The hornworts: Important advancements in early land plant evolution

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Review

The hornworts: important advancements in early land plant evolution

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In the last few years, a number of high-profile studies focused on land plant evolution have highlighted novel aspects of hornwort biology and the crucial position of this group among early land plants. For example, the widely accepted comprehensive molecular analysis of Qiu *et al.* (2006) which robustly recovered hornworts as sister to tracheophytes has recently been challenged. Using a phylotranscriptomic approach, Wickett *et al.* (2014) analyzed up to 852 nuclear genes from 92 taxa, and resolved hornworts as sister to land plants, sister to tracheophytes, or part of a bryophyte clade. Similarly, Cox *et al.* (2014) reanalyzed the Qiu *et al.* (2006) and other published data and recovered monophyly of bryophytes. These most recent studies have rekindled the debate about bryophyte interrelationships, while other comprehensive studies on hornworts have advanced the molecular and evolutionary frontiers in unexpected ways. In this review, we discuss emerging lines of inquiry that place hornworts in the centre stage and concentrate on pervasive and long-standing evolutionary issues. The five aspects of the review are: 1) the contentious position of hornworts in the global phylogeny of land plants, 2) revolutionary findings on horizontal gene transfer, 3) evolution of the peculiar hornwort plastid, 4) symbiotic relationships with fungal and cyanobacterial endophytes, and 5) new insights on hornwort stomata and clefts in the gametophyte thallus.

Keywords: Horizontal gene transfer, Plastids

Introduction

Although the least speciose and diverse of the three bryophyte groups, the hornworts have a perplexing combination of seemingly ancestral and derived features that have intrigued biologists since the days of Linneaus. Solitary chloroplasts with pyrenoids are found in no other embryophyte group and suggest affinities with green alga. The presence of a sporophytic basal meristem and asynchronized meiosis, in contrast, are unique to hornworts and unparalleled in extinct and extant plant taxa alike. During the advent of molecular phylogenetic studies in the late 20th century, hornworts were included in broad scale analyses but with little representation. Then in 2004, the first comprehensive phylogenetic analysis of hornworts based on *rbc* L provided novel insights on the relationships within the group (Duff *et al.*, 2004). In 2006, the group was placed in the centre stage of evolutionary debate following a comprehensive broad scale phylogenetic study of land plants that robustly recovered hornworts as the sister group to tracheophytes (Qiu *et al.*, 2006). However, the unusually poor fossil record and the lack of shared morphological features between hornworts and tracheophytes did nothing in the way of resolving character evolution and precluded the placement of hornworts within a precise evolutionary timescale (Renzaglia *et al.*, 2009; Villarreal & Renner, 2012; Figures 1–5).

It was not until 2007 that a molecular phylogenetic analysis was conducted solely on hornworts that sampled the full range of diversity in the group (Duff *et al.*, 2007). This study alone disrupted most hypothesized in-group relationships and dissolved long-standing affinities among genera (Hässel de Menéndez, 1988; Hasegawa, 1994). Almost overnight, the number of genera changed from 5 or 6 to 14. Continued analyses incorporating more taxa and sequence data (Villarreal & Renner, 2012; Villarreal & Renner, 2013) have firmed-up relationships without modifying the basic topology of this pioneering molecular work. The resolution of phylogeny within hornworts opened the way for in depth analyses on character state transformation, providing insight on the evolution of some of the unique hornwort morphological features (*e.g.* Villarreal & Renner, 2012).

The most recent studies that have focused on pervasive issues related to hornwort biology have

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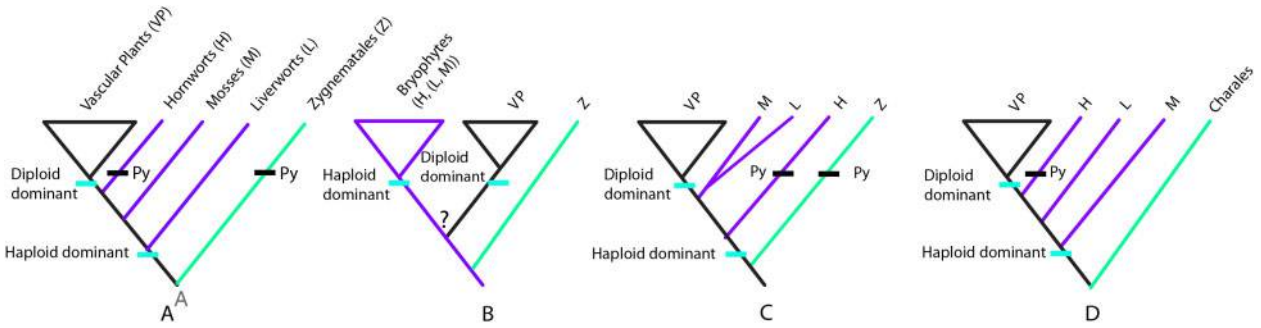

Figure 1 Early land plant topologies. (A) A liverwort-sister hypothesis with hornworts sister to vascular plants (e.g. Qiu *et al.*, 2006). In this scenario, the haploid dominant life cycle is plesiomorphic with a derived diploid-dominant life cycle found in vascular plants (in black). The pyrenoid evolved independently in hornworts and algae (see Villarreal & Renner, 2012). (B) Under the scenario of bryophytes monophyletic and sister to vascular plants (Cox *et al.*, 2014; Wickett *et al.*, 2014) it is impossible to know the state (haploid or diploid dominant) of the ancestor to all land plants. (C) A hornwort-sister hypothesis with mosses sister to vascular plants (*e.g.* Renzaglia *et al.*, 2000; Wickett *et al.*, 2014). In this scenario, the haploid dominant life cycle is plesiomorphic with a derived diploid-dominant life cycle found in vascular plants (in black). The pyrenoid is a plesiomorphic trait and it seems to be inherited from green algae. (D) A moss-sister hypothesis recovered in some analyses presented by Liu *et al.* (2014, see section 1).

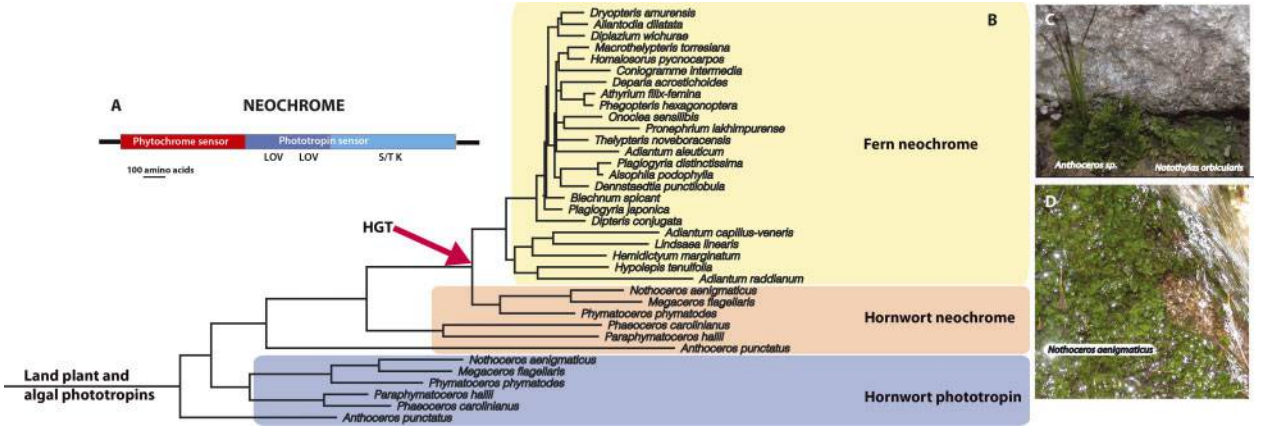

Figure 2 (A) Protein structures of the neochrome photoreceptor. This molecule fuses portion of the phytochrome (red) and a nearly full-length phototropin with the LOVs (dark blue) and S/TK (lighter blue) domains. (B) Bayesian tree based on hornwort phototropin and neochrome alignment (modified from Li *et al.*, 2014, their Figure S1 for support values). Most streptophyte phototropins were trimmed and also the neochrome from Zygnematales. The tree was downloaded from dryad (http://dx.doi.org/10.5061/. dryad.fn2rg). (C) A typical hornwort habitat in a Neotropical cloud forest. In the picture, ***Anthoceros*** **sp. "*punctatus* group" (left, with long sporophytes) and *Notothylas orbicularis* (right with small and horizontally placed sporangia). (D) One of the phenotypes of *Nothoceros aenigmaticus*. The plant is found submerged in creeks in the Southern Appalachian region, United States.**

provided exciting new insights on early land plant evolution, advancing frontiers in unexpected ways (Desirò *et al.,* 2013; Cox *et al.*, 2014; Li *et al.,* 2014; Pressel *et al.*, 2014; Sayou *et al.,* 2014; Wickett *et al.*, 2014). This review summarizes these studies, placing them into an historical context and identifying their impact on evolutionary thought. We start with the most recent work on the ever-contentious position of hornworts in the global phylogeny of land plants, followed by revolutionary findings on horizontal gene transfer and the evolution of the peculiar hornwort plastid. After an exploration of symbiotic relationships of hornworts with fungal and cyanobacterial endophytes, we finish with new insights on hornwort pores, namely stomata and ventral clefts. We conclude each section by identifying basic biological questions and fruitful areas of future research, sometimes with cautionary remarks coming from these studies.

Origin and diversification of early land plants and the phylogenetic position of hornworts

Goffinet (2000) summarized all the topologies that were posited to explain the phylogeny of early land plants. The two main hypotheses at that time were that bryophytes (liverworts, mosses and hornworts) were paraphyletic, and either liverworts (Figure 1A) (Qiu *et al.*, 1998) or hornworts, form the earliest divergent group of land plants. The "hornworts basal" topology (Figure 1C) was based on morphology, ribosomal 18S and mitochondrial ribosomal 19S sequence data (Hedderson *et al.*, 1996; Duff & Nickrent, 1999; Garbary & Renzaglia, 1998; Renzaglia *et al.*, 2000)

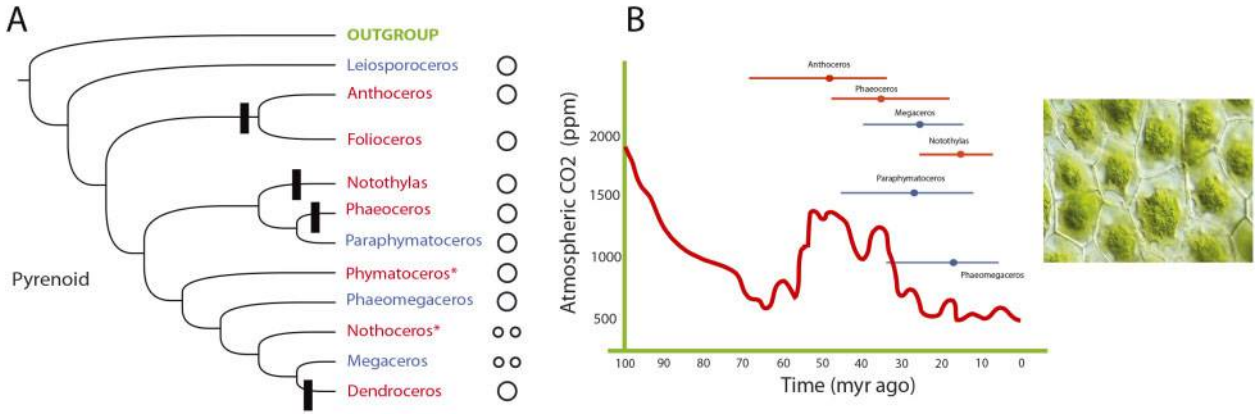

Figure 3 (A) Phylogeny of hornworts based on Villarreal & Renner (2012) and evolution of the hornwort plastids. *Leiosporoceros*, the sister taxon to all hornworts, is pyrenoidless. Genera with pyrenoids (red) and without pyrenoids (blue) are shown. Pyrenoid bearing genera with one or more species lacking pyrenoids have an asterisk (*). Multiplastidic genera are represented by two small circles and those that are typically uniplastidic are shown with one circle. The polarity and evolution of the hornwort pyrenoid strongly depends on the outgroup choice, see Figure 1. (B) Average values of atmospheric CO_2 (ppm) levels during the last 100 mya (Berner & Kothavala, 2001; Beerling & Royer, 2011). The highest posterior density age inferred for some pyrenoid-bearing clades: *Anthoceros*, *Phaeoceros* and *Notothylas* (red) and their pyrenoidless sister clades, *Paraphymatoceros, Phaeomegaceros* and *Megaceros* (blue). Fresh plastids of *Phaeoceros carolinianus* are shown, and the plastids are full of starch surrounding a conspicuous pyrenoid. Ages of the clades come from Villarreal *et al.* (in press).

(Figure 1). In 2004, Nishiyama and colleagues proposed that bryophytes are monophyletic (Figure 1B), based on 51 plastid genes (26,937 bp) for 14 green plants. Their analyses used amino acid sequences and the bryophytes-monophyletic topology was recovered with strong statistical support. The authors demonstrated that analyzing data at the nucleotide level would not resolve the deep relationships among plant groups, and argued that nucleotide composition bias may be responsible for alternative topologies using nucleotide data. The monophyly of bryophytes was previously supported by analyses of sperm cell ultrastructure and development (Garbary *et al.*, 1993; Renzaglia *et al.,* 2000).

Over the past 8 years, the most widely accepted hypothesis on bryophyte phylogeny is that they form a grade, with liverworts basal and hornworts sister to tracheophytes (Qiu *et al.*, 2006; Karol *et al.*, 2010; Chang & Graham, 2011) (Figure 1A). In 2006, Qiu *et al.* sampled 193 green plants using six markers (13,631 nucleotides), in 2010, Karol *et al.* analyzed 43 taxa and 49 protein-coding plastid genes (35,382 nucleotides), and in 2011, Chang & Graham sampled 23 bryophyte species for 14–16 plastid genes (25,064 nucleotides); all three nucleotide analyses recovered hornworts sister to tracheophytes with strong bootstrap values. Prior to that time, mosses were widely accepted as occupying this position (Mishler & Churchill, 1984). An additional analysis using amino acid sequences (Karol *et al.*, 2010, their Additional file 3) recovered bryophytes as a clade, but that analysis was largely discredited due to highly incongruent relationships among tracheophytes, particularly with regard to the position of monilophytes (including bryophytes) as sister to all land plants. The incongruence between analyses of nucleotide and amino acid sequences prompted a critical reexamination of both the Qiu *et al.* (2006) and Karol *et al.* (2010) datasets by Cox *et al.* (2014). These authors concluded that bryophytes are monophyletic (Figure 1B) and that the hornwort-tracheophyte clade is an artifact of mutation-driven compositional bias induced by synonymous substitutions in the nucleotide data (Figure 1B).

The contentious debate over early embryophyte relationships was further rekindled with a recent paper based on mitochondrial genes (Liu *et al.*, 2014). The authors sequenced entire mitochondrial genomes of mosses and liverworts and compiled a matrix of 41 protein-coding genes (31,467 bp). With published data, the final matrix included 27 bryophytes (including the hornworts *Nothoceros aenigmaticus* (R.M.Schust.) J.C.Villarreal & McFarland and *Phaeoceros* ref. *laevis,* probably *P. carolinianus* (Michx.) Prosk.), 28 tracheophytes and 5 algae. The authors did exhaustive analyses to quantify (and correct for) the effects of substitutional saturation, among-lineage specific codon-usage and among-taxa compositional heterogeneity biases on the phylogenetic reconstruction of early land plants. All of these analyses recovered a grade of bryophytes with hornworts sister to tracheophytes. A nucleotide analysis using a homogeneous composition model recovered mosses as sister to land plants (Figure 1D; their Figure 2a) independent of the partition scheme used. A similar analysis using amino acid data recovered liverworts as the sister group to land plants with equally high posterior probabilities and moderate maximum likelihood support (Figure 1A; their Figure 2B).

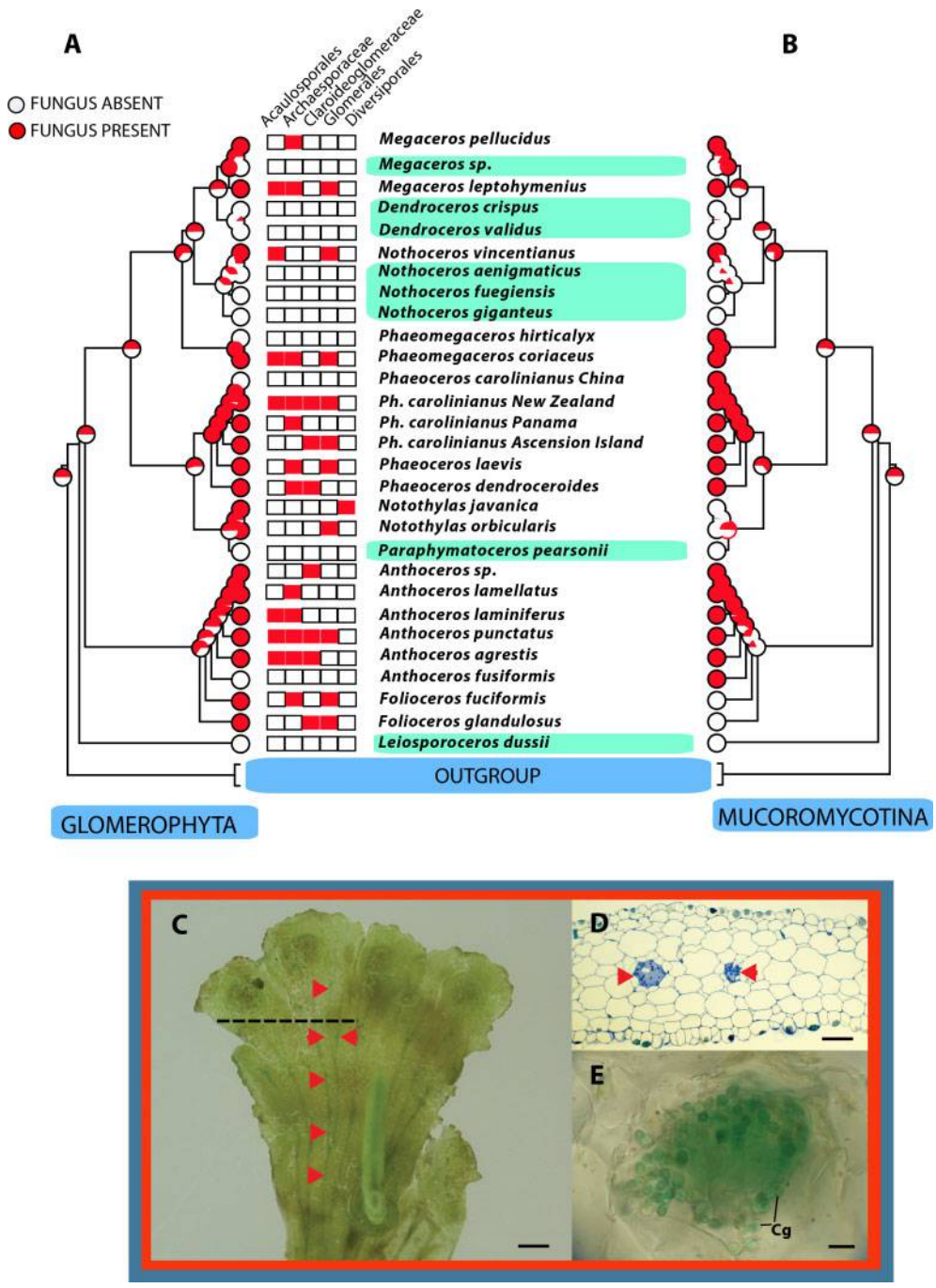

Figure 4 Ancestral state maximum likelihood (ML) reconstructions of the fungal symbionts of hornworts based on a ML *rbc*L tree (modified from Desiro *et al.*, 2013, Figure S5). Both trees are identical mirror images. Reconstructions for Glomeromycota (A) and Mucoromycotina (B) were done separately. Fungal absence is denoted by open circles and fungal presence by filled red circles; the pie diagrams represent the probability of either character. There is uncertainty in the backbone of the tree with 0.50 probability of fungal presence in the most recent common ancestor of the hornworts. The backbone in both reconstructions is ambiguous, suggesting the need of further resolution of the outgroup (see section 1) and fungal data from lycophytes, ferns and liverworts. (A) Glomeromycota. The reconstruction shows several losses of fungi in hornworts. To the right of the tree are the different Glomerophyte fungal clades present in each hornwort; open squares represent fungal absence and filled red squares fungal presence. (B) Mucoromycotina. The reconstruction shows several losses of fungi in hornworts. Glomeromycota and Mucoromycotina fungi are absent in *Leiosporoceros dussii*, *Paraphymatoceros pearsonii*, *Dendroceros*, *Nothoceros aenigmaticus*, *N. fuegiensis*, *N. giganteus* and *Megaceros* sp. (green highlighted boxes). (C) *Leiosporoceros dussii* (Steph.) Hässel. Bifurcating strands of *Nostoc* parallel the main axis of the thallus (red arrows) in a female plant with one sporophyte. A dashed line across represent the transverse section presented in Figure 4D, two opposite arrows represent the two individual strands in Figure 4D. Bar = 2 mm. (D) *Leiosporoceros dussii* (Steph.) Hässel. Transverse section of a mature thallus with two central Nostoc canals (red arrows) at the level of represented by dashed lines in Figure 4C. Bar = 75 μm. (E) Higher magnification of a canal with *Nostoc* (blue-green filaments), note the abundant cyanophycian granules (Cg) in the individual cells (see Villarreal & Renzaglia, 2006 for ultrastructural features). Bar = 10 μm.

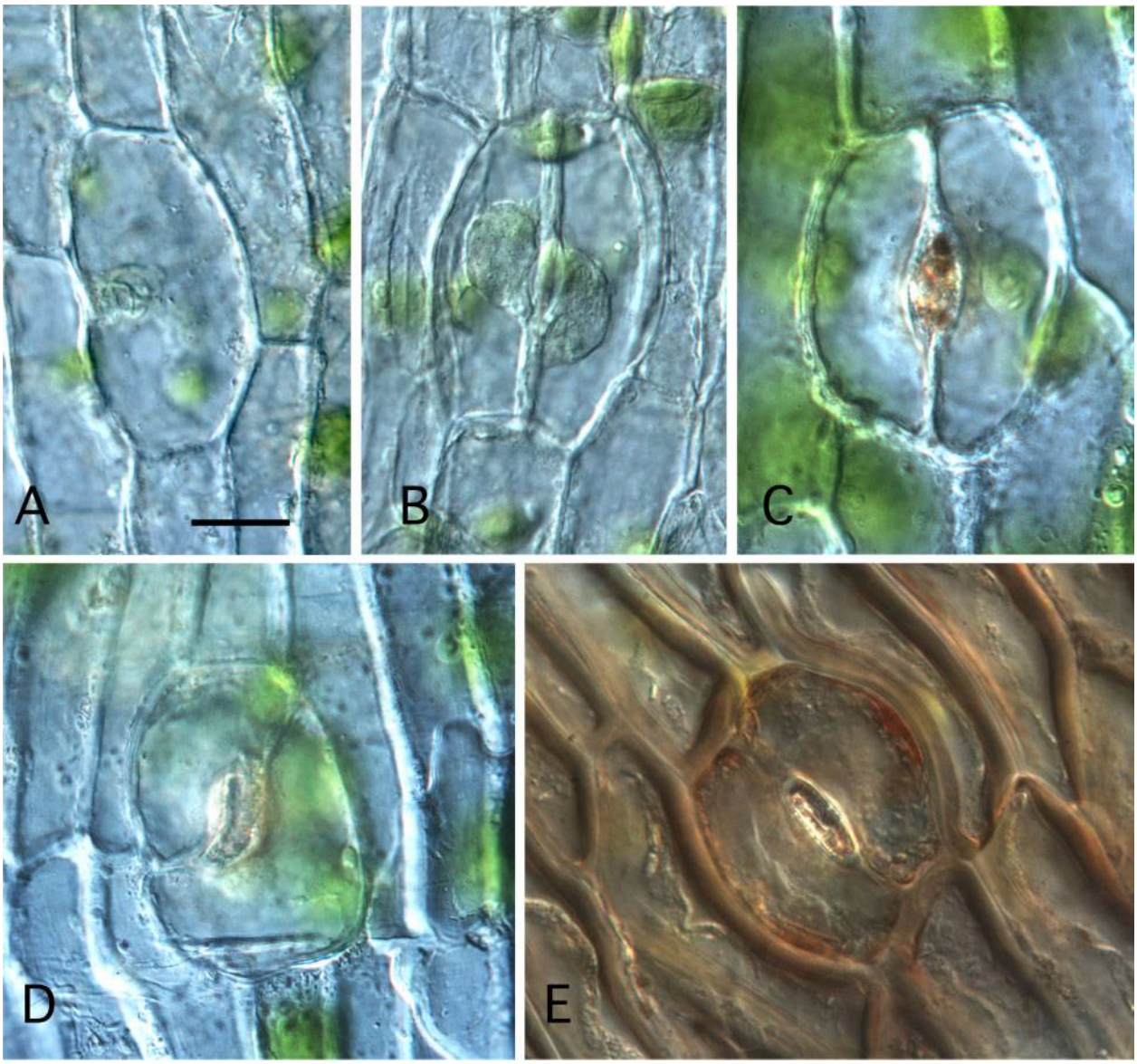

Figure 5 DIC images showing development of stomata in *Phaeoceros carolinianus*. Bar = 20 μm. (A) Guard mother cell differentiates from an epidermal cell that does not elongate and swells in width. (B) Guard mother cell divides symmetrically to produce two guard cells each with a large central plastid adjacent to developing aperture in ventral walls. (C) Stoma with developed aperture containing waxy material. Note increased size of plastids (green) in subtending assimilative cells and thicker cell walls throughout. (D) Asymmetrical stoma near sporophyte suture. (E) Stoma on brown part where sporophyte dehisced showing open aperture, thick walls and fragmented cytoplasm in guard cells and epidermal cells.

In contrast, a nucleotide Bayesian analysis using PhyloBayes with a site-heterogeneous composition model (CAT + GTR + Γ recovered mosses-sister, while an amino acid analysis failed to recover the liverworts-sister topology. The authors suggest that among-lineage compositional biases were responsible for the liverwort-sister relationship recovered under a homogeneous amino acid model. This study also investigated codon usage among plants, highlighting the similarity of codon usage in bryophytes, in contrast to polysporangiophytes and algae. Unexpectedly, mosses have a slightly more similar codon usage to algae, potentially biasing phylogenetic reconstruction. After correcting for all above-mentioned biases in phylogenetic reconstruction the analyses recovered paraphyletic bryophytes with hornworts sister to vascular plants and no support for a liverwort-basal topology (0.70 posterior probability). Liu *et al.* (2014) advised taking into account nucleotide composition, saturation in nucleotide substitution, and codon usage bias, and to present all phylogenetic scenarios using amino acid and nucleotide data. The results from this study strongly suggest that the question of early land plant branching is far from being settled. The authors caution that lack of mitochondrial data for ferns and Zygnematales may have had a profound impact on the results (Liu *et al.*, 2014).

The most recent report addressing land plant origin and diversification analyzed up to 852 nuclear

genes (1,701,170 aligned sites) from 92 species across streptophytes (from chlorophytes up to angiosperms) (Wickett *et al.*, 2014). The study included two hornworts (*Nothoceros aenigmaticus* and *N. vincentianus* (Lehm. & Lindenb.) J.C.Villarreal), six liverworts and eleven mosses. The authors undertook a wide range of analyses (69 in total, their Figure 4 and table S2) of nucleotide data, codon alignments (nucleotides forced to the amino acid alignment), codon analyses including only 1–2nd positions, amino-acid data of gene families and super matrices with different filtered databases (depending on the completeness of the genes used). All analyses were carried out using Randomized Axelerated Maximum Likelihood (RAxML) for nucleotide and amino acid analyses. The most extensively trimmed matrix consisted of 604 genes and 386,883 bp. They also used a site-heterogeneous composition model analysis implemented by PhyloBayes. Additional shortcut coalescent analyses (to account for incongruence due to incomplete lineage sorting) using the software ASTRAL were done in smaller datasets excluding fragmentary data and using gene trees only after long branches were removed. The most stringent dataset for coalescent analyses was reduced to 424 genes. The results were unexpected: all ML analysis using RAxML from concatenated analysis of first and second position and amino acid data for over 600 genes recover hornworts as the sister group to all other land plants (Figure 1C). A coalescent-based analysis from 424 genes from first and second position recovers bryophytes monophyletic with hornworts sister to a clade of liverworts plus mosses (Figure 1B; setaphytes *sensu* Renzaglia & Garbary, 2001). A third analysis of the super matrix of amino acids under a heterogeneous model using the software PhyloBayes recovers liverworts sister to mosses and hornwort sister to vascular plants (Figure 1A).

One concern in broad scale phylogenetic studies is that sampling within hornworts in many instances remains inadequate (systematic error), *e.g.* Wickett *et al.* (2014) sampled only two species of *Nothoceros*, a fairly derived hornwort. The large amount of available molecular data presents new challenges for theoreticians to develop more realistic substitution models and the implementation of shortcut coalescent approaches for such deep divergences should be re-evaluated (Gatesy & Springer, 2014). Other factors of potential influence are codon usage depending on the natural history traits such as sexual systems (Szövényi *et al.*, 2014), variable substitution rates, lack of information in short nuclear loci, conflation of concatenation and coalescence approaches and horizontal gene transfer plaguing super matrix, super tree and short-cut coalescence approaches (Lapierre *et al.*, 2012; Gatesy & Springer, 2014). The recent phylogenetic studies of Cox *et al.* (2014), Liu *et al.* (2014) and Wickett *et al.* (2014) serve to remind us that the branching order of the earliest diverging extant land plants is not settled and requires further scrutiny. We remain optimistic that with further taxon sampling (particularly in the hornworts), and the development of nucleotide substitution and amino acid models that take into account the effect of site heterogeneity for large datasets (Liu *et al.*, 2014; see Cooper, 2014 for a commentary), the seemingly intractable position of hornworts among embryophytes will be revealed.

Non-phylogeneticists may wonder why it is so important to know the position of hornworts in the overall phylogeny of land plant. Aside from satisfying an intellectual curiosity about how plants are interrelated, resolution of this problem provides a framework on which to evaluate the evolution of life history strategies, form, function and development. The liverworts-basal hypothesis is difficult to explain on morphological grounds, as there are no apparent morphological features exclusive to liverworts and Zygnematales, the presently accepted sister group to land plants (Wodniok *et al.*, 2011; Civáň *et al.*, 2014; Wickett *et al.*, 2014). In contrast, if hornworts are the sister group to land plants, the existence of a carbon-concentrating pyrenoid in their chloroplasts (see section 3) forms an intuitive connection with algal ancestors (Hanson *et al.*, 2014).

On a broader scale, if bryophytes form a grade (*i.e.*, bryophytes are paraphyletic) then a haploid-dominant life cycle is unequivocally resolved as ancestral in land plants. The evolutionary trajectory of plants can be viewed as progressing from haploid in charophytes, through the diminutive sporophytes of the bryophyte grade, culminating in a diverse tracheophyte clade with dominant indeterminate sporophytes (Figure 1A, C). If on the other hand, bryophytes are monophyletic, as put forward by Nishiyama *et al.* (2004), Cox *et al.* (2014) and some analysis by Wickett *et al.* (2014), the ancestral nature of the embryophyte life cycle cannot be determined based on extant plants. Only with a robust resolution of bryophyte interrelationships are "evo-devo" studies in land plants possible because they rely on understanding the directionality of change from 'ancestral' (bryophyte) to 'derived' (angiosperm) to ascertain changes in the genetic control of plant form through time (*e.g. Physcomitrella* rhizoids, Menand *et al.*, 2007). With the recent sequencing of a hornwort, liverwort and additional moss genomes, evo-devo studies within bryophytes will become commonplace and the issue of resolving the branching order of bryophytes becomes even more important.

As exemplified by a recent analysis of the transcription factor *LEAFY (LFY)* across green plants, the converse to analyzing results from evo-devo studies on robust phylogenies is to use such results to inform phylogenetic relationships (Sayou *et al.*, 2014). LEAFY is vital for flower development in angiosperms, and in *Physcomitrella patens* is required for the first zygotic division and expressed during sporophyte development (Tanahashi *et al.*, 2005). The function of LEAFY in hornworts is at present unknown. LEAFY is a single-copy gene in all land plants, except gymnosperms and the moss *Physcomitrella,* where two paralogs occur. The *LFY* protein attaches to DNA through a conserved dimeric binding domain (DBD). Sayou *et al.* (2014) demonstrated radial shifts in DNA-binding motifs from green algae (Type III) to land plants (Types I and II). Motif I binding is that of *Arabidopsis thaliana* (L.) Heynh. (AtLFY), which is found in other angiosperms, gymnosperms, ferns and liverworts. Despite possessing the same 15 DNA binding amino acids as AtLFY, the moss gene copy PpLFY1 binds to Motif II, which shares a similar overall organization with Type I**,** consisting of two 8-bp inverted half-sites separated by three nucleotides. Type III motif is found in green algae and resembles motif II but lacks a central 3-bp spacer. LEAFY in hornworts uniquely binds to all three DNA motifs showing versatility in expression of LFY target genes while remaining a single copy gene. The LFY amino acid phylogeny recovers hornworts as sister to land plants, and emphasizes the transitional role of the hornwort LFY with its promiscuous binding to algal and land plant DNA motifs (Sayou *et al.*, 2014).

Horizontal gene transfer (HGT) to and from hornworts

Independent acquisitions of the ribosomal intron or horizontal gene transfer

The newly assembled plastid genome of *Nothoceros aenigmaticus* (Villarreal *et al.*, 2013) coupled with that of *Anthoceros angustus* Stephani (Kugita *et al.*, 2003) represent two of the main clades of hornworts. A major finding in both studies is the presence of an intron at the same position in the ribosomal rrn23 gene, an occurrence restricted to the Anthocerotaceae and to select chlorophycean algae, the non-streptophyte green algal line distant from land plants. The insertion site of the intron in *Anthoceros* is identical to that in *Chlorella* and *Chlamydomonas* and sequence identity is significant ($\sim$50%), particularly near the splice junctions of the intron (Kugita *et al.*, 2003). The intron is absent from the plastid genomes of the charophytes *Chaetosphaeridium* and *Zygnema,* although a smaller intron (351 bp) is present in a different position in the plastid rrn23 gene of *Chara*. The presence in *Chlorella* and *Chlamydomonas* of an endonuclease-like open reading frame suggests a homing intron (Wakasugi *et al.*, 1997). In *Anthoceros angustus,* a small open reading frame (55 amino acids) within the *rrn23* intron shares only 40% pairwise similarity with the *Chlamydomonas* endonuclease. The presence of the intron may be explained by independent acquisitions in chlorophytes and *Anthoceros* or by horizontal transmission from green algae to *Anthoceros*. The semiaquatic habitats of some hornworts and *Chorella* often place them in proximity, lending plausibility to the HGT hypothesis. Although difficult to support conclusively, this *Anthoceros*/chlorophyte intron may be one of the few cases of HGT between plastomes, especially in light of the report described in the section that follows.

HGT of an adaptive chimeric photoreceptor from hornworts to ferns

Plant photoreceptors have two major action peaks in the light spectrum, phototropins absorbing the UV-blue light (320–500 nm) and phytochromes absorbing mostly red and far-red regions (600–800 nm). Phytochromes are involved in regulating seed germination, seedling development, and transition to flowering, among other processes (Hughes, 2013). Phototropins regulate phototropism, stomatal opening, leaf expansion, and chloroplast movement and accumulation (Christie, 2007). The role of blue light inducing chloroplast accumulation and avoidance has been documented in mosses, ferns and angiosperms (Christie, 2007; Königer, 2014). The two main domains of the phototropin are two light, oxygen and voltage sensors (LOV) and a serine/threonine kinase domains. (S/TK) (Figure 2A).

A novel type of light receptor is found in green organisms, the neochrome. This molecule fuses portion of the phytochrome and a nearly full-length phototropin with the LOVs and S/TK domains (Christie, 2007; Königer, 2014; Figure 2A), enhancing light sensing capabilities. Neochromes have been viewed as independently derived in *Mougetia,* a zygnematalean green alga, in the tree ferns (Cyatheales) and in the crown-group of (mostly epiphytic) ferns (Polypodiales) (Suetsugu *et al.*, 2005; Li *et al.*, 2014). The macro-evolutionary impact of neochrome is noteworthy: it has been suggested that this chimeric gene allowed the diversification of ferns during the Cretaceous literally in the shadow of angiosperms (Schneider *et al.*, 2004; Li *et al.*, 2014). Li *et al.* (2014) analyzed 434 transcriptomes and 40 whole genomes of plants and algae (including a low coverage draft genome of *Anthoceros punctatus* L.). Among land plants, they found neochrome only in ferns and all seven hornworts examined: *Anthoceros punctatus,*

Phaeoceros carolinianus, Paraphymatoceros hallii (Austin) Hässel, *Phymatoceros phymatodes* (M.Howe) R.J.Duff, J.C.Villarreal, Cargill & Renzaglia, *Megaceros tosanus* Stephani, *Nothoceros aenigmaticus* and *N. vincentianus* (Figure 2B). The authors concluded that the neochrome gene was transferred from hornworts to ferns and that this event occurred ~179 Ma, more recently than the last common ancestor of ferns and hornworts, precluding an alternative scenario of multiple neochrome losses in all other land plants. All hornworts examined to date seem to have a single intron-free phototropin gene and it is more similar to the terminal end of both fern and hornwort neochromes. Phototropins across land plants possess multiple introns. The lack of introns in the hornwort phototropin may have facilitated the fusion with a phytochrome gene. The widespread occurrence of neochrome in hornworts, however, provokes further questions on the role of this photoreceptor in these plants (Figure 2C). Many hornworts grow in open areas, while few genera (*Megaceros*, *Nothoceros* and *Dendroceros*) are exclusively found in shaded highly humid forest habitats (Figure 2D). Plastids in hornworts are large and structurally different from all other embryophytes, suggesting the acquisition of an equally unique biochemistry and means of coping with physiological stress.

The enigmatic hornwort plastid and the evolution of the pyrenoid

Hornwort plastids are unique among land plants in the presence of pyrenoids in uniplastidic mature cells (Renzaglia *et al.*, 2009). Pyrenoids are proteinaceous bodies consisting of up to 90% RuBisCO, and are otherwise restricted to algae (including chlorophytes, charophytes, dinophytes and euglenoids) (Badger *et al.*, 1998). In algae, the internal concentration of CO_2 in the vicinity of RuBisCO is nearly 40 times greater than surrounding ambient concentration, enhancing photosynthesis in aqueous environments where CO_2 diffusion is limited (Badger *et al.*, 1998; Meyer & Griffiths, 2013). The advantage of a pyrenoid-style carbon concentrating mechanism (CCM) in hornworts is puzzling, since pyrenoid-containing and pyrenoid-lacking species occur in a range of terrestrial environments (Figure 3). The riparian and sometimes submerged *Nothoceros aenigmaticus* lacks pyrenoids and more than one plastid is often present in each cell. In contrast, the vast majority of hornwort species are terrestrial, growing on soil banks or epiphytic on trees and leaves (e.g. *Dendroceros*), and these are the taxa that possess extremely large, solitary, pyrenoid-containing plastids (Figure 3). The function of the pyrenoid remains one of many unanswered ecophysiological questions concerning the unique hornwort plastid, especially given no other extant land plants occupying similar habitats travelled down that evolutionary path.

Plastid and pyrenoid microanatomy show considerable variability across hornworts, to the extent that this feature alone is diagnostic of some clades. Most notable are the pyrenoid-less plastids with extensive grana in *Leiosporoceros* (see section on pores), the sister to other hornworts, and the star-shaped plastids of *Dendroceros* (Renzaglia *et al.*, 2009; Schuette & Renzaglia, 2010). The latter contain peculiar pyrenoids with spherical inclusions. As the only epiphytic hornwort, *Dendroceros* is exposed to intermittent drying and wetting conditions not experienced by other genera. Thus, it has been postulated that the reappearance of pyrenoids in this taxon may be related to the ability of these plants to undergo cycles of hydration and dehydration with minimum impact on the photosynthetic machinery (Schuette & Renzaglia, 2010). A mechanism for desiccation tolerance is particularly important in this plant because the majority of the photosynthetic surface of the thallus extends across wings that are only one cell thick.

A recent study on the evolution of hornwort pyrenoids (Villarreal & Renner, 2012) suggests that the ancestor of all hornworts, about 300 Ma, lacked pyrenoids and that pyrenoids were gained and lost over the last 100 Ma under varying concentrations of atmospheric CO_2 (Figure 3B). The ecological impetus for the acquisition of the hornwort pyrenoid remains speculative, especially since pyrenoids were lost during periods of low CO_2 and re-appeared during times of high atmospheric CO_2. It is true that taxa with pyrenoids are more speciose than those without, suggesting an adaptive advantage to their presence. However, the adaptive role of the demonstrated carbon concentrating ability of hornwort pyrenoids (Hanson *et al.*, 2014) is difficult to make sense of given the 10 or so gains and losses of pyrenoids in this small group. The answer may lie in a more careful evaluation of the microhabitats of these plants with an eye on alternative factors, such as an opportunistic CCM depending on water availability or circadian rhythms that may have influenced the evolution of pyrenoids within some hornwort clades.

In green algae, and putatively hornworts, one of the main components of the CCM is the pyrenoid. Mutagenic *Chlamydomonas* lines that contain a hybrid RUbisCO holoenzyme with a small subunit from flowering plants recovered a pyrenoidless phenotype (Meyer *et al.*, 2012). When two exposed α-helices from *Chlamydomonas rbc*S were engineered back into the hybrid enzyme the pyrenoid was restored (Meyer *et al.*, 2012). More recently, mutants deficient in the protein LCIB recovered small and numerous pyrenoids (Yamano *et al.*, 2014). These results point to

the role of α-helices and LCIB in pyrenoid development. The ability to engineer a pyrenoid-based CCM into higher plants seems an achievable goal, and indeed tobacco plants have been engineered using cyanobacterial carboxysome proteins (analogous to the algal/hornwort pyrenoid) (Whitney & Sharwood, 2007; Lin *et al.*, 2014). Because of their phylogenetic proximity, hornworts present a more appropriate system from which to introduce a pyrenoid-based CCM into higher plants than algae.

Fungal and cyanobacterial endophytes

Fungal symbiosis allowed plants to successfully colonized bare habitats during early land plant invasion in the Ordovician. Symbiotic mycorrhizal genes have been found across land plants and Wang *et al.* (2010) elegantly demonstrated the conserved function of those genes in liverworts and hornworts. Mosses, however, are devoid of any mycorrhizal partners. Glomerophytes have been traditionally claimed to be the partners of non-vascular and vascular plants. The widespread association of species of the genus *Glomus* with bryophytes and tracheophytes cemented the idea of an ancient association between this fungal phylum and land plants (Krings *et al.*, 2007; Wang *et al.*, 2010). However, molecular data helped to discover a peculiar association of members of Mucoromycotina with the earliest divergent liverworts, *Treubia* and *Haplomitrium* (Bidartondo *et al.*, 2011). In addition, recent evidence of hyphae and fungal clumps has been discovered in the Devonian *Horneophyton* with striking similarities with living members of Mucoromycotina (Strullu-Derrien *et al.,* 2014). These findings prompted further research on the identity and function of the fungal partners across land plants (Field *et al.*, 2014). Desirò *et al.* (2013) conducted a worldwide study of fungal endosymbionts in hornworts that revealed the presence of diverse members of Glomeromycota and Mucoromycotina. In total, 120 out of 199 accessions, representing ~20 species, were found to have symbionts. Fifty accessions contained symbiotic fungi from both Glomeromycota and Mucoromycotina (Figure 4A, B). Among glomerophytes, four orders are represented, Glomerales (present in 65 samples), Archaeosporales (30 samples), Diversiporales (17 samples) and Paraglomales (1 sample). Mucoromycotina was represented by 13 clades, including members of the Endogonales (Figure 4A). The high diversity of fungal strains is unrivalled in any other land plant studied so far. The only genera, so far, without fungal symbionts are the Neotropical *Leiosporoceros*, the epiphytic *Dendroceros* and species of *Nothoceros* and *Megaceros*, typically associated with wet habitats (Figure 4A, B, green highlight). Additionally, one collection of *Phaeomegaceros hirticalyx* (Steph.) R.J.Duff, J.C.Villarreal, Cargill & Renzaglia lacks fungal endophytes. More recently, Desirò *et al.* (2014) characterized *Mollicutes*-related endobacteria within *Endogone* Mucoromycotina. They demonstrated that these are unrelated to endobacteria found in Glomeromycota, adding a new dimension to the intricate endophytic associations in early land plants. A novel finding of Desirò *et al.* (2013) is the intimate association of fungal hyphae with hornwort cyanobacterial colonies.

In land inhabiting green plants, symbiotic associations with cyanobacteria are thought to have evolved *ca* 500 Ma (Raven, 2002). In angiosperms (mostly legumes) the association with root nodule-forming cyanobacteria has a single origin (Werner *et al.*, 2014) and the "symbiotic genes" involve in nodule formation have been identified. Unlike nodule-forming symbioses, obligate cyanobacterial partnerships are found sporadically in all major plant lineages, particularly in all hornworts, two genera of liverworts, the fern *Azolla*, all cycads and the angiosperm *Gunnera*. The heterocyst-forming genera *Nostoc* and *Anabaena* are the most commonly reported symbionts. In *Azolla* the symbiosis is maintained perpetually (even through sexual reproduction) and in addition to cyanobacteria, entire communities of other bacteria have been found, including *Arthrobacter* and *Agrobacterium* (Lindblad *et al.*, 1991; Li & Pryer, 2014).

Hornworts are arguably the oldest land plant lineage with a widespread symbiosis with cyanobacteria. Most hornwort species develop globose *Nostoc* colonies inside their thalli and multiple re-infections occur throughout their life span. The sole exception is *Leiosporoceros* that has bifurcating strands of *Nostoc* locked inside the thallus, potentially enhancing the transfer of metabolites between partners (Villarreal & Renzaglia, 2006) (Figure 4C–E). The unique association of the cyanobiont with the basal hornwort *Leiosporoceros* has been equated to the association with cyanobacteria in the Devonian vascular plant *Aglaophyton*, yielding insights on the evolution of symbiosis in early land plants. (Krings *et al.*, 2007). The symbiotic relationship between hornworts and cyanobacterial has been subject of intense research, both the morphology and ultrastructure (Duckett *et al.*, 1977; Renzaglia, 1978; Adams, 2000). The functional and genomic aspects of the infection process, chemotaxis between partners, and hormogonial motility have been uncovered (Adams & Duggan, 1999; Meeks & Elhai, 2002; Meeks, 2003; Adams & Duggan, 2008; Adams & Duggan, 2012). However, aspects of genomic control of the symbiosis within the plant have not been explored due to the lack of an annotated hornwort genome.

In the fern *Azolla*, the obligate cyanobiont present within the leaf cavity presents signs of genomic erosion

with a large proportion of pseudogenes ($\sim$30%) and a high frequency of transposable elements in the genome (Ran *et al.*, 2010). The eroded state of the cyanobiont genome may be the initial road to the loss of its autonomy and becoming a strict symbiotic organelle. (Ran *et al.*, 2010). The complex genomics interactions between hornworts and cyanobacteria have not been explored. Consequently, candidate "symbiotic genes" have not been identified and the potential horizontally transferred genes between partners are unknown. We hypothesize a higher transfer of genes and high genomic erosion between the semi-permanent symbiosis in *Leiosporoceros* in comparison with the more transient symbiosis in other hornworts. The small genome size of *Leiosporoceros* (0.16–0.19 pg =160–184 Mbp; Bainard & Villarreal, 2013) facilitates transcriptomic and genomic sequencing. The presence of intertwined fungal hyphae with cyanobacterial colonies within hornworts has ignited questions on the genomic basis and the benefits of a tripartite mutualism within this metacommunity.

Hornwort pores

Bryophytes are key to deciphering the origin and evolution of stomata (Figure 5A), yet few studies have focused on hornwort stomata (Hartung *et al.*, 1987; Lucas & Renzaglia, 2002). To fill this void, Pressel *et al.* (2014) described the development of stomata and subtending tissue in two hornworts, *Phaeoceros carolinianus* and *Anthoceros punctatus*. They demonstrated that stomata of hornworts are formed from a median longitudinal division of sporophyte epidermal cells that are morphologically similar to the surrounding cells, confirming previous work by Campbell (1895) (Figure 5B–D). This is in contrast to the asymmetrical divisions that characterize stomatal development in angiosperms. As surrounding epidermal cells elongate, the guard mother cell ceases elongation and expands in width. These authors also reveal that plastid behaviour and morphology serves to differentiate guard cells from other epidermal cells, even prior to cell wall thickening (Figure 5). The guard mother cell contains a single plastid that divides once, thus young guard cells each contain a single large spherical plastid. A second cycle of plastid divisions results in newly opened stomata each containing two large spherical plastids. In contrast, epidermal cells contain fragmented plastids. Typically, hornwort stomata are solitary, symmetrical and longitudinally oriented, but with unusual dextral and sinistral asymmetry predominating near dehiscence furrows (Figure 5D).

Using cryoSEM, Pressel *et al.* (2014) reveal that substomatal cavities in hornworts are fluid-filled (likely with mucilage) when first formed, which is in stark contrast to the air-filled substomatal cavities in tracheophytes. Following pore formation in stomata, the fluid dries down in the intercellular spaces, while the matrix around developing spore tetrads remains. This observation led the authors to conclude that these stomata are instrumental in sporophyte drying and dehiscence as first postulated by Lucas & Renzaglia (2002). They also note that hornwort stomata are much less dense than those of tracheophytes, a similarity shared with early fossil plants. A general conclusion from the developmental studies of Pressel *et al.* (2014) is that there is no evidence of homology between hornwort and tracheophyte stomata.

Our observations of the early development of hornwort stomata for the most part agree with Pressel *et al.*, but our interpretations are very different. We suggest that the symmetry, longitudinal orientation and anomalous asymmetry of stomata near the suture are a function of the unique development of the hornwort sporophyte and therefore would not be expected to resemble other plants. The hornwort sporophyte, which is really an elongating sporangium, is a cylindrical structure that is produced from a basal meristem with nearly the same diameter. Most of the growth in epidermal cells in this structure is in length, with only slight increases in width, and no further cell divisions. Stomata develop directly from the basal meristem from protodermal cells that divide once and expand mostly in width. Examination of stomata in similar terete organs of tracheophytes, such as the aerial axes of *Psilotum*, reveals a similar association and development of stomata and epidermal cells (Mickle *et al.*, 2012). Stomata on planar leaves and expanding apophyses of mosses develop in synchrony with the growth and expansion of the organ on which they occur, processes that, unlike in hornworts, involve continued cell division and cell expansion in many directions. These are fundamental differences that are important to consider when examining cell lineage patterns leading to guard cell formation. We therefore interpret the asymmetrical stomata near the sporophyte suture as a normal feature of development in hornworts rather than an irregularity due to abnormal development. Given the ubiquity of their occurrence across taxa and their consistent location near the dehiscence line, it is likely that the asymmetry is instrumental in splitting and twisting of the two valves of the sporophyte. The oblique wall separating these guard cells is contiguous with wall patterns in adjacent epidermal cells leading to the suture, suggesting the asymmetry is an integral feature of these plants (Figure 5D).

We concur with Pressel *et al.* that stomata in hornworts play a major role in drying of the sporophyte and are not involved in gas exchange or water loss

through regulation of stomatal closing and opening. However, we find evidence in chloroplast ultrastructure for a major role of hornwort stomata in gas exchange that enhances photosynthesis up to spore maturation. As is typical in leaves of tracheophytes, guard cell chloroplasts are large and starch-filled while epidermal cells contain small often non-photosynthetic plastids (Crum, 2001). Chloroplasts are large in assimilative cells next to intercellular spaces as mucilage progressively disappears from the substomatal cavity (Pressel *et al.*, 2014). Ultrastructurally, these plastids in *Leiosporoceros* are identical to those in the gametophyte in that the central region contains numerous large grana while starch occupies the periphery of the organelle. As the sporophyte ages (or higher on the sporophyte), chloroplasts increase in size and contain extensive central grana and even more abundant starch. These observations support an active role in carbohydrate assimilation during this phase of development.

Pressel *et al.* (2014) missed a number of critical issues related to hornwort stomata that provide key comparisons with extant and extinct taxa. For instance, the development of guard cell and epidermal cell walls continues long after stomatal pores open, thus these authors did not follow stomata through maturation. The study also suffers from the lack of sectioned material at the level of guard cells, making some of their illustrations inaccurate and misleading. The differentiation of hornwort guard cell walls and fate of stomata are crucial processes in understanding the early function and evolution of land plant stomata. Because of the importance and complexity of these processes, a complementary manuscript is in preparation (Renzaglia *et al.*, unpublished) that picks up where Pressel *et al.* (2014) left off and comes to very different conclusions. The current hypotheses of early land plant branching suggest that hornworts are either sister to all other land plants or a member of a bryophyte monophylum. In either case, we see strong support for a single origin for the stomata in land plants based on the entire process of sporophyte development and the earliest plant fossil stomata.

To test the responsiveness of recently opened stomata of hornworts to environmental and exogenous factors Pressel *et al.* (in review) conducted extensive experiments on two hornwort taxa and compared responses to angiosperms. They once again demonstrated that stomata open apertures early in development and thereafter remain so. No changes in apertures were elicited following darkness treatments and slight reduction in aperture dimensions were noted after desiccation and plasmolysis, attributed to reduction in width of all epidermal cells, impacting guard cells. Surprisingly, ABA treatments brought about slight changes in aperture width. In contrast, all these treatment resulted in complete stomatal closure in tracheophytes. Potassium concentrations were measured by x ray microanalysis; no differences were detected between hornwort guard cells and epidermal cells under all treatments at all times. The lack of physiological regulation of opening and closing of stomata in hornworts compared with tracheophytes, supports accumulating evidence that stomata in hornwort are involved in sporophyte desiccation and spore discharge. The authors suggest that stomata first evolved on sporangia as structures facilitating dehiscence, and that regulation of stomatal movement was acquired subsequently with the evolution of planar leaves.

This interpretation is further supported by experimental studies probing the impact of elevated CO_2 on stomatal density, aperture size and guard cell length in mosses and hornworts (Field *et al.*, 2015). When young sporophytes were exposed to increased levels of CO_2, the number and structure of stomata showed no significant changes when compared with those grown at ambient CO_2 levels. This is compelling evidence that the regulation of development and physiology via CO_2 concentration that occurs in tracheophytes does not exist in hornworts.

In additional to sporophytic stomata, hornworts possess mucilage clefts on the gametophyte. These typically 2-celled pores resemble sporophytic stomata but do not have their complex pore anatomy, development and function. The mucilage clefts provide an entry route for cyanobacteria into the hornwort gametophyte. The homology of these structures (stomata and clefts) has been questioned (Villarreal & Renzaglia, 2006). Gametophytic stomata are absent in any extant land plant lineage and have only been reported in extinct lineages such as *Aglaophyton*. This leads to a number of intriguing questions: Might gametophytic fossil stomata be sites of entrance of symbionts as they are in hornworts? Do the gametophytic and sporophytic pores share similar genetic underpinnings? Is the genetic mechanism underlying the creation of the pore in hornworts co-opted in the sporophyte? With the release of a hornwort nuclear genome, at least some of these become testable questions.

Conclusions

The early history of plants on land set the stage for subsequent diversification of multicellular plants and animals, yet many aspects of the biology of one of the oldest lineages of land plants, the hornworts, remain unknown. Aside from the thalloid growth habit and the existence of stomata, it is difficult to identify features shared by hornworts and other land plants. Indeed, characteristics related to cell biology such as

plastid ultrastructure, spermatogenesis, cell division and sporogenesis serve to isolate the group from other plants or point to affinities with green algae. Thus, the availability of molecular data for phylogenetic analysis held great promise in regards to resolving the interrelationships across bryophyte groups. Great strides over the past decade were made using molecular data to resolve within hornwort relationships and these findings led to important contributions, including understanding the evolution of characters such as plastid microanatomy as described in this review. However, after two decades of study, the phylogenetic placement of hornworts within land plants remains elusive and will require rigorous analyses that include additional taxon sampling, and more complex and realistic models to resolve.

The recent studies of hornworts discussed herein have opened new avenues to explore and have pointed to the importance of including sufficient sampling of hornwort diversity in global evolutionary studies. With only a handful of hornwort biologists worldwide, the expertise to collect and identify these plants is scarce. We remain available to assist in providing hornwort diversity for molecular, evo-devo, physiological, and evolutionary studies, as do many in the bryological community. Only such collaboration will ensure that hornworts drop the stigma of being the least known, most neglected land plants. If history is predictive, the biological mysteries that await discovery in this obscure but intriguing group of bryophytes will be well worth the collective effort.

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