
	Rhizogoniales	*Orthodontium*	−	+LG	+
	Hypnales	*Amblystegium*	−	+LG	+
		Brachythecium	−	+LG	+
		Eurhynchium	−	+LG	+
		Hypnum	−	+LG	+
Leskea		−	+LG	+	
Fontinalis		−	+LG	−	
Leucodon		−	+LG	−	
Pteridophytes					
Lycopodiopsida	Lycopodiales	*Huperzia*	+L	+G	+
		Lycopodiella	+L	+G	+
		Lycopodium	+L	+G	+
Sellaginellopsida	Selaginellales	*Selaginella*	−	+G	+
Isoetopsida	Isoetales	*Isoetes*	−	+G	+
Equisetopsida		*Equisetum*	−	+G	+
Psilotopsida	Psilotales	*Psilotum*	−	+G	+
		Tmesipteris	−	+G	+
Polypodiopsida	Marattiales	*Marattia*	−	+G	+
		Pitsana	−	+G	+
	Ophioglossales	*Botrychium*	−	+G	+
		Ophioglossum	−	+G	+
	Hymenophyllales	*Cardiomanes*	−	−	−
		Hymenophyllum	−	−	−
		Trichomanes	−	−	−
	Osmundales	*Osmunda*	−	+G	+
	Dicksoniales	*Pteridium*	−	+G	+
	Pteridales	*Anogramma*	−	+G	+
		Ceratopteris	−	+G	+
	Blechnales	*Dryopteris*	−	+G	+
	Polypodiales	*Polypodium*	−	+G	+

weeks in *Campylopus* spp., *Dicranella heteromalla* and *Schistidium crassipilum*, four to six weeks in *Atrichum undulatum* and *Pogonatum aloides*.

Pteridophytes. Our CSEM observations on a range of *Hymenophyllum*, *Trichomanes* species plus *Cardiomanes reniforme* confirm earlier anatomical studies [55–58] that the absence of

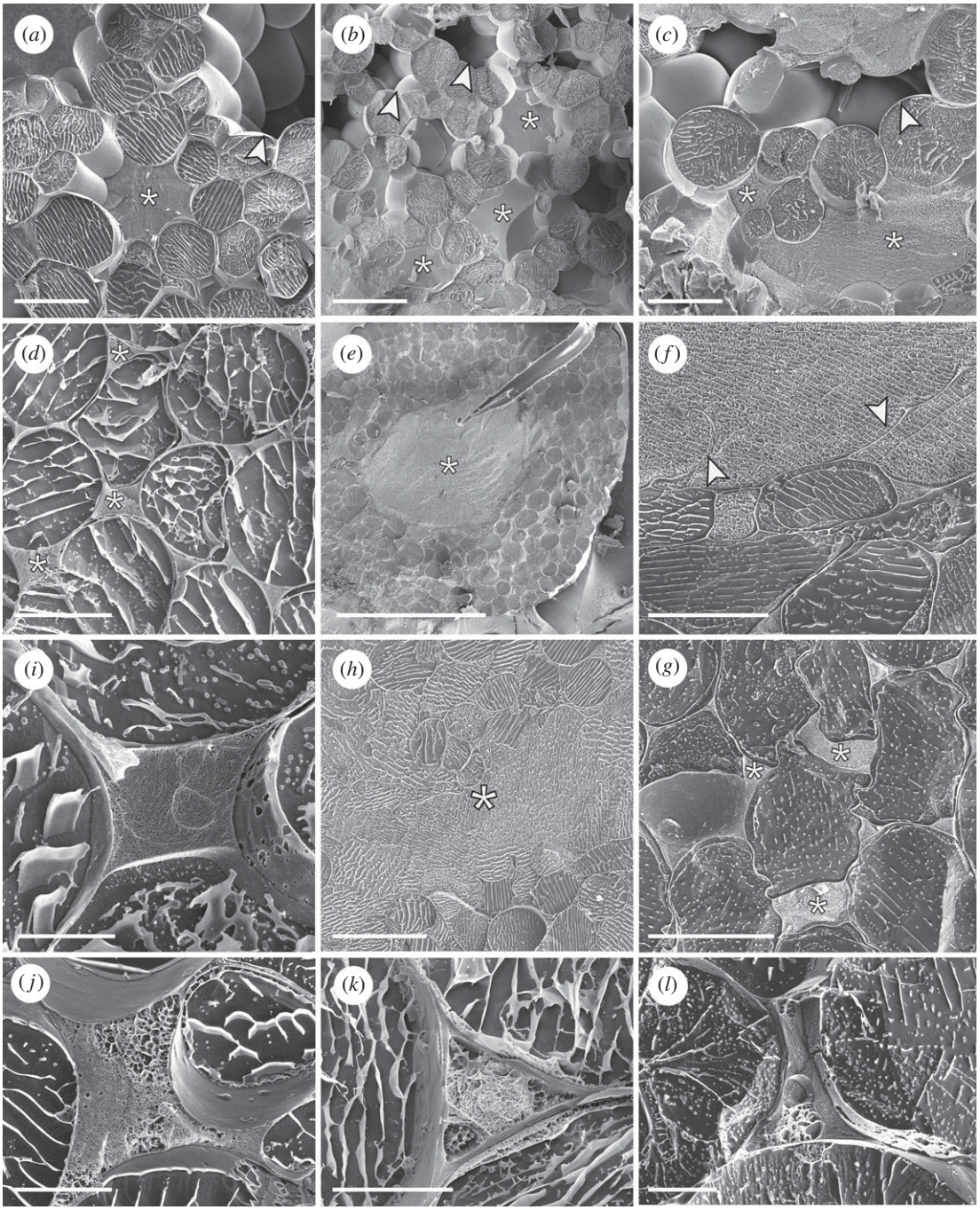

Figure 2. Cryo-scanning electron micrographs of freeze-fractured liverwort gametophytes (*a–c*) and sporophytes (*d–l*); *Neohodgsonia mirabilis* (*a*); *Athalamia hyalina* (*b*); *Riccia huebeneriana* (*c*); *Dumortiera hirsuta* (*d*); *Treubia lacunosa* (*e–g*); *Allisonia cockaynii* (*h*); *Haplomitrium gibbsiae* (*i*); *Monoclea forsteri* (*j*); *Pellia epiphylla* (*k*); *Wettsteinia schusteriana* (*l*). (*a–c*) Sections through thalli showing liquid- (asterisk) and gas-filled (arrowed) chambers. (*d–l*) Sections through carpocephalum stalk (*d*) and setae showing liquid-filled (asterisk) ICSs. The setae of some genera also contain a central fluid-filled lysigenous cavity (*e,f,h*) with remnants of broken cells around their periphery (arrowed in *f*). Scale bars: (*e*) 500 µm; (*b,h*) 100 µm; (*a,c,f,g*) 50 µm; (*d, j–l*) 20 µm; (*i*) 10 µm.

ICSs, even in the most robust genus *Cardiomanes* (figure 6*a*), sets the Hymenophyllales apart from all other pteridophytes. Under desiccating conditions the leaf lamina cells undergo cytorrhysis just like those in bryophytes [59,60,97–99].

(d) Potassium content of guard cells

Weights of potassium, determined by X-ray microanalysis, from the guard cells of open stomata and adjacent epidermal cells from wild-collected green sporophytes of seven mosses are summarized in table 3. Readings taken from the sporophyte surfaces (listed as the control for *Polytrichum juniperinum*) produced no or only a minute potassium reading. In only one species, *Philonotis fontana*, were the potassium readings the same from both the guard cells and adjacent epidermal cells. In the other six species, there was more potassium in the epidermal cells. In striking contrast, and in line with expectations, data from *Arabidopsis* show more potassium in the guard cells of open stomata than in the epidermal cells. In wilted leaves, potassium weights are the same in both kinds of cell [47].

(e) Rates of water loss from mosses

Rates of water losses are summarized in table 4. These data demonstrate major differences between the two generations in bryophytes. The 50% decrease in gametophytic fresh weights occurring between 45 min to 1 h and 3–3 h 15 min from full hydration is perhaps the clearest way to illustrate the well-documented initially exponential water loss from this generation. These water losses tend to be faster in taxa

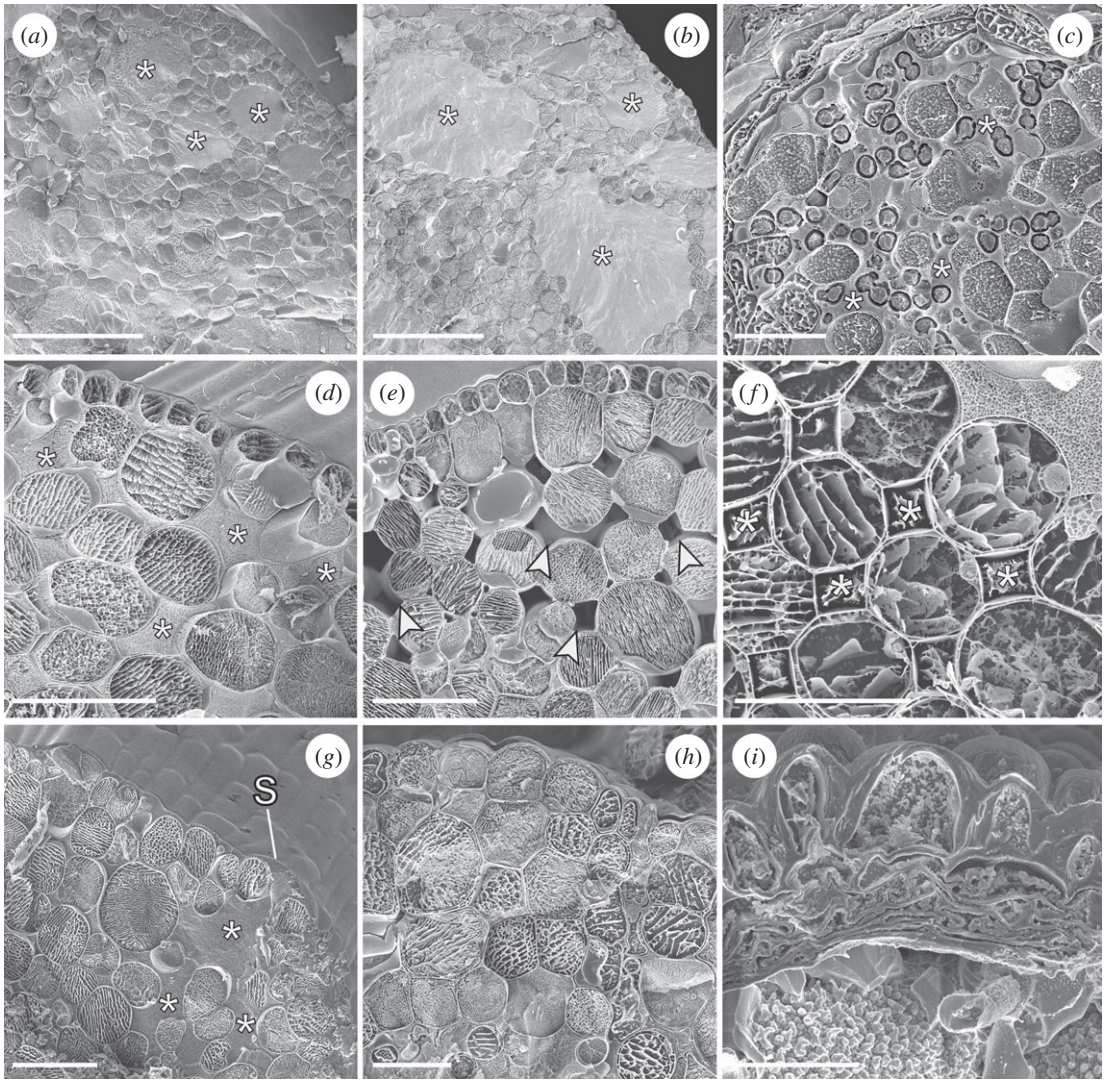

Figure 3. Cryo-scanning electron micrographs of freeze-fractured hornwort gametophytes (*a–c*) and sporophytes (*d–i*): *Anthoceros agrestis* (*a,c,d–f*); *Folioceros fusiformis* (*b*); *Leiosporoceros dussii* (*g*); *Megaceros enigmaticus* (*h*); *Dendroceros granulatus* (*i*). Sections through thalli showing mucilage-filled cavities (asterisk). (*c*) *Nostoc* colony. (*d,g*) Intercellular spaces are initially liquid-filled (asterisk) but become gas-filled (*e*, arrowed) following stomatal opening. (*f*) Columella with gas-filled (asterisk) intercellular spaces. (*h,i*) Young (*h*) and mature (*i*) sporophytes of astomate taxa, showing complete absence of intercellular spaces in the assimilatory layers which collapse and dry (*i*). Scale bars: (*a,b*) 200 µm; (*d,e,g*) 50 µm; (*c,f,h,i*) 20 µm.

with leaves with thin cell walls (e.g. *Tetraphis*, *Atrichum*, *Funaria*, *Physcomitrium*, *Bryum*, *Amblystegium*) and lower in those with thicker walls (e.g. *Polytrichum*, *Grimmia*, *Leskea*).

Moss sporophytes lose water much more slowly than the gametophytes; in fact, the rates were closely similar to those in excised evergreen xeromorphic vascular plant leaves and much slower than from mesic fern leaves; 24 h of drying resulted in a more than 50% water loss in only three taxa, *Mnium*, *Plagiomnium* and *Amblystegium*, while water loss remained less than 50% even after 72 h in five taxa. Light microscopy of specimens mounted in immersion oil [50] revealed that the stomata remained open throughout the experimental treatments.

(f) Sporophyte growth after stomatal opening

Table 5 shows the weight increases during sporophyte maturation in a range of mosses with widely different numbers of stomata and including two taxa where stomata are absent.

The stomata of most mosses develop and begin to open when the sporophytes have almost reached their full dimensions, except in *Funaria* where this takes place on much younger capsules approximately half their final diameters [30,100].

In 10 stomate genera the dry weights increase by 14% (*Brachythecium*) to 32% (*Tortula*) between the onset of stomatal opening (determined by light microscope observations on expanding capsules) and its completion, whereas the increase is over 117% for the same developmental stage in *Funaria*. Thereafter, until spore maturation, a period lasting from four to 16 weeks depending on the species, weight increases range from 25% (*Atrichum*) to 46% (*Funaria*). From stomatal initiation to spore maturation the weight increase in *Funaria* (217%) far exceeds that in all the other genera where the maximum is 81% in *Mnium hornum*. The weight increases in the astomate *Atrichum* and *Dicranella* lie within the range for the stomate genera and there is no relationship between the increases in sporophyte biomass and the number of stomata in the latter.

4. Discussion

The new data presented here on ICSs, the potassium content of moss stomatal guard cells, and the water relations and growth of moss sporophytes, add completely new dimensions to our understanding of stomatal evolution and function in

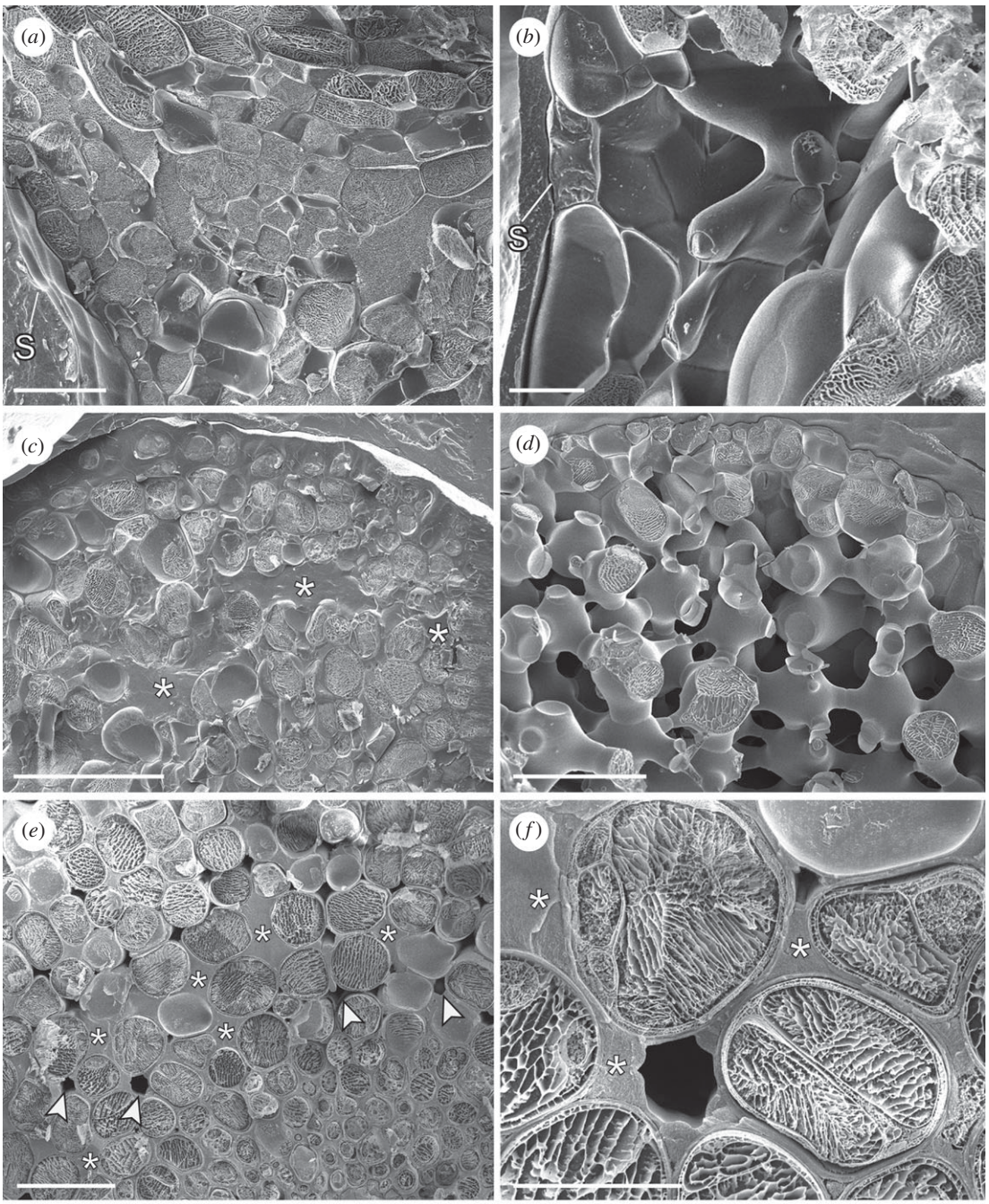

Figure 4. Cryo-scanning electron micrographs of freeze-fractured moss sporophytes: *Physcomitrella patens* (*a*,*b*); *Physcomitrium pyriforme* (*c*,*d*); *Lyellia crispa* (*e*,*f*). (*a*,*c*) Young sporophytes with liquid-filled (asterisk) intercellular spaces. (*e*) Gas (arrowed) gradually replaces their initially liquid-filled content following stomatal opening, as evidenced by the presence of intercellular spaces only partially filled with liquid (asterisk in *f*). Liquid is first lost from the substomatal cavities (*b*; S, stoma) until the entire intercellular space system becomes gas-filled (*d*). Scale bars: (*c*,*d*) 100 µm; (*a*,*e*) 50 µm; (*b*,*d*) 20 µm.

bryophytes particularly when considered within the framework of current schemes of bryophyte phylogeny (figure 7).

On the one hand, the presence of ICSs in both generations in liverworts and lycophytes is strong evidence that these were a very early acquisition by early land plants. On the other hand, their lacunae, fluid-filled at the outset throughout bryophytes, sets bryophyte ICSs apart from those in vascular plants. Gametophytic ICSs always remain liquid-filled but liquid is replaced by gas in hornwort and moss sporophytes. This major difference in ICSs ontogeny points to separate origins. Identification of possible specific ICSs genes, biochemical analyses of their liquid content and cell wall composition—to determine whether changes in wall chemistry are involved in the schizolytic processes on a par with vascular plants—are now required to test this hypothesis. Supporting the notion of independent origins is the fact that pectic strands are almost invariably visible traversing nascent ICSs in vascular plants (figure 6*b*) [43,44,101] but we rarely saw these in bryophytes. In addition, their very different distributions both between and within the three bryophyte lineages plus their varied functions all point to multiple rather than unitary origins.

In liverworts, the most prominent ICSs in both generations are at the base of the phylogeny and have three different functions. In liverwort setae ICSs are most likely a key structural component maintaining and augmenting the hydrostatic skeleton solely determined by the turgidity of its constituent thin-walled cells [102–104], whereas the most likely role for the presence of liquid-filled cavities in liverwort (and hornwort) thalli is as a buffer against rapid desiccation. It is noteworthy, however, that these occur in both highly

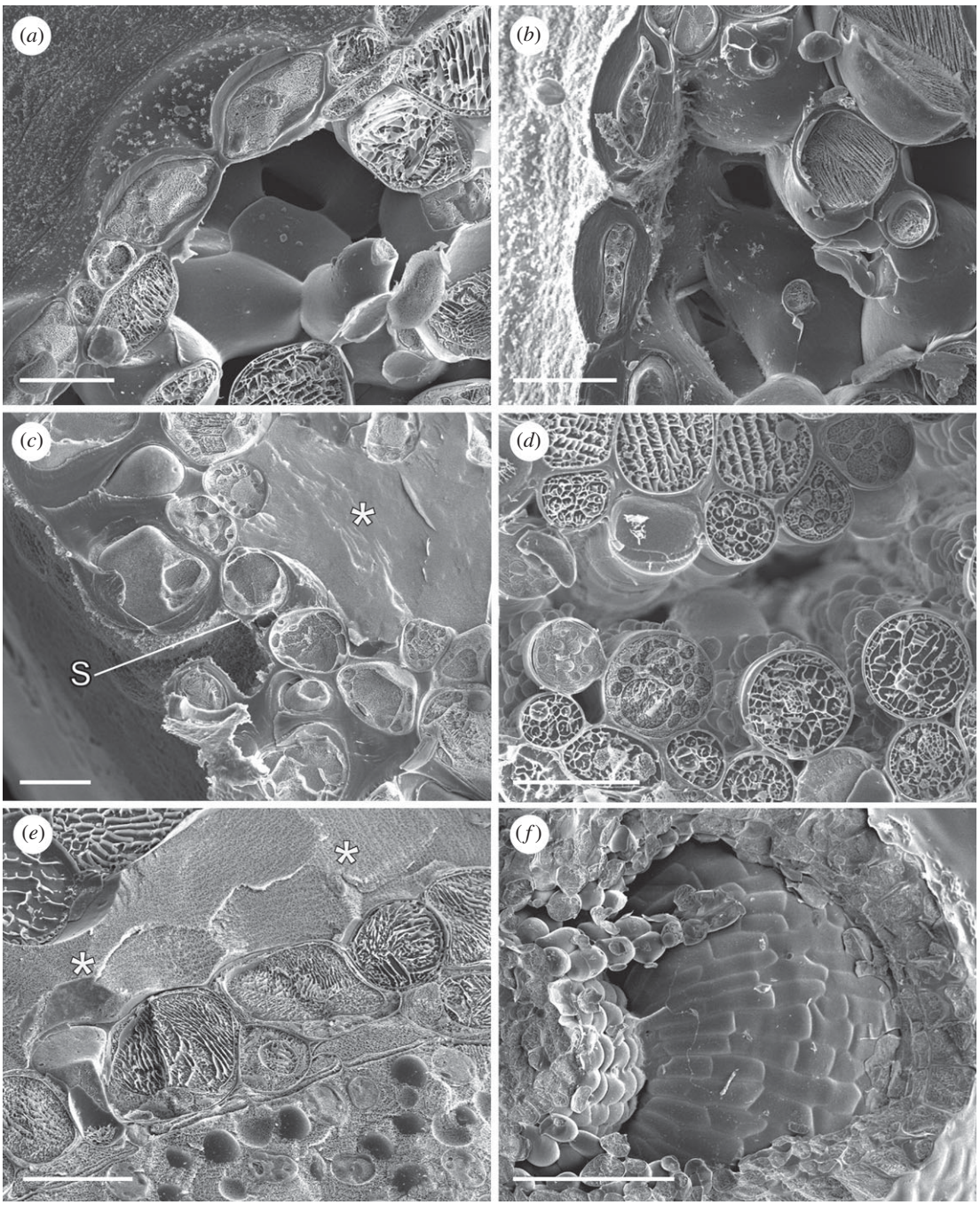

Figure 5. Cryo-scanning electron micrographs of freeze-fractured moss sporophytes: *Polytrichum juniperinum* (*a,b*); *Mnium hornum* (*c*); *Atrichum undulatum* (*d*); *Pogonatum aloides* (*e,f*). (*a,b*) Unopened (*a*) and open (*b*) stoma subtended by a gas-filled intercellular space. (*c*) Sunken stoma subtended by a liquid-filled intercellular space. (*d–f*) In astomate taxa, intercellular spaces are also initially liquid-filled (asterisk, *e*) and the same process of liquid replacement by gas occurs in their fully expanded capsules (*d,f*). Scale bars: (*f*) 200 µm; (*a–e*) 20 µm.

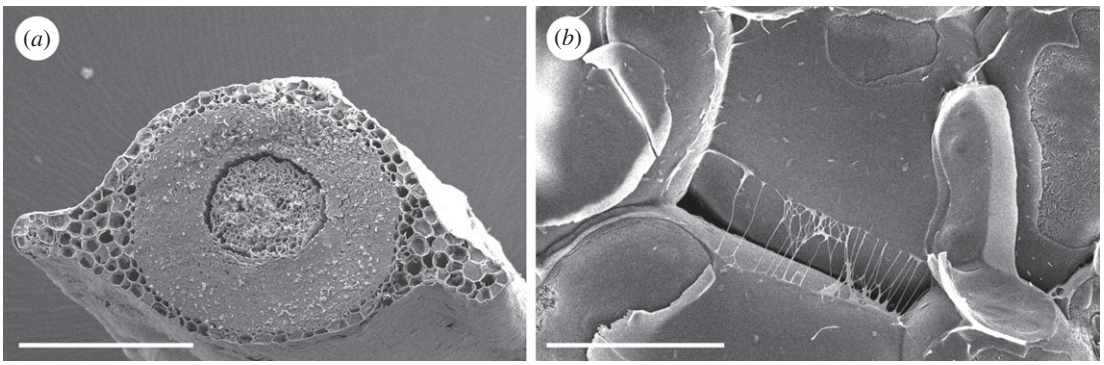

Figure 6. Cryo-scanning electron micrographs of leaves of *Cardiomanes reniforme* (*a*) and *Podocarpus nivalis* (*b*). Note the complete absence of intercellular spaces in (*a*). (*b*) Fine threads of wall material extending over a gas-filled intercellular space. Scale bars: (*a*) 500 µm; (*b*) 20 µm.

Table 3. Means from eight to 20 readings of the percentage weights of potassium from X-ray microanalysis. All the data are from fully expanded green capsules.

order	species	no. stomata	readings	potassium weights (g)
Sphagnales	*Sphagnum subnitens*[a]	c100	guard cells	1.14 ± 0.08
			epidermis	1.48 ± 0.06
			ratio of K guard cells to epidermal cells	0.77
			guard cell K relative to epidermis	lower
Polytrichales	*Polytrichum juniperinum*	80–120	guard cells	0.19 ± 0.05
			epidermis	0.39 ± 0.07
			sporophyte surface[b]	0–0.09
			ratio of K guard cells to epidermal cells	0.49
			guard cell K relative to epidermis	lower
Funariales	*Funaria hygrometrica*	160–220	guard cells	0.43 ± 0.09
			epidermis	0.56 ± 0.2
			ratio of K guard cells to epidermal cells	0.77
			guard cell K relative to epidermis	lower
	Physcomitrella patens	10–13	guard cells	1.42 ± 0.42
			epidermis	3.34 ± 0.67
			ratio of K guard cells to epidermal cells	0.43
			guard cell K relative to epidermis	lower
Pottiales	*Tortula muralis*	6–8	guard cells	0.5 ± 0.03
			epidermis	0.6 ± 0.03
			ratio of K guard cells to epidermal cells	0.83
			guard cell K relative to epidermis	lower
Bryales	*Bryum radiculosum*	80–120	guard cells	0.43 ± 0.06
			epidermis	0.94 ± 0.39
			ratio of K guard cells to epidermal cells	0.46
			guard cell K relative to epidermis	lower
	Philonotis fontana	>100	guard cells	1.7 ± 0.29
			epidermis	1.7 ± 0.09
			ratio of K guard cells to epidermal cells	1
			guard cell K relative to epidermis	same

[a]From Duckett *et al.* [47].
[b]Control.

desiccation-tolerant (*Cryptomitrium oreoides*, *Mannia californica*, *Plagiochasma rupestre*, *Athalamia hyalina*) and less desiccation-tolerant (*Neohodgsonia*, *Asterella australis*, *A. tenera*, *Peltolepis quadrata*, *Riccia huebeneriana*, *R. crystallina*, *Sauteria alpina*) complex thalloid liverwort taxa. In this context, it is most striking that *Dendroceros*, the only desiccation-tolerant hornwort genus, has many species with fenestrated thalli rather than mucilage cavities. This feature, unique to *Dendroceros*, is most likely related to rapid de- and rehydration. The third function of liquid-filled chambers is forming the domatia for symbiotic fungi [105] and *Nostoc* [86].

Perhaps the most striking feature of ICSs in bryophytes is their absence in moss gametophytes (figure 7*b*). One possible, but perhaps only partial explanation may lie in the fact that the majority of moss stems have peripheral cell layers with thickened walls whereas most liverwort stems and thalli do not. This feature is particularly characteristic in the early moss lineages—Takakiopsida, Andreaeopsida, Polytrichopsida, Tetraphidopsida—whereas in liverworts it is prominent only in a few later divergent leafy clades, e.g. Pleuroziales, some Porellales, Ptilidiales, Mastigophoraceae, Herbertaceae, Gymnomitriaceae, and is rare across thalloid taxa. Curiously, none of the aforementioned liverworts have fungal endophytes [106]. It may well be that external cells with thickened walls, which appear intrinsic to the basic construction of moss stems, were a major factor alongside the evolution of multicellular rhizoids that by-passed the establishment of fungal symbioses in the group [105]. Placing this in the Rhynie chert context, many of the fossils here resemble mosses in having peripheral rather than central vascular supporting tissues in their axes.

Whatever future molecular and biochemical analyses might reveal, the data in tables 1 and 2 highlight that the nascence of ICSs in bryophyte and pteridophyte gametophytes had nothing to do with gaseous regulation or with stomata in liverwort sporophytes. The associations between ICSs and

Table 4. Summary of weight losses from the gametophytes and sporophytes with green fully expanded capsules of a range of mosses, a hornwort and vascular plant leaves.

order	species	no. stomata	sporophyte weights 6 h drying % FW	sporophyte weights 24 h drying % FW	sporophyte weights 72 h drying % FW	gametophytes time (h) to 50% water loss
Mosses						
Tetraphidales	*Tetraphis pellucida*	0	80	65	44	1.15
Polytrichales	*Atrichum undulatum*	0	75	64	46	1.30
	Polytrichum formosum	180–200	82	67	41	1.30
	P. juniperinum	80–120	90	78	61	2.30
Funariales	*Funaria hygrometrica*	160–220	73	56	27	1.30
Grimmiales	*Grimmia pulvinata*	6–10	86	78	67	1.45
	Schistidium crassipilum	0	84	79	73	1.30
Dicranales	*Dicranella heteromalla*	0	67	61	60	2.0
	Ceratodon purpureus	8–10	73	58	43	1.45
Pottiales	*Tortula muralis*	6–8	82	74	67	1.45
Bryales	*Bryum capillare*	70–120	89	82	55	1.0
	Mnium hornum	20–40	81	48	30	2.15
	Plagiomnium cuspidatum	40–60	73	42	33	1.30
Hypnales	*Amblystegium serpens*	28–52	62	42	38	1.0
	Brachythecium rutabulum	20–30	62	55	48	1.0
	Hynum cupressiforme	6–8	72	68	54	2.0
Hornworts						
Anthocerotales	*Anthoceros punctatus*	n.a.	79	62	42	1.45
Vascular plants						
Polypodiales	*Scolopendrium*	n.a.	63	23	18	n.a.
Osmundales	*Osmunda*	n.a.	67	41	27	n.a.
Podocarpales	*Podocarpus*	n.a.	91	86	76	n.a.
Pinales	*Taxus*	n.a.	92	81	71	n.a.
Apiales	*Hedera*	n.a.	90	84	74	n.a.

stomata would appear to be a much later acquisition or acquisitions. Gas replacement of fluid-filled ICSs is absent in liverworts but is ubiquitous in moss sporophytes whether or not these have stomata and is the rule in stomate hornworts.

Whereas sporophytic ICSs are present at the base of the liverwort and hornwort lineages (figure 7), their appearance much later in the moss tree is perhaps the clearest signal of independent evolution. In the three moss lineages lacking ICSs (Sphagnopsida, Takakiopsida, Andreaeopsida), schizolytic phenomena appear to be very rare. The gas-filled cavities in their maturing sporophytes are all lysigenic and the splits in the capsule walls at dehiscence in Takakiopsida and Andreaeopsida (and in liverworts and hornworts) are the result of cell breakdown. However, lid shedding in *Sphagnum* is schizogenic [107] as is also the case in *Plagiomnium* [108,109] and *Bryum* [50], but in *Funaria* [110] and Polytrichopsida this involves cell breakdown.

The late acquisition of ICSs in moss sporophytes coincides with the appearance of stomata and, even following their loss in all the taxa we have examined where stomatal absence is almost certainly a secondary loss (*Atrichum, Pogonatum, Tetraphis, Scouleria, Schistidium crassipilum, Seligeria carniolica, Archidium, Campylopus, Dicranella heteromalla, Cinclidotus fontinaloides, Micromitrium, Fontinalis, Leucodon*), ICSs are retained. Even more remarkably, they become gas-filled as the capsules mature, whereas stomatal losses in two separate hornwort lineages, *Phaeoceros/Notothyas* and *Dendroceros/Megaceros/Nothoceros/Phaeomegaceros* [88,111], also see the loss of ICSs. It is most curious, therefore, that only in hornwort sporophytes is there complete congruence between the presence of sporophytic ICSs and stomata. A possible explanation for this major difference between hornworts and mosses is that double losses in hornworts have much more ancient origins than multiple losses in mosses, which are almost all in derived clades in their respective families [112].

Overall therefore in bryophytes there is now substantial evidence for the multiple evolution of ICSs and secondary losses only in estomate hornworts. This finding also points, strongly, to the multiple rather than the unitary origin of stomata. Whatever the ultimate configuration of the bryophyte part of the tree of life, adherence to the notion of unitary origin requires secondary losses and reacquisitions. We now highlight the issues. The placement of hornworts at the base of the land plant tree [113] presents what would appear to be insurmountable problems for unitary origin as this requires stomatal loss in liverworts and reacquisition in mosses. The

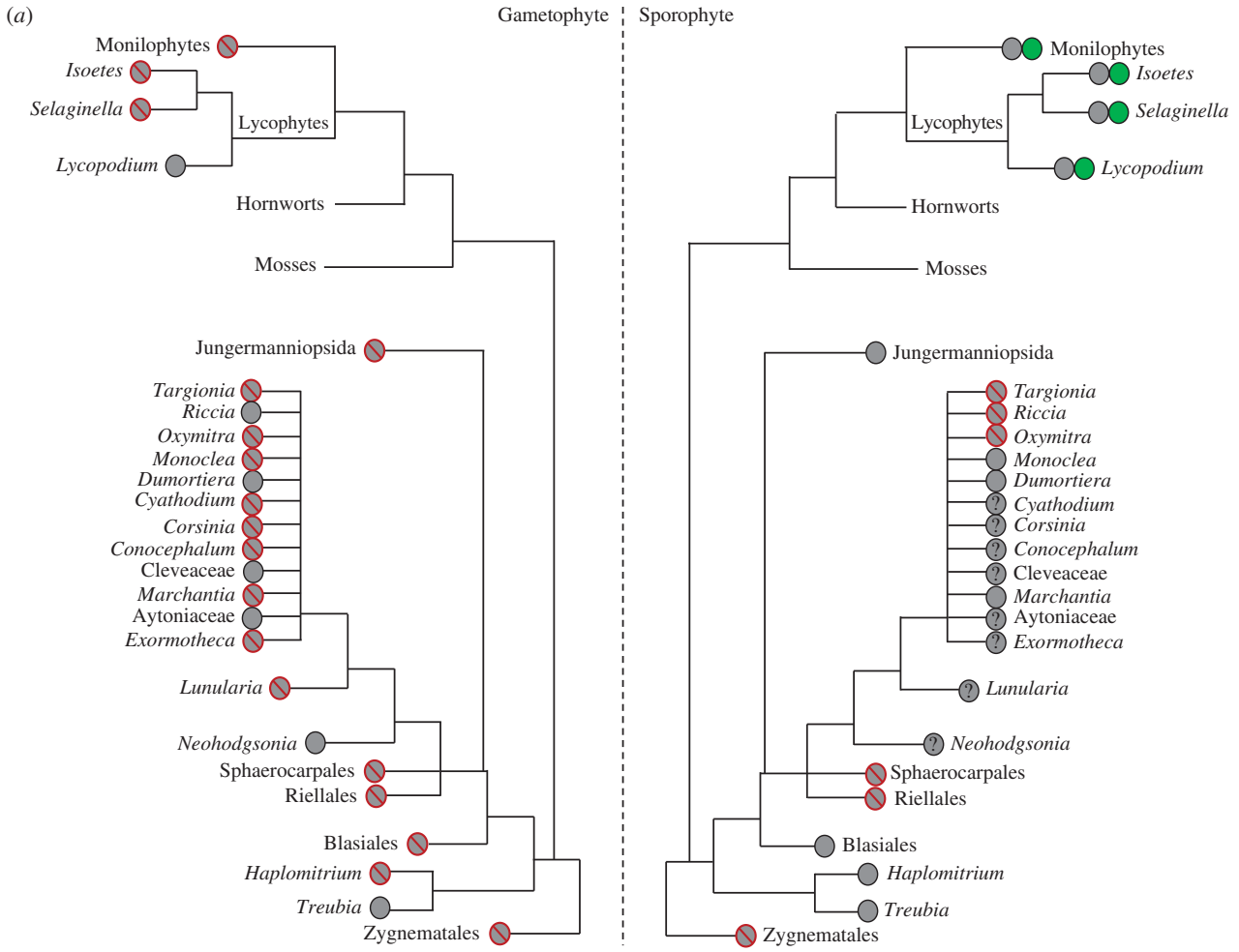

Figure 7. Simplified phylograms illustrating the distribution of intercellular spaces and stomata in the major lineages at the base of the land plant tree of life: (*a*) liverworts (gametophyte and sporophyte generations); (*b*) mosses (sporophyte only); (*c*) hornworts (gametophyte and sporophyte generations). This underlines the likely multiple evolution of both features. Stomata present, Stomata absent, Stomata +/−, ICSs present, ICSs absent, ICSs presence/absence not known.

Table 5. Changes in sporophyte dry weights from stomatal opening until spore maturation. Data from 10 (*Atrichum, Brachythecium, Mnium, Polytrichum*), 25 (*Amblystegium*, *Bryum, Funaria, Dicranella,Rhynchostegium*) or 50 (*Ceratodon, Grimmia, Tortula*) sporophytes. Approx. duration of stage in weeks in brackets.

order	species	no. stomata	% weight increase during opening	% increase after open for two weeks/ full size	% increase from open stomata to spores mature	% increase from stomata initiated to spores mature
content of intercellular spaces			*liquid*	*gas*	*gas*	*liquid to gas*
Polytrichales	*Atrichum undulatum*	0	n.a.	17	25 (4–8)	n.a.
	Polytrichum juniperinum	80–120	19 (4–6)	12	39 (8–16)	65
Funariales	*Funaria hygrometrica*	160–180–220	117 (2–4)	28	46 (4–6)	217
Grimmiales	*Grimmia pulvinata*	6–10	22 (4–6)	25	27 (6–12)	55
Dicranales	*Dicranella heteromalla*	0	n.a.	23	33 (4–8)	n.a.
	Ceratodon purpureus	8–10	29 (4–6)	19	39 (4–8)	79
Pottiales	*Tortula muralis*	6–8	32 (4–6)	17	34 (4–8)	77
Bryales	*Bryum capillare*	70–90–120	23 (2–4)	28	34 (6–16)	65
	Mnium hornum	20–30–40	31 (2–4)	33	38 (4–8)	81
Hypnales	*Rhynchostegium confertum*	10–15	26 (2–4)	16	29 (4–6)	54
	Amblystegium serpens	28–44–52	24 (2–4)	16	28 (4–8)	59
	Brachythecium rutabulum	20–30	14 (2–4)	21	32 (4–8)	51

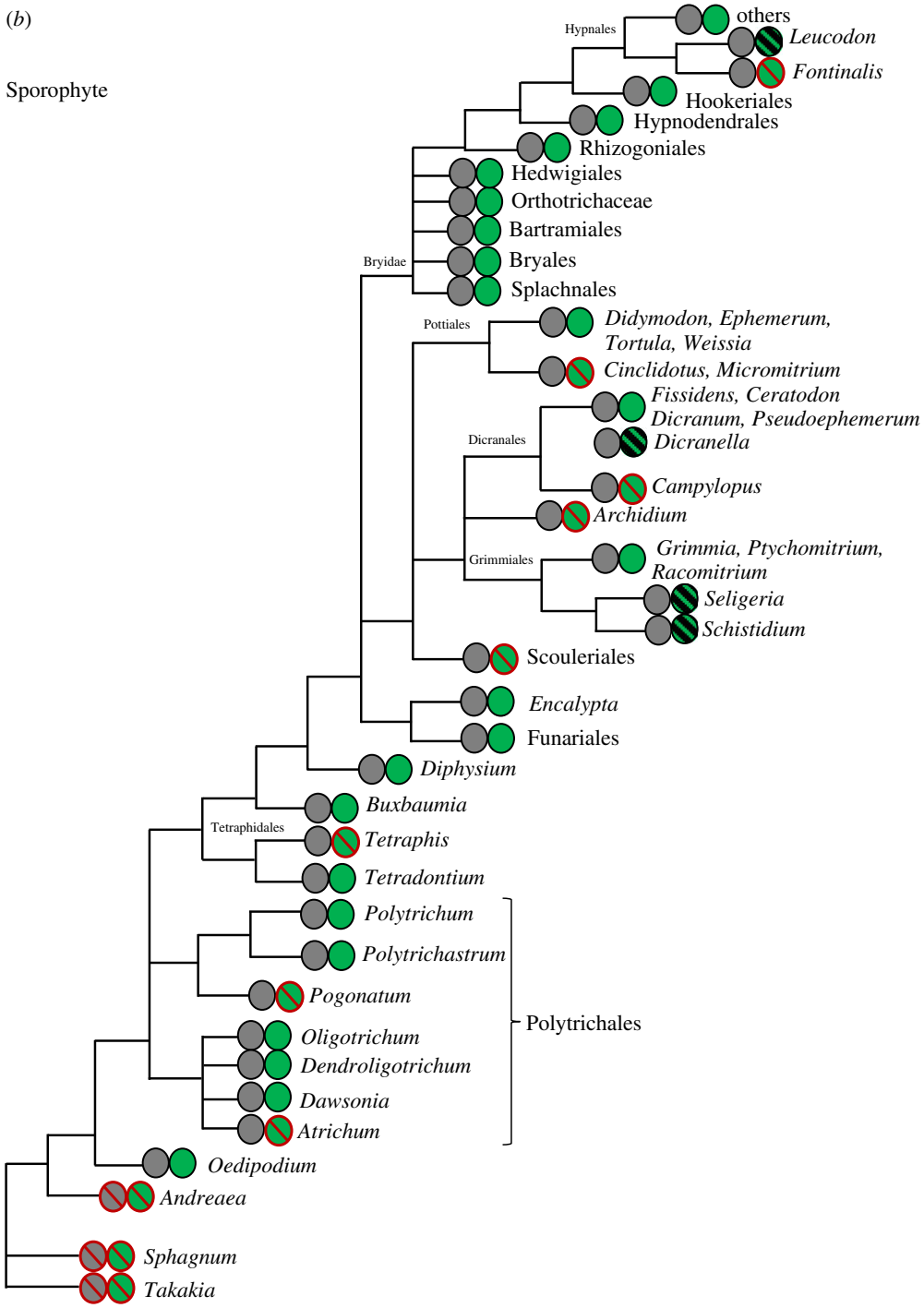

Figure 7. (*Continued.*)

strongest case for a single origin in a common ancestor of mosses, hornworts and tracheophytes—whether liverworts are sister to all other land plants [114–116] or liverworts, mosses and hornworts are successive sister groups to vascular plants [115,117–119]—now rests with multiple common genes [12] and the single demonstration of active control from measurements of small aperture changes in *Funaria* and *Physcomitrella* [13]. We are wary of an earlier demonstration of a linear stomatal closure response to increasing ABA concentrations in the hornwort *Anthoceros* [25] as we have been unable to replicate these findings in the same taxon and a range of other stomate hornwort genera (JG Duckett, KS Renzaglia, S Pressel 2017, unpublished data) and given more recent works revealing fixed apertures from very early in stomatal ontogeny in hornworts [21,51]. None of the other papers on possible stomata-related genes in *Physcomitrella* [2,4–6] present any data on whether these directly affect apertures in this moss. All other evidence is strongly stacked against early acquisition of active control and apertures that can actually change reversibly [7,12], including the present demonstration of absence of potassium pumps and our data on water relations. Our results showing lower potassium content in the guard cells than adjacent epidermal cells in moss sporophytes make any involvement of potassium ions either in the opening or maintenance of the open status of moss stomata extremely unlikely. The most probable mechanism for initial stomatal opening is an increase in guard cell osmoticum from mobilization of starch reserves [51]. Turning to rates of water loss, it is most striking that there appears to be no discernible relationship between these and stomatal numbers, indeed we measured a faster rate of water loss in astomate *Atrichum* than in *Polytrichum formosum* and *P. juniperinum*, both of

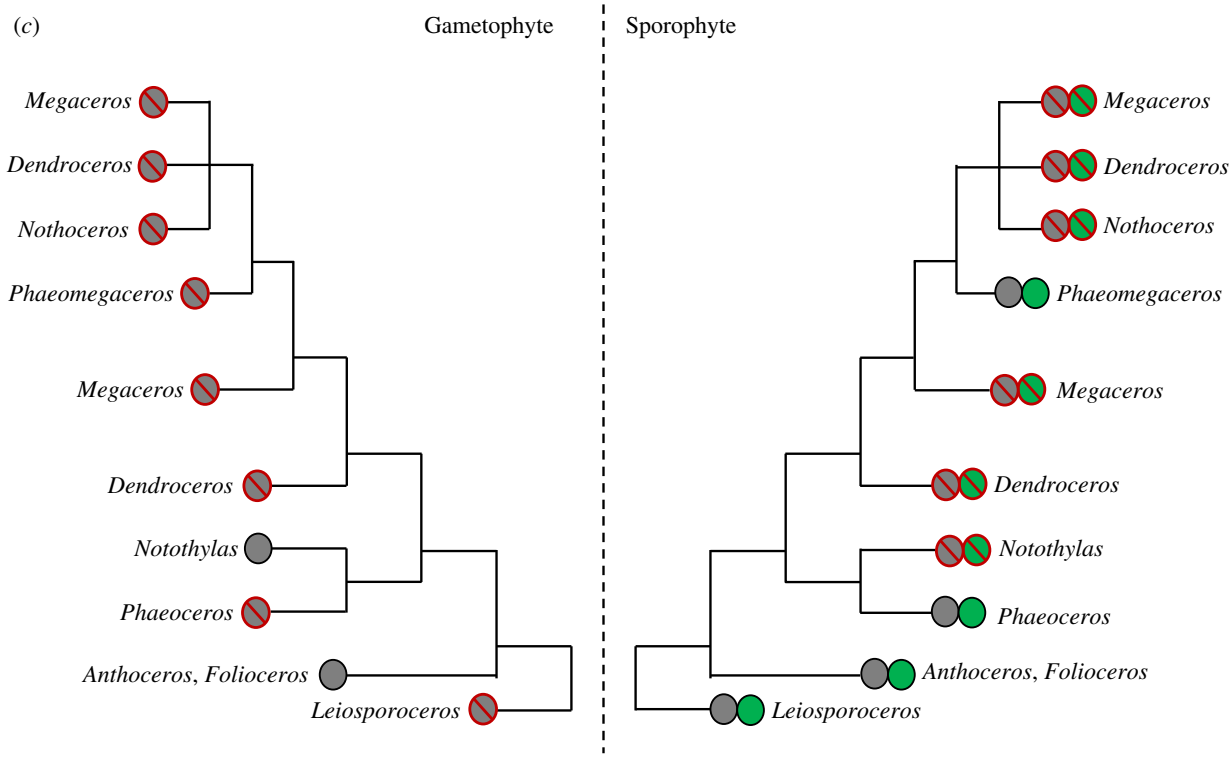

Figure 7. (*Continued.*)

which have numerous stomata and very similar ecologies (table 4). Further, in our experiments, stomata remained open throughout the 24-h drying period, just as they do in nature after periods of desiccation [50]. In addition, our data showing no relationship between increases in sporophyte biomass and number of stomata, and similar weight increases in estomate and stomate taxa, suggest that CO_2 entry through the stomata, possible only after fluid in the ICSs has been replaced by gas, makes at best only a minor contribution to sporophyte nutrition. This finding is very much in line with previous physiological studies showing that the bulk of sporophyte biomass derives from the gametophytes [74,75]. The early opening of the stomata in *Funaria* relative to capsule expansion, a phenomenon we have also noted in other funarialian genera (*Entosthodon, Physcomitrium*), suggests that the single guard cell characteristic of this order of mosses is a consequence of sporophytic neoteny, a feature which appears to be unique to this order of mosses.

These latest results, coupled with the growing suite of developmental structural data on guard cell walls indicating that these become immobile [21,48,49] and the fact that astomatal mutant sporophytes in *Physcomitrella* are the same size as wild-type plants [6], all argue against the notion of early acquisition of active stomatal control. One further factor that has received but scant attention in relation to stomatal function in both hornworts and mosses is pore obstruction by wax-like materials [26,49,76]. Not only can obstructed pores be seen near open pores in hornworts and in mature moss capsules, but Merced & Renzaglia [76] have now illustrated their occurrence in a capsule of *Physcomitrella* with nearly mature spores but still with liquid-filled ICSs.

None of the rules about stomatal numbers and spacing in tracheophytes [49,76,120–122] can be applied to mosses and, simply in terms of mechanical constraints [8], it is difficult to envisage how the widths and lengths of the apertures in the single guard cells in the Funariales might be able to change under different conditions. The present finding from sporophyte weights that funarialean stomata open much earlier in sporophyte development than in other mosses provides an explanation for the single guard cell.

With hornworts sister to tracheophytes [115,117–119] there would now appear to be a stronger case that stomata evolved twice, once in an ancestor of polytrichopsid and bryopsid mosses and once in an ancestor of hornworts and tracheophytes ([47,123]; figure 2*b*). However, problems with this dual origin scenario are the major functional and structural differences between hornwort and tracheophyte stomata [21,51,88]. Perhaps most striking is the early death and collapse of hornwort guard cells prior to spore maturation as highlighted by Merced & Renzaglia [76]. The homology between hornworts and tracheophyte stomata, and hence the possibility of a third origin, remains an open question.

Mirroring the debunking of a unitary origin for water-conducting cells in bryophytes [32], the recent statement by Sussmilch *et al.* [12, p. 249] 'Given the clear evolutionary advantages of stomatal pores for plant success, it is conceivable that these structures may be the result of convergent evolution due to strong developmental bias' receives considerable support from the present study.

Aside from these evolutionary and functional considerations on ICSs and stomata, this study provides the first unequivocal evidence confirming field observations that moss and hornwort sporophytes have strongly homiohydric attributes. Underlining the close association between the presence of stomata, ICSs and homiohydry is their absence in Hymenophyllaceae. However, whether it is strict homiohydry, and with this the implication of stomatal regulation of water content, or simply a case of very low rates of transpiration as evidenced by the data on rates of water loss (table 4) which appear unaffected by the numbers and presence or absence of stomata, remains an open question. For the future, this invites physiological studies on how far

moss sporophytes might maintain active assimilation under desiccating conditions and how far the presence or absence and numbers of stomata might affect this. A further unknown dimension is replenishment of capsule water via the setal hydroids which collapse under desiccating conditions [32]. It would also be pertinent to compare the desiccation biology of moss sporophytes with that of 'resurrection' *Selaginella* species and cheilantoid ferns [124], both of which do have ICSs and stomata.

Overall, our results provide further insights into possible roles of stomata in Rhynie chert plants. Based on striking morphological and architectural similarities with extant counterparts, especially those of mosses and some ferns [125], Devonian stomata are generally assumed to have functioned, both physiologically and mechanically, like those of modern land plants in gaseous exchange—in line with the paradigm that stomata evolved once in the common ancestor of land plants and that their role and regulation are conserved across all lineages [16]. However, whether ancient stomata had a key role in CO_2 acquisition and photosynthesis or functioned mainly in the supply of water and mineral nutrients to targeted plant tissues, remains controversial [125]. The intercellular space system subtending ancient stomata was generally smaller than that in modern angiosperms, possibly resulting in smaller stomatal conductance and thus conservation of water [125], while high photosynthetic rates, even in the absence of stomata, have been implied for Devonian fossils [125–127]. Our demonstration of key differences in the ontogeny of ICSs [51], water relations and guard cell physiology between the early divergent bryophytes and angiosperms, together with previous evidence of dramatic developmental changes in the bryophyte guard cell walls [49,51,76] that would render these immovable, all point to multiple origins of the stomatal apparatus. We do not know whether Devonian ICSs had an initial liquid-filled developmental stage, like those of bryophytes. An intriguing, alternative scenario to those proposed thus far [125] is that the ancient stomatal apparatus shared a similar development with that of extant bryophytes, culminating in a single, maturational, opening event with stomata remaining open thereafter. Possible functions then would include sporangium dehydration leading to dehiscence and spore discharge and/or maintenance of a transpiration flow for nutrient supply [76].

Data accessibility. This article has no data.

Authors' contributions. J.G.D. and S.P. conceived of and designed the research, and conducted the ultrastructural analyses and X-ray microanalyses. J.G.D. conducted all the weight measurements. J.G.D. led the writing, S.P. contributed to the writing, edited the manuscript and prepared all the figures.

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