

## Viewpoint

# A stomate by any other name? The open question of hornwort gametophytic pores, their homology, and implications for the evolution of stomates

### Summary

Advances in bryophyte genomics and the phylogenetic recovery of hornworts, mosses, and liverworts as a clade have spurred considerable recent interest in character evolution among early embryophytes. Discussion of stomatal evolution, however, has been incomplete; the result of the neglect of certain potential stomate homologues, namely the two-celled epidermal gametophytic pores of hornworts (typically referred to as ‘mucilage clefts’). Confusion over the potential homology of these structures is the consequence of a relatively recent consensus that hornwort gametophytic pores (‘HGP’s – our term) are not homologous to stomates. We explore the occurrence and diverse functions of stomates throughout the evolutionary history and diversity of extinct and extant embryophytes. We then address arguments for and against homology between known sporophyte- and gametophyte-borne stomates and HGPs and conclude that there is little to no evidence that contradicts the hypothesis of homology. We propose that ‘intergenerational heterotopy’ might well account for the novel expression of stomates in gametophytes of hornworts, if stomates first evolved in the sporophyte generation of embryophytes. We then explore phylogenetically based hypotheses for the evolution of stomates in both the gametophyte and sporophyte generations of early lineages of embryophytes.

### Background – a hole in our discourse

Most plant biologists know stomates as two-celled epidermal pores that actively regulate water loss and facilitate gas exchange for photosynthesis in the sporophytes of vascular plants. Not all stomates and stomate homologues perform these functions, however – for example, the two-celled pores of angiosperm hydathodes (Belin-Depoux, 1989) and of some nectaries (e.g. Fahn, 1952) – which prompts the question of how we should define a ‘stomate’. Progress in bryophyte genomics now allows comparative studies in bryophytes of the genes that are associated with

stomatal development in model angiosperms, while the recent (and unanticipated) recovery of monophyletic bryophytes (de Sousa *et al.*, 2018; Puttick *et al.*, 2018; Leebens-Mack *et al.*, 2019; Sousa *et al.*, 2020; Su *et al.*, 2021) has strongly renewed interest in character evolution, including stomate evolution, among ancient lineages of land plants (e.g. Chater *et al.*, 2017; Kenrick, 2017; Merced & Renzaglia, 2017; Puttick *et al.*, 2018; Harris *et al.*, 2020; Li *et al.*, 2020; Donoghue *et al.*, 2021; Chang *et al.*, 2023). This recent spate of papers exploring stomatal evolution prompts us to clarify what ‘stomate’ means.

We see a terminological and conceptual issue in the fields of bryology and early land plant evolution with important implications for the study of early embryophyte character evolution. The problem we identify is an apparent consensus on what stomates are *or are not* in hornworts. This consensus denies the field an opportunity to carefully investigate the evolutionary origin of stomates and their subsequent diversification, yet it seems to lack solid evidence. Based partly on a notion that, if two structures are homologous, they ought to have similar functions (e.g. Duckett & Ligrone, 2003; Szövényi, 2016), many researchers have rejected the possibility that the two-celled pores, often referred to as ‘mucilage clefts’, on the gametophytes of hornworts might properly be considered homologous to the stomates of all stomate-bearing land plants (Villarreal & Renzaglia, 2006; Renzaglia *et al.*, 2008), although interestingly, Villarreal & Renzaglia (2015) seem open to this potential homology (to be described later). More often and perhaps more problematically, authors simply do not discuss this potential homology (Table 1).

While it is unquestionably true that most stomates are involved in the diurnal regulation of carbon-dioxide uptake and water-vapor loss (Clark *et al.*, 2022), it is also unquestionably true that many groups of plants bear stomates that do not function in the exchange of gases and that stomates have been evolutionarily and developmentally recruited to play a variety of functions. Many plants release water through hydathodes, which in angiosperms consist of permanently open stomates (Belin-Depoux, 1989; Mehlreter *et al.*, 2022), and many nectaries (both floral and extrafloral) exude nectar via modified stomates (Prantl, 1872; Von Goebel, 1915–1918; Fahn, 1952; Kirchoff & Kennedy, 1985; Davis *et al.*, 1986; Belin-Depoux, 1989; Davis, 1997; Moran, 2022). The leaves of many plants can even absorb water through hydathodes or through more-typical ‘gas-exchange’ stomates (Martin & von Willert, 2000; Schreel & Steppe, 2020; Fradera-Soler *et al.*, 2024). Aguirre *et al.* (2023) recently provided evidence that stomates are also involved in the detection of volatile compounds released by plants during herbivory.

Among bryophytes, most hornwort and moss sporophytes bear stomates that appear capable of opening once but not of closing except by occlusion with wax (Merced & Renzaglia, 2017; Pressel *et al.*, 2018; McAdam *et al.*, 2021), although in a few taxa stomates

**Table 1** Recent papers (from the last 20 years) on stomate evolution, bryophyte stomates, and/or hornworts that do not mention hornwort gametophytic pores (HGPs).

Renzaglia <i>et al.</i> (2007)	Bryophyte phylogeny: advancing the molecular and morphological frontiers
Rudall <i>et al.</i> (2013)	Several developmental and morphogenetic factors govern the evolution of stomatal patterning in land plants
Pressel <i>et al.</i> (2014)	Stomatal differentiation and abnormal stomata in hornworts
Szövényi (2016)	The genome of the model species <i>Anthoceros agrestis</i>
Kenrick (2017)	Changing expressions: a hypothesis for the origin of the vascular plant life cycle
Merced & Renzaglia (2017)	Structure, function and evolution of stomata from a bryological perspective
Renzaglia <i>et al.</i> (2017)	Hornwort stomata: architecture and fate shared with 400-million-year-old fossil plants without leaves
Pressel <i>et al.</i> (2018)	Hornwort stomata do not respond actively to exogenous and environmental cues
Harris <i>et al.</i> (2020)	Phylogenomic evidence for the monophyly of bryophytes and the reductive evolution of stomata
Li <i>et al.</i> (2020)	<i>Anthoceros</i> genomes illuminate the origin of land plants and the unique biology of hornworts
Donoghue <i>et al.</i> (2021)	The evolutionary emergence of land plants
McAdam <i>et al.</i> (2021)	Stomata: the holey grail of plant evolution
Clark <i>et al.</i> (2022)	The origin and evolution of stomata
Chang <i>et al.</i> (2023)	Liverwort bHLH transcription factors and the origin of stomata in plants
Renner & Sokoloff (2024)	The sexual lability hypothesis for the origin of the land plant generation cycle

have been reported to be at least briefly capable of reversible aperture adjustments (Garner & Paolillo Jr., 1973; Hartung *et al.*, 1987; Chater *et al.*, 2011; Merced & Renzaglia, 2014). Moss and hornwort stomates on sporophytes have thus been viewed as functioning primarily to help dry the sporangium and so promote the dispersal of spores (Pressel *et al.*, 2014, 2018; Merced & Renzaglia, 2017; Clark *et al.*, 2022). This stomatal function has an interesting parallel in that of permanently open stomates on the anthers of many flowering plants, which can facilitate anther dehiscence and pollen dispersal (Keijzer *et al.*, 1987). It has also been suggested that, at least in mosses, the transpiration stream that bryophyte stomates create draws water and dissolved mineral nutrients up from the gametophyte through the dependent sporophyte (Edwards *et al.*, 1998; Ligrone *et al.*, 2012; Haig, 2013).

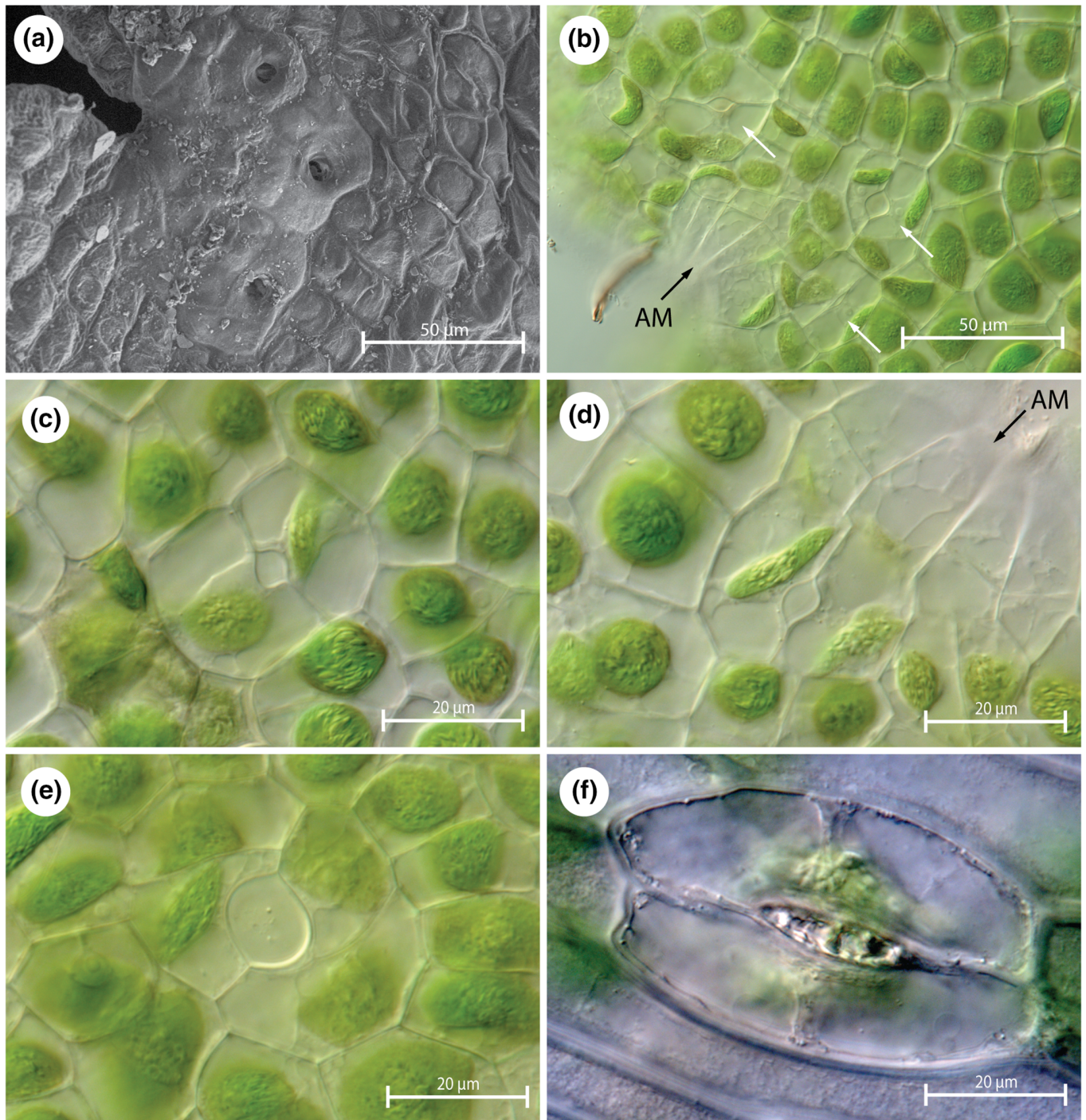
All these diversely functioning stomates occur in the sporophyte generation of extant land plants, yet the fossil record reveals even greater diversity. Stomates have been found in a fossilized Lower Devonian polysporangiophyte sporophyte (*Aglaophyton*) in association with subdermal cavities containing nitrogen-fixing cyanobacteria (Krings *et al.*, 2008). But have stomates occurred outside the sporophyte generation? If so, what have been their functions? All Lower Devonian Rhynie chert gametophytes so far discovered – *Lyonophyton* (the gametophyte of *Aglaophyton*), *Langiophyton* (the gametophyte of *Horneophyton*), *Kidstonophyton* (the gametophyte of *Nothia*), and *Remyophyton* (the gametophyte of *Rhynia*) – bore stomates as did their sporophytes (Remy

*et al.*, 1993; Kerp *et al.*, 2004). The roughly isomorphic (co-dominant) generations of these Devonian polysporangiophytes were likely the result of bulk expansions in gene expression from gametophyte to sporophyte and vice versa (Jones & Dolan, 2012; Kenrick, 2017). According to this hypothesis, gametophytes came to express stomates, which had probably been a sporophytic innovation (Ligrone *et al.*, 2012; Kenrick, 2017), although it is possible that these pores emerged in gametophytes first (Villarreal & Renzaglia, 2015; Chater *et al.*, 2017). Stomatal functions in the Rhynie Chert gametophytes remain enigmatic but could have ranged from gas exchange and maintaining a transpiration stream, to guttation, or to admitting symbionts (Edwards *et al.*, 1998; Adams & Duggan, 2008; Krings *et al.*, 2008; Villarreal & Renzaglia, 2015). Considering the breadth of functions stomates have assumed over the evolutionary history of plants in both sporophytes and gametophytes, why do so few researchers seem interested in the two-celled mucilage clefts of hornwort gametophytes as potential homologues of stomates, while some authors invoke functional differences to dismiss such a homology?

Hornwort gametophytic pores, ‘HGPs’ – our term in the interest of remaining open-minded (Fig. 1) are widespread among hornworts (Anthocerotophyta), with pores on the ventral (and in some species, also on the dorsal) epidermal surface of the thalli (Von Goebel, 1915–1918; Renzaglia, 1978; Schuster, 1992; Renzaglia *et al.*, 2000). Hornwort gametophytic pores appear to form from a longitudinal division of a single cell near an apical meristematic notch (Campbell, 1918; Schuster, 1992), and the opening of a pore between the two cells allows *Nostoc* cyanobacterial hormogonia to enter cavities in the gametophyte, where they symbiotically fix nitrogen while obtaining shelter and carbohydrates (Campbell, 1918; Schuster, 1992; Adams & Duggan, 2008; Renzaglia *et al.*, 2008; Chatterjee *et al.*, 2022). These cavities may be air-filled at first but are commonly observed full of mucilage (Renzaglia, 1978; Schuster, 1992; Renzaglia *et al.*, 2000, 2008; Villarreal & Renzaglia, 2006; Adams & Duggan, 2008). It is worth noting that the cavities underlying stomates in the hornwort sporophyte are also liquid-filled before the capsule dehydrates (Pressel *et al.*, 2014; Duckett & Pressel, 2017). Following colonization of the HGP cavity (and probably even if a cavity is not colonized), the pore closes, after (and often before) which further divisions of the original pore cells (Fig. 1e; Schuster, 1992: figs 1051–4, 1052–5) obscure the previous existence of a two-celled pore (Prantl, 1872; Von Goebel, 1915–1918; Schuster, 1992).

### By any other name: Defining a stomate

Recognizing the functional diversity among sporophytes of structures that are called stomates, we propose a minimal ontogenetic and anatomical definition of a ‘stomate’: a pair of epidermal cells derived from a single parent cell, capable of separating to create a pore that may or may not be able to close. The pore usually opens onto an intercellular space (a substomatal cavity or chamber) the contents of which may be liquid or gaseous (Kirchoff & Kennedy, 1985; Schuster, 1992; Rudall *et al.*, 2013; Duckett & Pressel, 2017). This definition covers the broad range of typical stomates, such as those on the sporophytes of



**Fig. 1** Hornwort gametophytic pores (HGPs) and sporophytic stomates in hornworts. (a) *Anthoceros agrestis*, three HGPs by an apical notch, taken with a scanning electron microscope (SEM). (b–f) *Phaeoceros carolinianus*, taken with a light microscope using differential interference contrast microscopy (DIC). (b) Three HGPs (white arrows) by an apical notch with apical meristem (AM). The lowermost (two-celled) HGP has a pore that appears to still be in the process of opening. The uppermost HGP is two-celled, while the 'guard cells' of the third pore (middle right) have divided secondarily into a four-celled structure. (c, d) Higher magnification of two-celled HGPs. (e) Higher magnification of four-celled HGP where the two original cells have divided secondarily. (f) Two-celled stomate on a sporophyte sporangium wall. Bars in (a) and (b) are 50  $\mu\text{m}$ , while those in (c–f) are 20  $\mu\text{m}$ .

mosses and hornworts, as well as those of modern vascular plants and diverse fossil plants, and also their various acknowledged homologues including the two-celled pores of hydathodes and of many nectaries. When viewed from this structural and ontogenetic

perspective, hornwort gametophytic pores might well be stomate homologues. Indeed, until roughly 30 years ago, many (if not most) plant biologists referred to HGPs as 'stomates' or duly considered the possibility that they were stomate homologues

(Prantl, 1872; Von Goebel, 1915–1918; Bower, 1935; Evans, 1939; Proskauer, 1951; Pant, 1965; Bold *et al.*, 1987; Schuster, 1992). Some even explored the implications of such a homology for the evolution of the two generations in hornworts (Von Goebel, 1915–1918; Bower, 1935).

Light microscopy and scanning electron microscopy (SEM) reveal that HGP look remarkably like stomates (Fig. 1a–e, *cf.* Fig. 1f; Von Goebel, 1915–1918: fig. 488 I–III, *cf.* fig. 488 IV; Campbell, 1918: figs 65D, 77B; Renzaglia, 1978: Plate 6, fig. 33; Schuster, 1992: figs 1042-6, 1051-3, 1053-3,6,7, 1056-3; Villarreal & Renzaglia, 2006: fig. 4–6,10,11; Frangedakis *et al.*, 2021: figs 1b, 8e,f), yet recent reviews of stomates and stomatal evolution overwhelmingly fail to mention HGPs (Table 1). A paper that does mention HGPs may still fail to discuss the possible homology between sporophytic stomates and these two-celled pores on the gametophytes of hornworts (Duckett & Pressel, 2017). In other recent bryological literature, HGPs have been either rejected as stomates (Villarreal & Renzaglia, 2006; Renzaglia *et al.*, 2008), mentioned without any acknowledgment that they might be homologous to stomates (Renzaglia *et al.*, 2000; Ligrone *et al.*, 2012; Desirò *et al.*, 2013), or dismissed as nonhomologous by omission – *e.g.*, ‘bryophyte stomata are located exclusively on the sporangium’, (Merced & Renzaglia, 2017; see also Renzaglia *et al.*, 2020; Donoghue *et al.*, 2021; Renner & Sokoloff, 2024). Among recent works, only Villarreal & Renzaglia (2015) and Pezeshki *et al.* (2022) considered the possibility that HGPs might be stomate homologues. The prevailing judgments rest on the assertions that (1) among extant embryophytes, stomates are found only on sporophytes, thus implying that HGPs are not stomate homologues (Ligrone *et al.*, 2012; Merced & Renzaglia, 2017; Renzaglia *et al.*, 2020), (2) they function to admit *Nostoc* for symbiosis and hence are not homologous to stomates (Villarreal & Renzaglia, 2006; Renzaglia *et al.*, 2008), and (3) there are slight anatomical differences between HGPs and the two-celled pores most authors deem stomates (Villarreal & Renzaglia, 2006; Frangedakis *et al.*, 2021).

We are concerned that the present consensus against homology leans too heavily on the first two arguments: HGPs’ presence on gametophytes and their function associated with cyanobacterial symbiosis. First, as mentioned, there is clear fossil evidence that stomates occurred on gametophytes during the Devonian (Remy *et al.*, 1993; Edwards *et al.*, 1998; Clark *et al.*, 2022). Second, the multitude of two-celled pores descended from the first stomates clearly have and have had many functions over their evolutionary history – this repertoire might easily include the function HGPs serve in hornworts. As Von Goebel (1915–1918) pointed out over a century ago in his argument that HGPs (‘Thalluspaltöffnungen’) could be homologous to the stomates (‘Spaltöffnungen’) on (hornwort) sporophytes, a fundamental aspect of the concept of homology is that functional divergence of two structures does not preclude shared ancestry between them.

There are, however, other arguments that could constitute a case against HGPs being homologous to stomates. Some have pointed out that structurally the HGP ‘guard cells’ lack the ledges and differential thickenings seen in most sporophytic stomates (Villarreal & Renzaglia, 2006). We might expect such differences

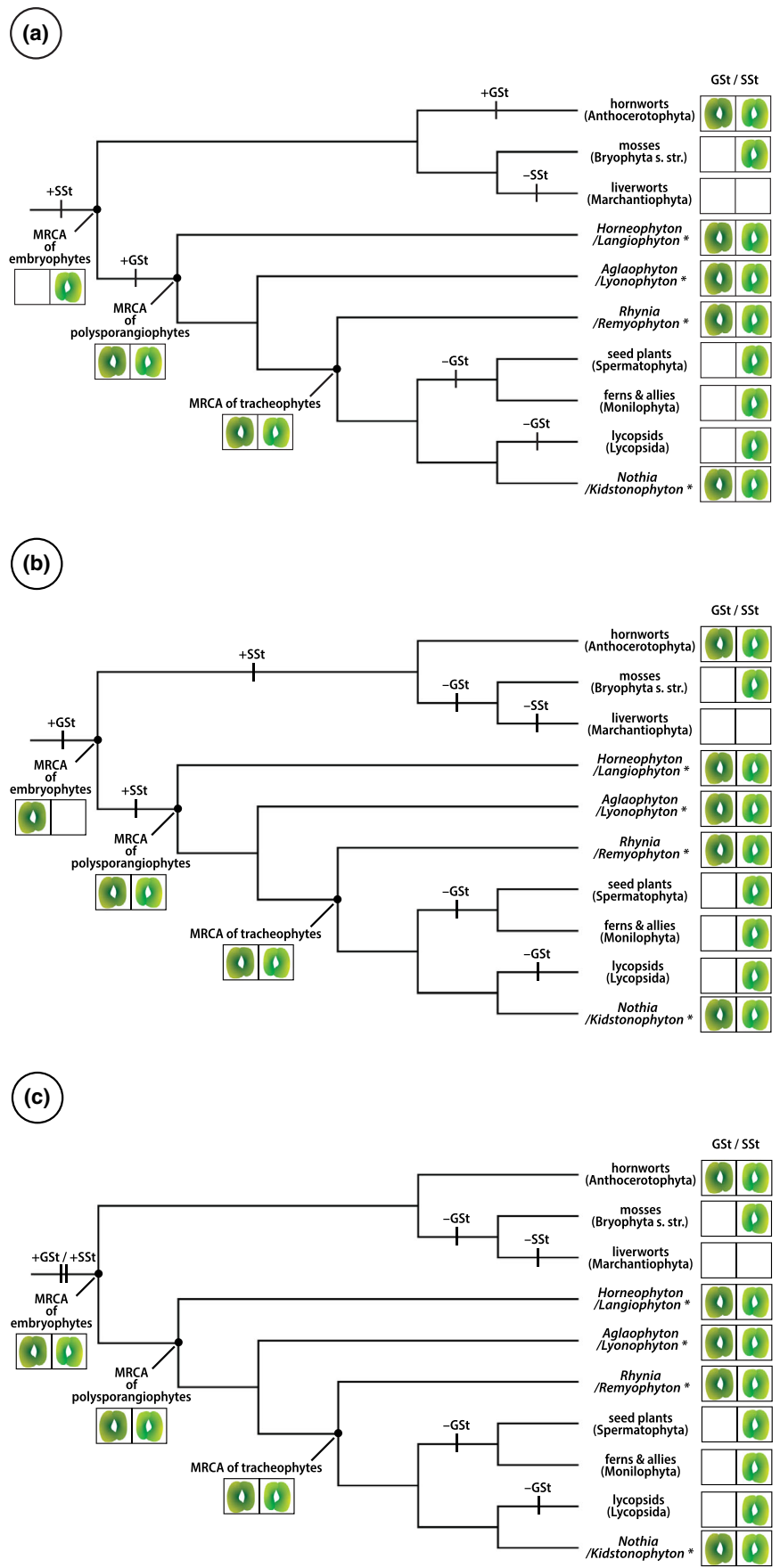
among two-celled epidermal pores to arise from differences in function. For example, ledges are absent in the ‘pseudostomates’ of the *Sphagnum* sporophyte, which have a distinct pattern of wall thickening yet are widely viewed as homologous to stomates (Pant, 1965; Kenrick & Crane, 1997; Pressel *et al.*, 2014; Merced, 2015 – although see Duckett *et al.* (2009), who disagreed with that homology assessment). If the primary purpose of ledges is to prevent liquid water from entering a pore (Edwards *et al.*, 1998), they would likely be superfluous in fluid-filled HGPs and might hinder *Nostoc*’s access to the pores and subdermal intercellular chambers. Moreover, guard-cell shape, ornamentation, and wall thickness exhibit considerable variation among extinct and extant land plants (Edwards *et al.*, 1998; Duckett & Pressel, 2017; Merced & Renzaglia, 2017; Renzaglia *et al.*, 2017, 2020; Frangedakis *et al.*, 2021). A second point that could be invoked against homology is that HGP ontogeny has elements unusual among familiar stomates, namely the additional cell divisions of the original two ‘guard cells’ and permanent closure. Secondary divisions generating three to five guard cells, however, are common in the stomates of diverse mosses (Field *et al.*, 2015; Merced & Renzaglia, 2017). Another notable aspect of HGP ontogeny is that these structures can form immediately adjacent to one another, an arrangement generally rare among stomate-bearing plants but not unusual among bryophytes, including hornwort sporophytes (Schuster, 1992: fig. 1052-5; Rudall *et al.*, 2013; Chater *et al.*, 2017; Merced & Renzaglia, 2017).

Despite some anatomical differences between HGPs and the diverse structures we currently call stomates, the idea that these two-celled structures with pores are homologous to stomates is reasonable, even appealing.

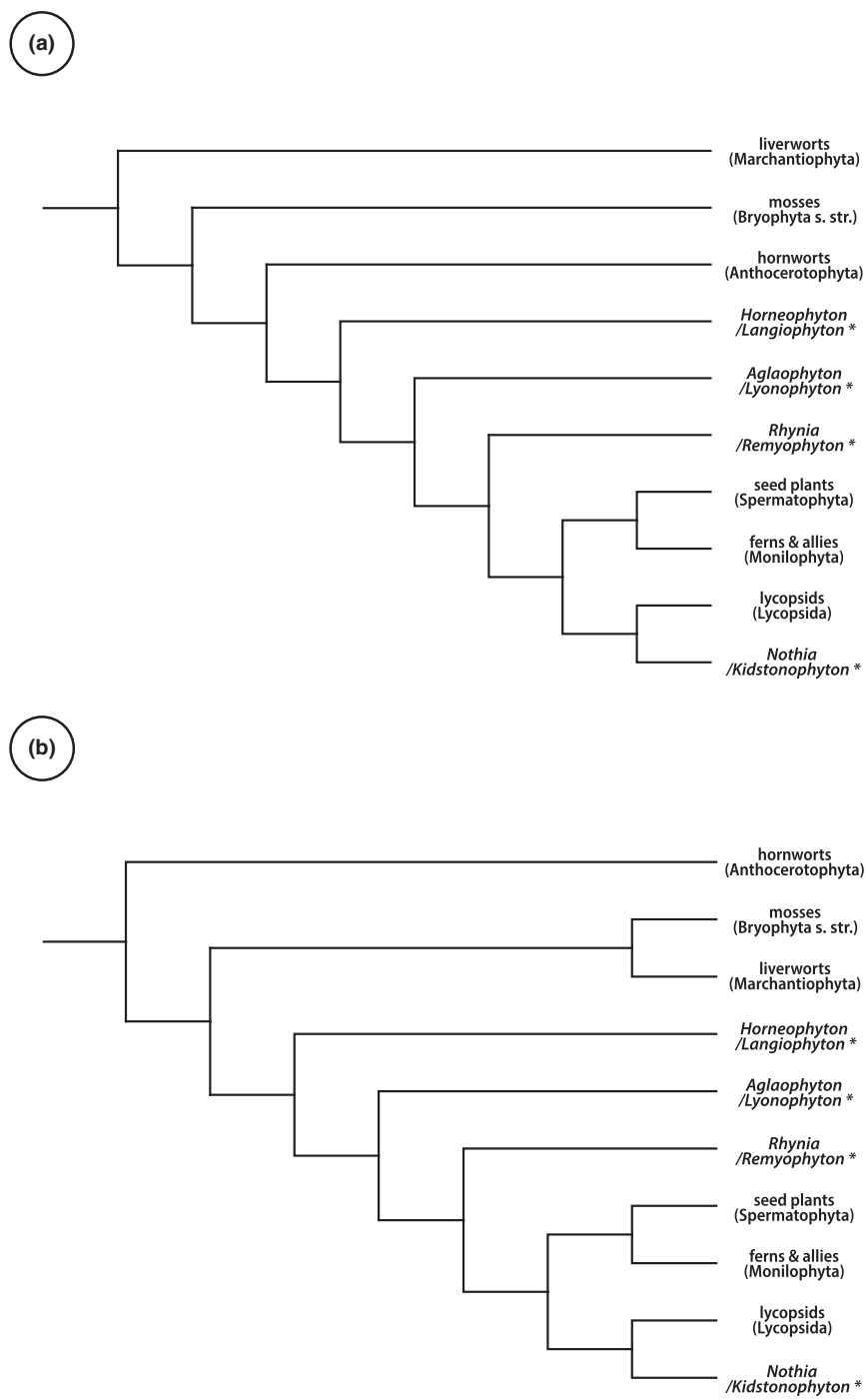
### Implications of HGPs for the evolutionary history of stomates

Recent phylogenetic studies have reported strong support for the monophyly of bryophytes (*e.g.* de Sousa *et al.*, 2018; Puttick *et al.*, 2018; Leebens-Mack *et al.*, 2019; Sousa *et al.*, 2020; Su *et al.*, 2021), although other topologies may not yet be entirely ruled out (see Bell *et al.*, 2020; Qiu & Mishler, 2024 for thoughtful discussions of why definitively resolving the interrelationships of the three bryophyte lineages and vascular plants is challenging). If we hypothesize that the gametophytic pores of hornworts are stomate homologues, the interesting question is whether the last common ancestor of embryophytes bore stomates on just the sporophyte, just the gametophyte, or on both the sporophyte and the gametophyte.

Assuming that extant bryophytes are monophyletic, if the last common ancestor of embryophytes bore stomates only on the sporophyte (Fig. 2a), the appearance of stomates on the gametophytes of ancient hornworts, like that in extinct gametophytes including *Lyonophyton* (*Aglaophyton*) and others in the Rhynie chert, could be considered a form of what we are calling ‘intergenerational heterotopy’ within the context of the alternation of generations (Fig. 2) (see Jones & Dolan, 2012; Kenrick, 2017 for other proposed examples of the ‘transfer’ of developmental programs between the gametophyte and sporophyte generations



**Fig. 2** Phylogenies depicting three alternative patterns of stomatal evolution across embryophytes assuming the monophyly of extant bryophytes and the homology of hornwort gametophytic pores (HGPs) with sporophytic and gametophytic stomates. Boxes containing representations of stomates by nodes and tips indicate the presence of stomates on the gametophyte ('GSt,' left) or of stomates on the sporophyte ('SSt,' right) in a clade or common ancestor. Backbone phylogeny for fossil lineages (\*) based on Kenrick & Crane (1997) (but note that other workers have questioned the placement of *Nothia* within the Lycopside: Kerp *et al.*, 2001; Edwards, 2004; Niklas & Crepet, 2020). (a) The most-recent common ancestor (MRCA) of extant embryophytes possessed stomates only on the sporophyte. The expression of stomates has expanded twice through intergenerational heterotopy: once in hornworts in the form of HGPs and once in the common ancestor of polysporangiophytes. Among tracheophytes, gametophytic stomates were lost twice, while sporophytic stomates would have been lost in the common ancestor of extant liverworts. (b) The most recent common ancestor (MRCA) of all extant land plants bore stomates solely on the gametophyte. Two separate instances of intergenerational heterotopy led to the presence of stomates on the sporophytes of bryophytes (with subsequent loss in liverworts) and polysporangiophytes. Setaphytes (mosses plus liverworts), lycopsids, and the ancestor of monilophytes and seed plants each lost gametophytic stomates. (c) The most recent common ancestor (MRCA) of embryophytes bore stomates on both generations, reflecting one instance of intergenerational heterotopy prior to the last common ancestor of extant embryophytes. Gametophytic stomates disappeared three times: once in the setaphytes (mosses and liverworts), once in the common ancestor of monilophytes and seed plants, and once in the ancestor of lycopsids (which include all extant lycopside). Gametophytic stomates were present in the diverse polysporangiophytes of the Rhynie Chert flora – and therefore in the last common ancestor of polysporangiophytes – and exist as HGPs in extant hornworts. Sporophytic stomates occur generally in extant plants, although they have been lost many times, including in the common ancestor of extant liverworts and many times within mosses. The pattern in (c) could be true whether the first stomates appeared on a sporophyte or on a gametophyte.



**Fig. 3** Alternative hypotheses for the phylogeny of land plants in which bryophytes are paraphyletic (e.g. Bell *et al.*, 2020; Qiu & Mishler, 2024). \* denotes a fossil lineage. (a) Liverworts are sister to all other embryophytes, while mosses are the sister group to a clade consisting of hornworts and polysporangiophytes. (b) Hornworts are sister to a clade containing both the setaphytes and the polysporangiophytes.

in early land-plant evolution). Novel expression of stomates on the gametophyte body at some point in a common ancestor of hornworts would then have allowed for the serendipitous acquisition of new functions, including the ability to provide conditions suitable for the attraction of cyanobacterial hormogonia and their subsequent lodging in a substomatal chamber filled with appropriate exudates. The fact that hornwort sporophytic substomatal cavities are mucilage-filled suggests that the first HGP's may have (at first incidentally) offered a favorable home for

nitrogen-fixing cyanobacteria. Given the wide phylogenetic distances inferred among Rhynie Chert species that bore gametophytic stomates – *Horneophyton/Langiophyton* (a nontracheophyte polysporangiophyte), *Aglaophyton/Lyonophyton* (another nontracheophyte polysporangiophyte), *Rhynia/Remyophyton* (a member of an early tracheophyte lineage whose origin predated the split between lycophytes and euphyllophytes), and *Nothia/Kidstonophyton* (Kenrick & Crane, 1997; *Nothia* was possibly an early lycophyte, although this placement is tentative: cf. Kerp *et al.*, 2001;

Edwards, 2004; Niklas & Crepet, 2020) – a second intergenerational heterotopy event would have occurred in the gametophyte of the common ancestor of polysporangiophytes, with subsequent losses in the gametophyte generations in the common ancestor of a clade that includes seed plants and monilophytes (and possibly all euphyllophytes) and in the common ancestor of lycopsids (Fig. 2a) (Kenrick, 2000, 2017). Additionally, sporophytic stomates would have been lost in the common ancestor of liverworts. (We note that although the gametophytic air pores occurring in a subclade of complex thalloid liverworts (Pant, 1965; Rudall *et al.*, 2013; Shimamura, 2016; Villarreal *et al.*, 2016) intriguingly share two regulatory genes with stomates (*SMF* and *SCRM*; Chang *et al.*, 2023), liverwort pore ontogeny differs markedly from that of acknowledged stomates (Apostolakos *et al.*, 1982), and these pores have traditionally been viewed as nonhomologous with stomates.)

Alternatively, if the most recent common ancestor of embryophytes bore stomates only on the gametophyte generation (Fig. 2b), intergenerational heterotopy must be invoked twice to result in the presence of stomates on the sporophyte: once in the common ancestor of polysporangiophytes and separately in the common ancestor of bryophytes (with a subsequent loss in the common ancestor of liverworts). As in the sporophyte-first scenario (Fig. 2a), losses of gametophyte-borne stomates would have occurred in the common ancestor of seed plants plus monilophytes and in the common ancestor of lycopsids. Additionally, the common ancestor of liverworts plus mosses would have lost gametophytic stomates.

Finally (and again hypothesizing that HGPs are homologous to stomates), if the most-recent common ancestor of embryophytes bore stomates on both the gametophyte and the sporophyte (Fig. 2c), gametophytic stomates would then have been lost in the common ancestor of liverworts plus mosses and in the common ancestor of seed plants plus ferns and fern allies (and possibly of all euphyllophytes), as well as in the common ancestor of lycopsids. In this scenario, no intergenerational heterotopy events are invoked subsequent to the last common ancestor of embryophytes. In this case, gametophytic stomates are plesiomorphic among modern plants, despite their occurrence today solely in the hornworts as HGPs. The hypothesis that the most recent common ancestor of extant embryophytes bore stomates on both generations prompts the intriguing question of whether the first stomates might have appeared in the gametophyte before expanding through intergenerational heterotopy into the sporophyte, as some have suggested (Villarreal & Renzaglia, 2015; Chater *et al.*, 2017). In our view, assuming the most recent common ancestor of embryophytes had stomates on both generations, it would be utter speculation to suggest in which generation stomates first appeared before that ancestor.

Obviously, if other embryophyte phylogenetic hypotheses that have received some support should ultimately prevail (see Bell *et al.*, 2020; Qiu & Mishler, 2024) – for example, liverworts sister to all other embryophytes with hornworts sister to polysporangiophytes (Fig. 3a) or hornworts sister to all other embryophytes with setaphytes (liverworts plus mosses) sister to polysporangiophytes (Fig. 3b) – each of the three scenarios for stomate evolution outlined above (sporophyte-first, gametophyte-first, and stomates present in both generations of

the most recent common ancestor of embryophytes) would be altered. Importantly, in the case with liverworts sister to all other embryophytes, the most recent common ancestor of embryophytes would be hypothesized to have lacked any stomates, and the basic stomatal structure would be hypothesized to have arisen in the common ancestor of mosses, hornworts, and polysporangiophytes.

Irrespective of preferred embryophyte phylogeny, an HGP-stomate homology will shape our understanding of how stomates have evolved across land plants. Also irrespective of preferred phylogeny, among the three bryophyte lineages, stomates on the sporophyte were lost at least 60 times in mosses (Renzaglia *et al.*, 2020) and at least three times in hornworts (in *Notothylas*, in the *Megaceros-Dendroceros-Nothoceros* clade, and in *Folioceros incurvus*; Duckett & Ligrone, 2003; Renzaglia *et al.*, 2008; Frangedakis *et al.*, 2021). Finally, irrespective of embryophyte phylogeny, we can conclude that the common ancestor of polysporangiophytes bore stomates on both the gametophyte and sporophyte generations.

### Final thoughts on the history and future of the HGP homology question

Three decades ago, Schuster (1992) – in the most recent publication we found that provided any discussion of HGPs while referring to them as gametophytic stomates – pointed out that these structures had been perennially understudied to the detriment of our understanding of hornworts and of stomatal evolution. Ignoring the possibility that HGPs are stomates (Table 1) risks closing avenues of research and presumably has done so. Hornwort gametophytic pores remind us to think carefully about the (many) terms we use. What opportunities for inquiry and discovery might be missed because of our choice of words?

The ‘guard cells’ of HGPs and the pores’ underlying cavities would benefit from a set of thorough developmental and comparative studies. Over the last century and a half, HGPs have been studied sporadically in various hornwort taxa, but with little attention to their developmental origin. While we are strongly inclined to view a pair of sister cells that opens to create a pore (and closes after cyanobacteria are inside) as a stomate homologue, cellular, molecular, and genomic approaches could yield critical clues to the question of HGP-stomate homology. A recent search for genes regulating stomatal development in hornworts revealed gametophyte-specific expression of an *Epidermal Patterning Factor-like* (*EPFL*) gene and expression of *ERECTA* in the gametophyte (Li *et al.*, 2020) but did not examine whether expression of either gene is associated with HGP formation (Frangedakis *et al.*, 2021).

We are now equipped with the tools needed to rectify a longstanding omission and untangle the question of homology between stomates and the two-celled gametophytic pores of hornworts. At the very least, we should presume that such a homology is possible, given the deficiency of current evidence in opposition to this hypothesis. Whichever way the evidence leads, studying HGPs will provide insight into the origin of a critical plant structure, the stomate, that has been and continues to be central to plant evolutionary history and diversification.

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## Competing interests

None declared.

## Author contributions

JPF and WEF wrote the manuscript.

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