Fern genomes elucidate land plant evolution and cyanobacterial symbioses

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Ferns are the closest sister group to all seed plants, yet little is known about their genomes other than that they are generally colossal. Here, we report on the genomes of Azolla filiculoides and Salvinia cucullata (Salviniales) and present evidence for episodic whole-genome duplication in ferns—once at the base of ‘core leptosporangiates’ and one specific to Azolla. One fern-specific gene that we identified, recently shown to confer high insect resistance, seems to have been derived from bacteria through horizontal gene transfer. Azolla coexists in a unique symbiosis with N2-fixing cyanobacteria, and we demonstrate a clear pattern of cospeciation between the two partners. Furthermore, the Azolla genome lacks genes that are common to arbuscular mycorrhizal and root nodule symbioses, and we identify several putative transporter genes specific to Azolla-cyanobacterial symbiosis. These genomic resources will help in exploring the biotechnological potential of Azolla and address fundamental questions in the evolution of plant life.

The relatively small genome (0.75 Gb) of Azolla is exceptional among ferns, a group that is notorious for genomes as large as 148 Gb and averaging 12 Gb. Azolla is one of the fastest-growing plants on the planet, with demonstrated potential to be a significant carbon sink. Data from the Arctic Ocean show that, ~50 Myr ago, in middle-Eocene sediments, an abundance of fossilized Azolla characterizes an ~800,000-year interval known as the ‘Azolla event’. This period coincides with the shift from the early Eocene greenhouse world towards our present icehouse climate, suggesting that Azolla had a role in abrupt global cooling by sequestering atmospheric carbon dioxide. Azolla is also remarkable in harbouring an obligate, N2-fixing cyanobacterium, Nostoc azollae, within specialized leaf cavities. Because of this capability, Azolla has been used as ‘green manure’ for over 1,000 years to bolster rice productivity in Southeast Asia.

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Asia. The Azolla symbiosis is unique among plant–bacterial endosymbiotic associations because the cyanobiont is associated with the fern throughout its life cycle, being vertically transmitted during sexual reproduction to subsequent generations. In all other land plant symbiotic associations, the relationship must be renewed each generation. The Nostoc symbiont is not capable of autonomous growth and its genome shows clear signs of reduction, with several housekeeping genes lost or pseudogenized. With a fossil record that extends back to the mid-Cretaceous period, Azolla probably shares a ~100-Myr-old co-evolutionary relationship with Nostoc.

To better understand genome size evolution in Azolla and its closely related lineages, we obtained genome size estimates for all five genera of Salviniales (Supplementary Table 1). We found them to be at least an order of magnitude smaller than any other fern species (Fig. 1a), and, most notably, the genome of Salvinia cucullata, which belongs to the sister genus to Azolla, is only 0.26 Gb, the smallest genome size ever reported in ferns. This unanticipated discovery afforded us the opportunity to include a second fern genome for comparison.

## Results

### Genome assembly and annotation

To gain insight into fern genome evolution, as well as plant–cyanobacterial symbioses, we sequenced the genomes of A. filiculoides (Fig. 1b) and S. cucullata (Fig. 1c) using Illumina and PacBio technologies. The assembled Azolla and Salvinia genomes have N50 contig size of 964.7 Kb and 719.8 Kb, respectively. The BUSCO (Benchmarking Universal Single-Copy Orthologs) assessment and Illumina read-mapping results indicate high completeness for both assemblies (Supplementary Fig. 1 and Supplementary Table 2). We identified 20,201 and 19,914 high-confidence gene models in Azolla and Salvinia, respectively, that are supported by transcript evidence or had significant similarity to other known plant proteins (Supplementary Figs. 1–3, Supplementary Table 3 and Supplementary Discussion). Salvinia genes are much more compact, with a mean intron length half of that in Azolla (Supplementary Fig. 1). In addition to introns, differences in the repetitive content explain some of the nearly threefold difference in genome size. Azolla has more of every major category of repeat, but 191 Mb of the 233-Mb difference in the total...
repetitive content are made of retroelements, especially Gypsy and Copia long terminal repeat retrotransposons (LTR-RTs; Fig. 1d and Supplementary Fig. 4). DNA transposon profiles are similar for the two ferns except that Azolla has substantially more SOLA elements than does Salvinia (Fig. 1e).

Insights into gene family evolution in land plants. The genomes of Azolla and Salvinia offer a new opportunity to examine the evolution of plant genes and gene families across all Viridiplantae (land plants plus green algae). We classified genes into orthogroups from 23 genomes (12 angiosperms, 2 gymnosperms, 2 ferns, 1 lycophyte, 2 mosses, 2 liverworts, 1 charophyte and 1 chlorophyte; Supplementary Table 5) and reconstructed the gene family evolution—gain, loss, expansion and contraction—across the green tree of life (Supplementary Fig. 5 and Supplementary Table 5). To investigate the origin of genes linked to seed development, we examined orthogroups containing 48 transcription factors that express exclusively in Arabidopsis seeds\(^{14}\). Homologues of 39 of them were detected in ferns or other seed-free plants, indicating that many seed transcription factors were present before the origin of seeds (Supplementary Table 6). Similarly, only a handful of transcription factor families arose along the branch that led to seed plants (Supplementary Table 7); rather than relying on entirely novel transcription factors, it seems instead that an expansion of pre-existing transcription factor families had a greater role in seed plant evolution\(^{14}\). Indeed, ancestral gene number reconstructions of MADS-intervening keratin-like and C-terminal (MIKC)-type MADS box genes (orthogroup 23) show that these important developmental regulators more than doubled in number from 15 in the ancestral vascular plant to 31 in the ancestral eukaryophyte (here, Salviniales plus seed plants; Supplementary Table 5).

In a recent study on the evolution of plant transcription-associated proteins, which include transcription factors and transcriptional regulators\(^{14}\), ferns were exclusively represented by the Pteridium aquilinum transcriptome. The finding that the transcriptional regulator Polycarya group EZ (Pg_EZ) was lost in ferns is corroborated here by our whole-genome data (Supplementary Table 8). Conversely, the transcription factor ULTRAPETALA, which originated at the base of eukaryophytes and is present in P. aquilinum, was apparently secondarily lost in Salviniales (Supplementary Table 8). YABBY, an important transcription factor that patterns leaf polarity in flowering plants, is absent in our fern genomes and in the genome of the lycophyte Selaginella moellendorffii\(^{17}\) (Supplementary Table 8). Interestingly, a YABBY homologue was recently identified in a separate lycophyte species—*Huperzia selago*\(^{16}\)—suggesting that YABBY has been lost at least twice in land plant evolution (in Selaginella and in ferns). How the differential retention of YABBY shaped the evolution of the vascular plant body plan requires further studies.

Among the orthogroups specific to seed plants, 1-aminoacyclopropane-1-carboxylic acid (ACC) oxidase is of special interest because it converts ACC to ethylene—the last step in the ethylene biosynthetic pathway (Fig. 2). Ethylene is a critical plant hormone that controls various important physiological responses (for example, fruit ripening, flowering time, seed germination and internode elongation). Because ethylene responses are known in bryophytes, lycophytes and ferns\(^{11}\), it is intriguing to find that ACC oxidase only evolved within seed plants, a result confirming that seed-free plants must possess an alternative ethylene-forming mechanism\(^{14}\). Two other mechanisms, found in bacteria and fungi, result in ethylene formation: one via the 2-oxoglutarate-dependent ethylene-forming enzyme and the other through the non-enzymatic conversion of 2-keto-4-methylthiobutyric acid (KMBA) into ethylene\(^{17}\). We did not identify ethylene-forming enzyme in seed-free plant genomes, suggesting the absence of the ethylene-forming enzyme-based biosynthetic pathway. Seed-free plants may possibly synthesize ethylene non-enzymatically via KMBA; however, further biochemical studies are needed to test this hypothesis. Interestingly, ACC synthase (upstream of ACC oxidase) is present in seed-free plants, albeit in a lower copy number (<3) compared to seed plants, which average 9.3 copies (Fig. 2 and Supplementary Fig. 6). Paralogues of ACC synthase in seed plants are differentially regulated in response to varying developmental or environmental stimuli\(^{18}\). Thus, it is plausible that the expansion of the ACC synthase family was coupled with the origin of ACC oxidase in seed plants to create a regulated ethylene biosynthetic pathway.

The history of whole-genome duplication in ferns. Our MultiTAXon Paleopolyploidy Search (MAPS)\(^{29}\) phylogenetic analyses of the Azolla and Salvinia genomes (Fig. 3a), together with all available transcriptome data from other ferns, support two whole-genome duplication (WGD) events: a recent WGD event occurring in Azolla following its divergence from Salvinia and an earlier WGD predating the origin of core leptosporangiates (sensu Pryer at al.\(^{21}\)), a large clade comprising the heterosporous, tree and polypod ferns. The observed peaks of duplication associated with the inferred WGDs exceeded the 95% confidence intervals of our birth and death simulations for gene family evolution in the absence of WGDs. This high number of shared gene duplications is readily explained by a significant episodic birth event, such as a WGD. The discovery that Azolla experienced a genome duplication independent of other heterosporous ferns is not entirely surprising because Azolla has nearly twice the number of chromosomes of other heterosporous ferns, including Salvinia and *Pilularia*\(^{30,31}\) (Fig. 1a).

To further substantiate the two WGD events identified by MAPS, we examined the distribution of synonymous distances (*Ks*) between syntenic paralogues within each of the genomes, as well as syntenic orthologues between Azolla and Salvinia. In the Azolla genome, we detected 242 syntenic blocks comprising 988 syntelog pairs. By contrast, only 83 syntenic blocks with 254 syntelog pairs could be found in Salvinia. Between Azolla and Salvinia, 3,587 pairs of syntenic orthologues were detected, clustering into 356 syntenic genomic blocks. We fit Gaussian mixture models to identify peaks in the Ks distributions (Fig. 3b and Supplementary Fig. 7). The main peak for Azolla–Salvinia orthologue pairs centres at ~1.0, which marks the species divergence between the two genera. To the left of this peak is the major Azolla intragenomic peak (~0.8), whose position confirms the Azolla-specific WGD event (Fig. 3b). To the right of the Azolla–Salvinia divergence peak is the Salvinia intragenomic Ks peak (~1.2–1.3), which matches a minor Azolla intragenomic peak, consistent with the proposed pre-core leptosporangiates WGD (Fig. 3b).

Moreover, despite the antiquity of the WGDs and species divergence (Fig. 1a), we were still able to detect Azolla–Salvinia syntenic regions in a 2:1 or 2:2 syntenic relationship (Fig. 3c), respectively, corroborating the Azolla-specific and the older WGD events. The confirmation of these two WGDs in ferns further allows us to characterize patterns of gene retention following WGD. We found that Azolla syntenic paralogues are enriched for transcription-related genes (Supplementary Table 9), similar to what was observed in Arabidopsis and other angiosperms\(^{16}\). Likewise, protein kinases, another functional category commonly retained after WGD in seed plants, are significantly enriched in Salvinia syntenic paralogues (Supplementary Table 9). Additional genomic data are needed to better characterize the distribution of WGD events across the fern tree of life and to compare patterns of post-WGD gene fractionation with those documented in seed plants.

The pentatricopeptide repeat family and RNA editing. The pentatricopeptide repeat (PPR) family is the largest gene family found in the Azolla and Salvinia genomes, with the Azolla genome encoding over 2,000 PPR proteins and the Salvinia genome over 1,700 PPR proteins. PPRs are implicated in organellar RNA processing\(^{32}\), and
the large repertoire of PPRs correlates well with the extensive RNA editing we observed in the organellar genomes of Salviniales: 1,710 sites in Azolla organelles and 1,221 sites in Salvinia (Supplementary Table 10). These editing events include both C-to-U conversions (~70%) and U-to-C conversions (~30%). The number of PPR genes and the degree of RNA editing greatly exceed that found in seed plants and most bryophytes. Of the sequenced plant genomes, only that of S. moellendorffii has more PPR genes, correlating with the hyperediting seen in lycophytes. However, there are no U-to-C editing events in Selaginella, making the Azolla and Salvinia genome sequences a novel and valuable resource for identifying the unknown factors catalysing these events.

More than half of the plastid transcripts and two-thirds of the mitochondrial transcripts in Azolla and Salvinia require start codon creation by C-to-U editing or stop codon removal by U-to-C editing before translation is possible. Most stop codon edits (76%) and start codon edits (62%) are shared between Azolla and Salvinia plastomes (as opposed to only 19% in internal ACG codons; Supplementary Fig. 10). This persistence of start and stop codon edits suggests that their loss is selected against, that is, creating the translatable sequence by RNA editing has an advantage over having it encoded by the genome. This argues that these particular RNA-editing events are not selectively neutral and supports editing as a control mechanism for gene expression in fern organelles.

Only ~55–60% of PPR proteins (1,220 in Azolla and 930 in Salvinia) contain domains associated with RNA editing in other plants. Although sufficient to account for the number of editing events observed (assuming each protein can specify one or a few sites as in other plants), this leaves a very large number of PPR proteins (~700 in Azolla and ~600 in Salvinia) with unknown functions. By comparison, flowering plants contain only 200–250 PPR proteins that lack editing domains.

**Origin and evolution of a fern insecticidal protein.** Ferns are remarkable for their high levels of insect resistance compared to flowering plants. Recently, Shukla et al. isolated a novel insecticidal protein, Tma12, from the fern Tectaria macrodonta. Transgenic cottons carrying Tma12 exhibit outstanding resistance to whitefly, yet show no decrease in yields, demonstrating tremendous agricultural potential. Tma12 has a high similarity to chitin-binding proteins (Pfam PF03067), but its evolutionary origin is unknown. Here, we found a Tma12 homologue to be present in the Salvinia genome (henceforth ScTma12), as well as in a few 1,000 Plants (1KP) fern transcriptomes, but not in Azolla or any other publicly available plant genomes. Phylogenetic analyses position the fern Tma12 sequences together with bacterial sequences, and are most closely related to the chitin-binding proteins from Chloroflexi (Fig. 4). We investigated whether this insecticidal protein was more likely a result of horizontal gene transfer (HGT) from bacteria to ferns or produced by fern-associated microorganisms. ScTma12 is in a 646,687-bp scaffold (Sacu_v1.1_s0099) and has a 247-bp intron. The genes upstream and downstream of ScTma12 are all clearly plant genes, and we found no abnormality in read-mapping quality, nor an abrupt change in read coverage (Supplementary Fig. 9), which together speak against the sequence being a contamination from a bacterial source. It has been argued that differential loss of genes in eukaryotes is the rule and gene acquisition by HGT rather rare. The concerted loss of Tma12 in each of the other Viridiplantae lineages is unlikely but cannot entirely be ruled out. However, functional HGT into eukaryotes does occur and ScTma12 might represent such a case that contributed to the well-documented resistance of ferns against phytophagous insects.

**Azolla–cyanobacterial symbiosis.** To explore the co-evolutionary history of the Azolla–Nostoc symbiosis, we resequenced five other Azolla species and assembled each of their cyanobiont genomes. We then compared the cyanobiont phylogeny to the host species phylogeny and found a clear cospeciation pattern, with just one exception (the placement of Azolla caroliniana; Fig. 5a). Although such a pattern has been hinted at before, we provide unequivocal...
Salvinia, CYCLOPS, and Azolla were found in other ferns, the VAPYRIN/SYMRK), Argolentia, and the COP10 clades, respectively. Density plots from fitting Gaussian mixture models to K divergence between Azolla and Salvinia regions, respectively. Each subpanel represents a genomic region in Azolla or Salvinia, with gene models on both strands shown above and below the dashed line. High-scoring sequence pairs (HSPs) in protein-coding sequences are marked by short vertical bars above the gene models. Selected HSP links between genomic regions are depicted as colored lines crossing the subpanels, whereas others (for example, the HSP links between the two Azolla genomic regions in the left panel) are left out for clarity. Collinear series of HSPs across genomic regions indicates a syntetic relationship between the regions concerned. Genomic regions conserved in duplicate after the WGD that occurred prior to the divergence between Azolla and Salvinia should show a 2:2 syntetic relationship, whereas regions conserved in duplicate after the Azolla-specific WGD should show a 2:1 syntetic relationship with Salvinia regions. The left and right panels can be regenerated at https://genomevolution.org/r/ujll and https://genomevolution.org/r/ukys, respectively.

Fig. 3 | The history of WGD in Azolla and Salvinia. a, MAPS analysis identified two WGD events: one specific to Azolla (orange circle) and one predating the core leptosporangiates (green circle). The blue line illustrates the percentage of subtrees indicative of a gene duplication shared by the descendants at each node. The grey lines display the gene birth–death simulation results without WGD. The species divergence dates are from Testo and Sundue. b, Density plots from fitting Gaussian mixture models to K divergence between Azolla and Salvinia regions, respectively. Each subpanel represents a genomic region in Azolla or Salvinia, with gene models on both strands shown above and below the dashed line. High-scoring sequence pairs (HSPs) in protein-coding sequences are marked by short vertical bars above the gene models. Selected HSP links between genomic regions are depicted as coloured lines crossing the subpanels, whereas others (for example, the HSP links between the two Azolla genomic regions in the left panel) are left out for clarity. Collinear series of HSPs across genomic regions indicates a syntetic relationship between the regions concerned. Genomic regions conserved in duplicate after the WGD that occurred prior to the divergence between Azolla and Salvinia should show a 2:2 syntetic relationship, whereas regions conserved in duplicate after the Azolla-specific WGD should show a 2:1 syntetic relationship with Salvinia regions. The left and right panels can be regenerated at https://genomevolution.org/r/ujll and https://genomevolution.org/r/ukys, respectively.

Evidence from whole-genome data. The genetic basis for this persistent symbiosis is still under investigation. In plants, two other mutualistic associations—the arbuscular mycorrhizal (AM) and the nitrogen-fixing root nodule (RN) symbioses—have been well characterized. Whereas the AM symbiosis is formed between almost all land plants and a single fungal clade (Glomeromycota), the RN symbiosis is restricted to a few angiosperm lineages (mostly legumes) that associate with various nitrogen-fixing bacterial symbionts (for example, Rhizobium and Frankia). Despite these distinct differences, both symbioses require that a common symbiosis pathway (CSP) be established. This pathway is highly conserved in all land plants, except for those that have lost the AM symbiosis or have multiple symbiotic partners, such as A. thaliana and other aquatic angiosperms.

We investigated whether the CSP might have been co-opted during the evolution of the Azolla–Nostoc symbiosis by searching for six essential CSP genes in the Azolla and Salvinia genomes, as well as in transcripтомic data from other ferns in the 1KP data set (Supplementary Table 11). Although DMI2 (also known as SYMRK), DMI3 (also known as CGaMK), IPD3 (also known as CYCLOPS) and VAPYRIN were found in other ferns, the Azolla and Salvinia genomes completely lacked orthologues (Fig. 5b). IPD3 and VAPYRIN do not belong to multigene families and homologues were not detected. Although homologues of DMI2 and DMI3 were identified, phylogenetic analyses confirmed that they are not orthologous to the symbiotic genes (Supplementary Data). In addition, for DMI3, we searched the Azolla and Salvinia homologues for two motifs (threonine 271 and the calmodulin-binding domain) that are critical for symbiosis. Both motifs are missing from these sequences, confirming the absence of DMI3. CASTOR and POLLUX are paralogues resulting from a gene duplication event in the ancestor of seed plants, and although pre-duplicated homologues are present in Salvinia and other seed-free plants, they are absent in Azolla (Fig. 5b). The co-elimination of the CSP genes suggests the lack of AM symbiosis in Azolla and Salvinia and that the nitrogen-fixing Azolla–Nostoc symbiosis does not rely on this pathway.

To identify genes important for the Azolla–Nostoc symbiosis, we treated A. filiculoides with erythromycin to remove the cyanobiont (AzCy–) and compared its gene expression patterns with the wild type (AzCy+). Experiments were carried out in conditions where the nitrogen nutrient (ammonium nitrate) was either supplied (N+) or withheld (N–) from the growth media. Results from nifH real-time PCR confirmed the complete absence of cyanobacteria in AzCy– and showed that the addition of the nitrogen nutrient suppresses symbiotic N fixation in AzCy+ (Supplementary Fig. 10),
consistent with an earlier study\(^{42}\). A large portion of the transcriptome is affected by the presence or absence of cyanobionts, with 6,210 and 2,125 genes being differentially transcribed under N⁻ and N⁺ conditions, respectively (Fig. 5c and Supplementary Discussion). Of these, over 33% have at least a twofold expression difference. In response to nitrogen starvation, the Azolla transcriptomes remained moderately stable when the cyanobiont was present, but shifted drastically once it was absent (Fig. 5d). This finding suggests that the presence of the cyanobiont buffers the transcriptomic profile of Azolla from fluctuations in environmental nitrogen availability.

We focused primarily on those genes that are differentially expressed between the nitrogen treatments when the cyanobiont is present, and to a lesser extent on when the cyanobiont is absent (Fig. 5e and Supplementary Discussion). Because the cyanobacterial N₂-fixation rate is strongly induced in the N⁻ condition, we expect these genes to be candidates involved in nutrient exchange or in communication with the cyanobiont to promote N₂ fixation. A total of 88 upregulated and 72 downregulated genes were identified (Fig. 5e). Among the upregulated genes is a parologue of the ammonium transporter 2 subfamily (\textit{AfMOT1}; \textit{Azfi_s0167.g054529}) that is most likely specialized for supplying molybdenum, a required co-factor for nitrogenase, to the cyanobiont. One of the legume \textit{MOTI} genes was recently found to facilitate nitrogenase activity in RN symbiosis\(^{44}\). In addition to these two transporters, we identified a chalcone synthase paralogue that catalyses the production of flavonoid biosynthesis. Interestingly, naringenin and naringin both inhibit differentiation\(^{47}\). Naringin is also a hormogonium-repressing factor\(^{46}\). Because hormogonia lack heterocysts and cannot fix nitrogen, naringin (or related flavonoids) could act as a plant signal to boost N₂ fixation in the cyanobiont (Supplementary Discussion).

Although the ancient and intimate nature of the \textit{Azolla–Nostoc} relationship suggests that gene transfer from \textit{Nostoc} to the \textit{Azolla} nuclear genome may have occurred over time, a thorough homology search found no evidence of \textit{Nostoc-to-Azolla} HGT. However, we did discover a cyanobacteria-derived gene in the \textit{Azolla} genome, but one that is shared with other ferns. This gene encodes a squalene–hopene cyclase (SHC), which mediates the cyclization of squalene into hopene, and is thought to be the evolutionary progenitor of many classes of eukaryotic and prokaryotic sterol cyclases.

### Fig. 4 | Origin of a fern insecticidal protein

Phylogenetic analysis of the chin-binding domain Pfam PF03067 shows that the fern Tma12 insecticidal protein was probably derived from bacteria through an ancient HGT event. The numbers above the branches are bootstrap (BS) support values (BS = 100), and the thickened branches indicate BS > 70. The tree is rooted based on the result from a broader phylogenetic analysis of PF03067 and PF08329 (Supplementary Data). The pink star denotes the sequence from the \textit{S. cucullata} genome.

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[42]: http://www.nature.com/natureplants/issue/vol4/iss4/460/full/460465a.html
[43]: http://www.nature.com/natureplants/issue/vol4/iss4/460/full/460465a.html
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**ARTICLES**
Fig. 5 | Azolla–cyanobacterial symbiosis. a, The cyanobiont phylogeny largely mirrors the host species phylogeny, indicating a convincing cospeciation pattern between the two partners. All nodes received a maximum likelihood bootstrap support of 100% and for the host phylogeny, all nodes also received a local posterior probability of 1.0 from the ASTRAL analysis. Both the nuclear and the plastome data sets gave the same topology for the host, and the branch lengths shown here were from the plastome tree. Scale bars represent 0.01 substitutions per site. b, The CSP genes were lost in the Azolla and Salvinia genomes (empty boxes), whereas orthologues can be found in other fern transcriptomes (red boxes). *Arabidopsis* lacks the CSP genes and does not have AM symbiosis. c, Cyanobionts have a large effect on the Azolla transcriptome. d, The Azolla transcriptome responds to nitrogen starvation more significantly when cyanobionts are absent than when they are present. PC, principal component. e, Candidate genes involved in nutrient transport and communication with cyanobionts.

no homologue can be found in seed plants or in green algae, the SHC is also present in bryophyte (moss and liverwort) genomes and transcriptomes. Interestingly, these bryophyte SHCs are not related to those of ferns but are embedded in other bacterial SHC lineages (the monophyly of land plant SHCs is rejected by the Swofford–Olsen–Waddell–Hillis test, \( P < 0.005 \)). This finding implies a complex evolutionary history for SHCs in land plants, possibly featuring independent transfers of SHC from different prokaryotic lineages to mosses, liverworts and ferns. We are confident that these SHC genes are not from contaminants because the gene phylogeny largely mirrors the species phylogeny; furthermore, the SHC genes were not assembled into stray scaffolds in the genomes of Azolla, Salvinia, Physcomitrella or Marchantia. In addition, we detected the triterpene products of SHC, hop-22(29)-ene, diploterol and tetrahydropipecol, in S. cucullata biomass, providing direct evidence for SHC activity in this fern (Supplementary Fig. 13). Similar observations of SHC-synthesized triterpenes have been made in polypod ferns and mosses. Because hopene has an important role in plasma membrane stability in prokaryotes (similar to steroids in eukaryotes) and have been shown to confer low-temperature adaptation and stress tolerance, it is plausible that the convergent evolution of hopene biosynthesis in seed-free plants, through independent HGTs from bacteria, might have contributed to the early adaptations of land plants to diverse and adverse environments. Functional studies are needed to confirm this hypothesis.

We anticipate that the availability of the first genomic data from ferns will continue to lead to vital insights into the processes that govern the evolution of plant genes and gene families. The implementation of fern data into the existing comparative genomic framework will enhance our understanding the plant tree of life.

**Methods**

**Flow cytometry and genome size estimation.** To estimate the genome sizes of *S. cucullata, P. americana, Regnellidium diphyllum* and *Marsilea minuta* (Supplementary Table 1), we used the Beckman chopping buffer to extract nuclei from fresh leaves, following the protocol of Kuo and Huang. The nuclei extractions were mixed with those from standards, stained with 1/50 volume of propidium iodide solution (2.04 mg ml\(^{-1}\)) and incubated at 4 °C in darkness for 1 h. For each species, three replicates were performed on the BD FACSCan system. For *S. cucullata*, we used *A. thaliana* (0.165 pg per C) as the standard, and for all other samples, we used *Zea mays* CE-777 (2.785 pg per C). For each peak (in both standard and sample), over 1,000 nuclei were collected with cross-validation values lower than 5%, except for those of *A. thaliana* 2n nuclei peaks, which ranged from 5.5% to 5.9%. To calculate the 2C-value of *S. cucullata*, we used a formula of:  

\[
\text{2C-value} = \text{peak value} \times \text{standard value} / \text{sample value}
\]
manner using HISAT2 (v2.0.4) and StringTe (v1.2.2), except for nine libraries published in de Vries et al. for which only a reference-guided approach was used. All programs used default parameters, and Trinity was run with the additional --archismetic option. StringTe results were merged using StringTe --merge, combined with the Trinity output, and were purged of redundant sequences using the GenomeTools sequniq utility.

Putative centromere sequences were first identified by searching the genome assemblies with Tandem Repeat Finder to identify very high-copy (>100 repeats) tandem repeats with a motif length in the range of 185–195 bp. Motif sequences were extracted from the Tandem Repeat Finder output and clustered using USEARCH. A single major cluster was identified for each species and the sequences were separately aligned using MAFFT. Multiple sequence alignments for each species were used to generate a profile HMM representing the putative centromere sequences. We next used himsearch to search the genome assemblies against this HMM to identify all MAPS-extracted tetranucleotide HMMs. Genomic regions with significant HMM matches were identified and these regions were annotated in a GFF3 format.

Gene prediction. Protein-coding genes were predicted using MAKER-P (v2.3.18), and three MAKER-P iterations were performed: (1) repeat masking and creation of initial gene models from transcript and homologous protein evidence; (2) refinement of initial models with SNAP−1 ab initio gene predictor trained on initial models; and (3) final models generated using SNAP and the ab initio gene predictor AUGUSTUS trained on gene models from the second iteration.

Masking was performed by RepeatMasker (v4.0.5) using the previously described species-specific repeat regions and repeat libraries and the full Repbase v22.0 database. After masking, gene models were inferred from transcripts and homologous protein sequences by first aligning to the genomes using BLAST+ (v2.2.31) blastn or blastp, and then refined using the functions est2genome and protein2genome from the splice-site aware alignment program Exonerate (v2.2.0). We included the previously described A. 2; 1 genome RNA from the flowering and submerged leaves was separately extracted using the Sigma Spectrum Plant Total RNA kit, each with three biological replicates. To examine patterns of RNA editing, one library per leaf type was prepared by the Illumina Ribozero Plant kit (that is, poly-A enriched), whereas the other two were done by the Kapa Stranded mRNA-seq kit. These six RNA libraries were pooled and sequenced in one lane of Illumina HiSeq2000 (125 bp paired-end).

For the null simulations, we first estimated the mean background gene count distribution across all non-plant genomes using PBcR and the resulting drafts were then polished by WGDgc. Gene count data were obtained from OrthoFinder clusters associated with each species tree. We discarded the remaining OrthoFinder clusters. We only kept gene families that contained at least one gene copy from each taxon in a given MAPS analysis. We translated each transcriptome into amino acid sequences using the TransPipe pipeline. Using these translations, we performed reciprocal protein BLAST (blastp) searches among datasets for each MAPS analysis using an E-value cut-off of 1×10−10. We clustered gene families from the BLAST results with the default parameters and only kept gene families that contained at least one gene copy from each taxon in a given MAPS analysis. We discarded the remaining OrthoFinder clusters. We used PASTA for automatic alignment and phylogenetic reconstruction of gene families, employing AUGUSTUS for constructing alignments, MUSCLE for merging alignments and RAXML for tree estimation. The parameters for each software package were the default options for PASTA. For each gene family phylogeny, we ran PASTA until we reached three iterations without an improvement in the likelihood score using a centroid breaking strategy. We used the best-scoring PASTA tree for each multi-species nuclear gene family to infer and locate WGDs using MAPS.

For the null simulations, we first estimated the mean background gene duplication rates and the gene loss rate, with WGDs. Gene count data were obtained from OrthoFinder clusters associated with each species tree. We chose a maximum gene family

| (0.66 × 10−6 × F − S2n) + 0.33 pg × (S4n − F1)/(S4n − S2n) | For all other samples, we used: 5.57 pg × F/S2n, where 0.66, 0.33 and 5.57 pg are the 4-value of A. thaliana, the 2-value of A. thaliana and the 4-value of Z. mays CE-777, respectively. S2n, S4n and F are the relative fluorescence amount (that is, the peak mean value) of the standard 2n nuclei, standard 4n nuclei and the sample 2n nuclei, respectively.

Genome and transcriptome sequencing. A. filiculoides was collected from the Galgenwaard ditch in Utrecht, the Netherlands, and propagated directly or sterilized as described in Dijkhuizen et al. A. filiculoides (sterilized without cyanobionts) DNA was extracted, then sequenced on PacBio RSII at 51x coverage and Illumina HiSeq2000 (100 bp paired-end; --86x coverage; Supplementary Table 12) with library insert sizes of 175 bp and 340 bp. RNA sequencing (RNA-seq) data from A. filiculoides of the Galgenwaard ditch used for annotation included the following RNA profiles: (1) at four time points during the diel cycle of fern sporophyte leaves, (2) for ornamental ferns without 2n ammonium nitrate for 1 week; (2) of different reproductive stages comparing fern sporophytes, microsporocarps and megalosporocarps collected at noon; (3) of roots treated with cytokinin, indole-3-acetic acid (IAA) or none; and (4) of sporophytes with or without cyanobacterial symbionts grown with or without ammonium nitrate for 2 weeks then collected at noon. Plant materials of A. filiculoides, Azolla coccinalta, Azolla nilotica and Azolla rubra were obtained from the International Rice Research Institute (Supplementary Table 1) and DNA was extracted by a modified cetyltrimethylammonium bromide (CTAB) procedure. Illumina libraries with a 500-bp insert size were prepared and sequenced on Illumina HiSeq2000 (100 bp paired-end; --50x coverage; Supplementary Table 2). S. cucullata was originally collected from Bangladesh and subsequently cultured at Taiwan Forestry Research Institute, Dr. Cecilia Koo Botanic Conservation Center and Duke University (Supplementary Table 1). Genomic DNA was purified using a modified CTAB procedure and sequenced on both PacBio RSII (10 SMRT cells; 46x coverage) and Illumina HiSeq2000 platforms (1 lane of 125 bp paired-end; --50x coverage; Supplementary Table 12). S. cucullata and S. filiculoides were obtained from the International Rice Research Institute (Supplementary Table 1) and DNA was extracted by a modified cetyltrimethylammonium bromide (CTAB) procedure. Illumina libraries with a 500-bp insert size were prepared and sequenced on Illumina HiSeq2000 (100 bp paired-end; -28x coverage; Supplementary Table 12). The genome of S. filiculoides was assembled using Mitobim and annotated in Geneious with manual adjustments. The PBCR contigs were filtered to remove plastome fragments. Although the A. filiculoides strain we sequenced was surface sterilized and treated with antibiotics to remove its associated microbe, other endophytes could still persist, as shown by Dijkhuizen et al. Thus, we thoroughly assessed the A. filiculoides and S. cucullata assemblies to filter out all possible non-plant contigs. We used BlobTools in combination with SILVA and UniProt databases to infer the taxonomy for each scaffold. We removed all scaffolds that were classified as bacteria or fungus and also those that had a skewed GC content and read coverage. The completeness of each final assembly was assessed by BUSCO with the Plants set, and by using RNA−1 and HISAT2−1 to map Illumina reads to the assemblies (Supplementary Table 2).

Repeat annotation. RepeatModeler was used to generate species-specific repeat libraries for masking and annotation. Consensus repeat sequences with homology to known plant genes were removed from the repeat libraries. Homology was defined as having a significant (E-value <1 × 10−10) blastn hit to a subset of the PlantTreev1.1 database that does not contain transposable element-related terms. Filtered RepeatModeler libraries were annotated with the name of the highest-scoring significant Repbasev22.04 full database sequence (tblastx, E-value <1 × 10−10) and the highest-scoring significant Dfamv2.0 profile hidden Markov model (HMM) (E-value <1 × 10−10) predictions.

LTR-RTs were discovered using structural criteria by the GenomeTools program LTRHarvest with the following modifications to the default settings: a LTR similarity threshold of 0.01, an allowed LTR length range of 100–250 bp, an allowed distance between LTRs of a single element range of 1,000–25,000 bp and the number of bases outside LTR boundaries to search for target-site duplications set to 10. The GenomeTools program LTRDive was used with a set of 133 transposable element-related Pfam profile HMMs to annotate protein-coding domains in the internal regions of LTR-RTs. We used 38 previously published A. filiculoides RNA-seq libraries and 6 S. cucullata libraries (see above) to assemble transcriptomes for facilitating gene model predictions. RepeatModeler, A. filiculoides and S. cucullata RNA-seq libraries were processed using a combination of Scythe and Sickle or SOAPouke, with adapter and contaminating sequences discovered using FastQC (v0.11.5). Approximately 627 million (A. filiculoides) and 259 million (S. cucullata) cleaned paired reads entered the assemblies. Libraries from experimental replicates were combined and assembled de novo by Trinity (v2.0.6) and in a reference-guided
size of 100 for parameter estimation, which was necessary to provide an upper bound for numerical integration of node states\(^{41}\). We provided a prior probability distribution of 1.3 on the number of genes at the root of each species tree, such that ancestral gene family sizes followed a shifted geometric distribution with a mean equal to the average number of genes per gene family across species.

Gene trees were then simulated within each MAPS species tree using the GuestTreeGen program from GenPhyloData\(^{39}\). We developed ultrametric species trees from the topological relationships inferred by the 1KP Consortium analyses and median branch lengths from TimeTree\(^{40}\). For each species tree, we simulate 4,000 gene trees with at least one tip per species: 1,000 gene trees at the \(\mu\) and \(2\mu\) maximum likelihood estimates, 1,000 gene trees at half the estimated \(\lambda\) and \(\mu\), 1,000 trees at three times \(\lambda\) and \(\mu\), and 1,000 trees at five times \(\lambda\) and \(\mu\).

Classification of syntropic duplicates and microsynten analysis. To distinguish gene duplicates as syntonic or tandem, we used the SynMap\(^{42}\) tool from the CoGe\(^{43}\) platform, with default parameters and the Quota Align algorithm to merge syntonic blocks. Sets of syntonic paralogues or orthologues (defined by a collinear series of putative homologous genes) were extracted using the DAGChainer algorithm, whereas duplicates within ten genes apart in the same genomic region were identified as tandem duplicates (Supplementary Discussion).

Results for within Azolla and Salvina genome comparisons, as well as between Azolla and Salvina, can be regenerated using the links https://genomevolution.org/r/toz7 and https://genomevolution.org/e/toy, respectively. Microsynten analyses were performed using the GEvo tool from CoGe\(^{43}\). We used an empirical setting to define the minimum number of column genes for two regions to be called syntonic. Non-coding regions were masked in the two genomes to include only the protein-coding sequences. The two example microsynteny shown in Fig. 5c can be regenerated at https://genomevolution.org/r/ri/eff and https://genomevolution.org/r/riyks.

Gaussian mixture model analysis of \(K\) \(K\) distributions. Estimates of \(K\) were obtained for all pairs of syntonic paralogous and orthologous genes using the CODEML program\(^{44}\) in the PAML package (v.4.8)\(^{45}\) on the basis of codon sequence alignments. We used the GY model with stationary codon frequencies empirically estimated by the F3 x 4 model. Codon sequences were aligned with PRANK (version 100701) using the empirical codon model\(^{46}\) (setting -codon) to align coding DNA, always skipping insertions (-F). Only gene pairs with \(K\) values in the range of 0.05–5 were considered for further analyses. Gaussian mixture models were fitted to the resulting frequency distributions of \(K\) values by means of the densityEMclust function in the R clusten version 5.3 package\(^{47}\).

The Bayesian inference criteria were used to determine the best-fitting model for the data, including the optimal number of Gaussian components to a maximum of nine. For each component, several parameters were computed including the mean and the variance, as well as the density mixing probabilities and the total number of gene pairs.

Gene family classification and ancestral reconstruction. The OrthoFinder\(^{48}\) clustering method was used to classify complete proteomes of 23 sequenced green plant genomes, including A. filiculoides and S. cucullata (Supplementary Table 5), into orthologous gene lineages (that is, orthogroups). We selected taxa that represented all of the major land plant and green algal lineages, including six core eudicotyledons (A. thaliana, A. thaliana, Populus trichocarpa, Solanum lycopersicum, Erythrina guntana and Vitis vinifera), five monocots (O. sativa, Sorghum bicolor, Musa acuminata, Zosteria marina and Spirodella polyrhiza), one basal angiosperm (A. trichopoda), two gymnosperms (Pinus taeda and Picea abies), two ferns (A. filiculoides and S. cucullata), one lycophyte (S. moellendorfii), four bryophytes (Siphagnum fallax, P. patens, Marchantia polymorpha and Funaria hydranthera) and two green algae (Klebsormidium, ScGus and C. reinhardtii). In total, 16,817 orthogroups containing at least two genes were circumscribed, 8,680 of which contain at least one gene from either A. filiculoides or S. cucullata. Of the 20,203 annotated A. filiculoides genes and the 19,780 annotated S. cucullata genes, 17,941 (89%) and 16,807 (84%) were classified into orthogroups, respectively. The details of orthogroup dynamics for the key ancestral nodes that include seed plants, such as Salviniaceae, euphyllophytes and vascular plants, are reported in Supplementary Table 5.

We used Wagner parsimony implemented in the program Count\(^{49}\) with a weighted gene gain penalty of 1.2 to reconstruct the ancestral gene content at key nodes in the phylogeny of the 23 land plants and green algae species (Supplementary Table 5). The ancestral gene content dynamics—gains, losses, expansions and contractions—are depicted in Supplementary Fig. 5. Complete details of orthogroup dynamics for the key ancestral nodes that include seed plants, such as Salviniaceae, eufylophytes and vascular plants, are reported in Supplementary Table 5.

Transcription-associated protein characterization. Transcription-associated proteins comprise transcription factors that bind in a sequence-specific manner to cis-regulatory DNA elements and transcriptional regulators that act via protein–protein interaction or chromatin modification. We conducted genome-wide, domain-based annotation of transcription-associated proteins according to previous studies\(^{50,51}\). A total of 1,206 (6%, Azolla) and 983 (7%, Salvina) proteins were sorted into families; this amount is similar to Selaginella but less than in gymnosperms or angiosperms (Supplementary Table 8).

PPR annotation. We conducted a targeted annotation for PPR genes because they are generally only weakly expressed and thus often lack transcriptome support. Open reading frames from the nuclear genome assemblies were translated into amino acid sequences using the "getorf" tool from the EMBOSS (v.6.5.7) package\(^{52}\) with a minimum size restriction of 300 nucleotides. These open reading frames were searched for PPR motifs using the hmmsearch tool from the HMMER3 package\(^{52}\). The PPR motif models and parameters used follow those of Cheng et al.\(^{53}\).

Motifs were assembled into full PPR tracts and the best model for each PPR was determined\(^{54}\).

To study the prevalence and location of RNA editing, non-poly(A)-enriched RNA data were filtered to remove adapters, low-quality reads and reads with 25%Ns. Clean reads were aligned against the assembled plastid and mitochondrial genome assemblies using TopHat 2.0 (ref\(^{55}\)). One of the inverted repeat regions in the plastid genomes was removed before mapping. Only uniquely mapped reads were retained as input for SAMtools\(^{56}\) to call mismatches between paired-end reads. Differences between corresponding RNA and DNA sequences were identified as the putative RNA-editing sites. The RNA-editing level was defined as the number of altered reads divided by the total mapped reads for each site.

Phylogeny of the insecticidal protein Tma12. We used BLASTp\(^{57}\) to search for Tma12 (Genbank accession: 3Q438776) homologues in Phytomyzoz\(^{58}\), 1KP transcriptomes\(^{59}\) and the NCBI Genbank non-redundant protein database. Although Tma12 homologues are present in fern transcriptomes and in the S. cucullata genome, no significant hit was found in any other plant genomes or transcriptomes. In addition, the majority of the Tma12 protein is composed of a circular coding domain that begins to the 5KF03067 Pfam family. This domain does not contain any plant genes but is predominantly represented in the genomes of Actinobacteria, insects and fungi. To trace the origin of fern Tma12 genes, we downloaded representative sequences containing Pf03067 and Pf08329 (as the outgroup) from UniProt and Genbank and reconstructed the phylogeny using IQ-TREE\(^{60}\). We then used this preliminary phylogeny (Supplementary Data) to construct a more focused data set to narrow down the phylogenetic affinity of Tma12. PartitionFinder\(^{61}\) was used to infer the optimal codon partition scheme and substitution models, and RAxML\(^{62}\) was used for maximum likelihood phylogeny inference and to calculate bootstrap branch support.

Azolla phylogeny. From the resequencing data (Supplementary Table 12), we compiled both plastome and nuclear phylogenomic data sets to infer the Azolla species phylogeny. S. cucullata was used as the outgroup. For the plastome phylogeny, we concatenated nucleotide alignments from 83 protein-coding genes and used PartitionFinder\(^{61}\) to identify the optimal data partition scheme and the associated nucleotide substitution models. RAxML\(^{62}\) was used for maximum likelihood phylogeny inference and to calculate bootstrap branch support. For the nuclear data set, we focused on genes that, based on the gene family classification results, are single copy in both A. filiculoides and S. cucullata genomes. We used HybPiper\(^{63}\) to extract the exon sequences from each of the resequenced species. The tree topology instead of the 1KP tree was used to estimate the best tree on the final alignment. To infer the species tree from these gene trees, we used the multi-species coalescent method implemented in ASTRAL-III (v.5.6.1)\(^{64}\). The tree topology from the plastome and nuclear data sets were identical, and all nodes received bootstrap support of 100 and a local posterior probability of 1.0.

Cyanobiont phylogeny. To compare the host and symbiont phylogenies, we assembled the cyanobiont genomes from five additional Azolla species (Supplementary Table 12) using the resequencing data generated from total DNAs, including sequences derived from both the host and the cyanobiont. To extract the cyanobiont genomes from each of the Azolla species, we first filtered out chloroplast sequences by using BWA (default parameters) to map the total clean DNA reads against each chloroplast genome reference. In this step, ~3–4% of the reads were filtered out, which is necessary to remove plastid ribosomal RNAs that are highly similar to ones in the cyanobionts. For each of the five Azolla species, we then mapped the filtered reads to the published cyanobiont reference (N. azollae 0708 isolated from A. filiculoides\(^{65}\); Genbank accession: NC_014248) using BLAT (alignment criteria: E-value ≤ 10\(^{-10}\), sequence similarity > 90%, and an aligned coverage of ≥0.90). Only the aligned reads were assembled by Mitobim\(^{66}\) (iterations = 5) using N. azollae 0708 (ref\(^{67}\)) as a reference. Gene prediction for each assembled cyanobiont was performed by the Prodigal program\(^{68}\). Transfer RNAs were predicted by tRNAscan-SE\(^{69}\) using a bacterial tRNA gene structure model. The presence of tRNA sequences (gene number and structure) for each cyanobiont appears to be more widespread in the Azolla species than in the cyanobionts of other genera.
was confirmed by mapping the rRNAs of *N. azollae* 0708 against each assembled genome cyanobiont sequence using BLAST. We used mugsy to generate the whole-genome alignment, which resulted in a nucleotide matrix of 5,354,840 characters. IQ-TREE was used for model testing and maximum likelihood tree inference. Because the *N. azollae* genome is reduced in size and is significantly diverged from other cyanobacteria, we could not find an appropriate outgroup to root the cyanobiont tree. To overcome this, we used STRIDE implemented in OrthoFinder to locate the root by reconciling gene trees. STRIDE was run with the default setting, except that MAFFT was used for alignment and RAxML for tree inference. The root was found to be the node placing the *A. nilotica* cyanobiont as sister to a clade comprising all other cyanobions. The reconciled species tree is identical to the tree reconstructed from the whole-genome alignment.

Identification of the CSP genes. The Medicago truncatula DM2, DM3, IPD3, CASTER/POLLUX and VAPYRIN sequences were used as queries, as in a previous study, to search against the genomes and transcriptomes from species listed in Supplementary Table 11 using BLASTn. For liverworts and ferns from the 1KP data set, non-annotated transcriptomes were used as targets, with the longest open reading frame of each contig extracted and translated. For *A. filiculoides* and *S. cucullata*, both the annotated genes and the unannotated genome contigs were used. All hits that matched already annotated gene models were discarded prior to subsequent analyses. No homologues were identified in the two fern genomes for IPD3 and VAPYRIN. Protein sequences for DM2/SYM/R, DM3/CaM/R and CASTOR/POLLUX were aligned using MAFFT. The best substitution model for each alignment (JTT for all alignments) was determined using MEGA6 (ref. 12). Phylogenetic trees were generated using RAxML on the CIPRES platform. Phylogenetic trees were generated using RAxML on the CIPRES platform. The reconciled species tree is identical to the tree reconstructed from the whole-genome alignment.

Quantitative real-time PCR of nifH. Quantitative real-time PCR for the *N. azollae* nifH gene was conducted using total RNA extracted from *A. filiculoides*. Primers were designed from *A. filiculoides* rRNA sequences. These highly redundant protein sequences were used for a DIAMOND BLASTx against a protein data set of 11 flowering plant species. This resulted in 30,312 Azolla genome contigs hitting 8,779 different cyanobacterial proteins that were used as a query in a BLASTP search against the Azolla genome; 340 Azolla contigs had reciprocal hits. To investigate whether these represent possible *Nostoc*–Azolla transfers or just examples of plastid-to-nucleus transfers, we used the 340 Azolla contigs for another BLASTx against the cyanobionts and extracted all 51,743 BLASTx-aligned Azolla contigs for analysis. These highly redundant protein sequences were used for a DIAMOND BLASTp against the non-redundant database of NCBI. Almost all of the sequences had streptophyte proteins as the top hit, and when not, phylogenetic analysis clearly placed them within streptophytes.

Phylogeny of SHC. Homologues of SHC and oxidosqualene cyclase were obtained by searching against Phytozome, 11K transcripts and the NCBI Genbank non-redundant protein database. Protein alignment was done in MUSCLE. We used IQ-TREE to find the best-fitting amino acid substitution model and infer the phylogeny using maximum likelihood. Bootstrap support was assessed with 1,000 pseudoreplicates. To test whether the monophyly of fern, lycophyte, moss and liverwort SHC could be rejected, we conducted a Swofford–Olsen–Waddell–Hillis test using SOWHAT. We compared the best maximum likelihood topology against the topology with all land plant SHC constrained to be monophyletic. SOWHAT was run with 1,000 replicates.

Detection of SHC-synthesized triterpenes. Freeze-dried *S. cucullata* biomass was Soxhlet extracted in a 9:1 DCM:MeOH mixture for 24 h. The total lipid extracts obtained were dried over Na2SO4 followed by evaporation of the solvent by a gentle stream of N2. Aliquots of the total lipid extracts were methylated with diazomethane to convert the acid groups into corresponding methyl esters, purified over a SiO2 column and silylated using bis(trimethylsilyl)trifluoracetamide (BSTFA) in pyridine at 60°C for 20 min to convert the hydroxy groups into the corresponding trimethylsilyl ethers. The total lipid extracts were on-column injected on a Thermo Trace GC Ultra Trace DSQ gas chromatography mass spectrometer (GC–MS) on a CP-sil SCB fused silica column (30 m × 0.32 mm internal diameter, film thickness: 0.10 μm). The GC–MS was operated at a constant flow of 1.0 ml min⁻¹. The GC oven was programmed starting at 70°C to rise to 130°C at a rate of 20°C per min and then to 320°C at a rate of 4°C per min, followed by an isothermal hold for 20 min.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The genome assemblies and annotations can be found at [www.fernbase.org](http://www.fernbase.org). The raw genomic and transcriptomic reads generated in this study were deposited in the NCBI SRA under the BioProject PRJNA340527 and PRJNA403459. The sequence alignments and tree files can be found in the Supplementary Data.

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Author contributions
F.-W.L., P.B., A.B., C.R., E.M.S., J.P.D., H.S., G.K.-S. and K.M.P. conceived the project. F.-W.L., J.P.D., H.S., G.K.-S. and K.M.P. coordinated the project. P.B., Y.-M.H., X.X. and H.S. provided plant materials. F.-W.L. and Y.-K. performed flow cytometry to estimate genome sizes. F.-W.L., S.C., B.H., X.L., Y.S., H.W. and X.X. undertook the sequencing activities. N.K. and A.B. assembled the Azolla genome. F.-W.L. and S.C. assembled the Salvinia genome. F.-W.L., S.C., X.L., Y.S., H.W. and X.X. assembled and annotated the symbiotic cyanobacteria genomes. M.S. and J.P.D. annotated the nuclear genomes. F.-W.L., T.R. and P.G.W. assembled and annotated the plastid genomes. M.S., S.A. and J.P.D. characterized the repeat content. L.C.-P., M.S., I.S., E.W., C.D., S.M., R.A.R., P.T., Y.V.D.P., P.K.W.J. and J.P.D. performed the gene functional annotation. L.C.-P., E.W., C.D., S.M., P.R.T. and Y.V.D.P. conducted the gene family classification. Z.L. and M.S.B. performed the MAPS analyses. L.C.-P., S.M. and Y.V.D.P. carried out the syntenic analyses. S.C., I.S., X.L., R.M., Y.S., H.W. and X.X. characterized the PPR gene family and RNA editing. J.D.V. and S.G. examined the cytochrome-b–Azolla HGT. P.M.D. characterized the common symbiosis genes. F.-W.L., S.C., A.E., X.L., Y.S., H.W. and X.X. carried out the RNA-seq analyses. F.-W.L. and P.B. conducted the phylogenetic analyses. P.B. and K.G.I.N. carried out the triterpene detection. P.H. and L.A.M. constructed FernBase. F.-W.L., P.B., L.C.-P., S.C., A.E., M.S., I.D., P.M.D., N.K., Y.-K., Z.L., I.S., E.W., J.P.D., H.S., G.K.-S. and K.M.P. contributed to writing the manuscript. F.-W.L. and K.M.P. organized the manuscript.

Competing interests
The authors declare no competing interests.

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The genome assemblies and annotations can be found in www.fernbase.org. The raw genomic and transcriptomic reads generated in this study were deposited in NCBI SRA under the BioProject PRJNA430527 and PRJNA430459. The sequence alignments and tree files can be found in Supplementary Data.

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Life sciences study design

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| Sample size | For phylogenetic analyses, bootstrapping datasets were sampled between 100 to 1000 times, which is the field standard. |
|-------------|-------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Gene models without transcript or homology supports were excluded (see the supplementary discussion). |
| Replication | The RNA-seq experiments were done with three biological replicates per treatment. |
| Randomization | The plant cultures for RNA-seq were placed on the same growth chamber shelf, but the positions were randomized in terms of nutrient treatments and symbiont types. |
| Blinding | Blinding is not applicable in this study. |

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| Materials & experimental systems | n/a | Involved in the study |
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| ☒ | ChIP-seq |
| ☐ | Flow cytometry |
| ☐ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials | The plant materials used in this study are available upon request (to F.-W. Li or Schluepmann) |
Flow Cytometry

Plots

Confirm that:

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Methodology

Sample preparation

We used flow cytometry to estimate the genome sizes of Pilularia americana, Regnellidium diphyllum, Marsilea minuta, Salvinia cucullata.

1. Prepare buffer for use.
   a. Allocate appropriate amount of Backmen stock buffer to a 50-ml tube based on an estimation of 1-1.5 ml per sample.
   b. Add 0.04 g PVP-40, 5 μl 2-mercaptoethanol, 1 μl RNase per ml of buffer.
2. Extract sample and standard nuclei by chopping leaf tissue
   a. Add 500 μl of buffer to a glass Petri dish.
   b. Add a (~400 mm²) piece of young leaf to the Petri dish, and chop it with a razor on ice until most tissue slices are less than 1 mm in size.
   c. Filter the chopped sample and standard into a 2.0-ml tube through a 30-μm nylon mesh.
   d. Add additional buffer to the sample, and ensure that the filtered leaf nuclei solution is greater than 500 μl in volume or more depending on need.
3. Staining nuclei solutions
   a. Mix sample nuclei and standard leaf nuclei solutions into a 500-μl volume in 2.0-ml tubes.
   b. Add 10 μl PI solution (2.04 mg/ml ) into each of mixed nuclei solutions.
   c. Incubate in the dark at 4 °C for 1 h for staining.

Recipes

Backmen stock buffer
1.0% Triton X-100
50 mM Na₂SO₃
50 mM Tris-HCl (pH 7.5)
ddH₂O (the solvent)

Note: Store at 4 °C up to 1 year.

Instrument

BD FACSCan system (BD Biosciences, Franklin Lake, NJ, USA)

Software

BD FACSCan system (BD Biosciences, Franklin Lake, NJ, USA)

Cell population abundance

Pilularia americana:
Replicate 1: sample peak particle number = 1514, standard1 peak particle number = 1154.
Replicate 2: sample peak particle number = 1834, standard1 peak particle number = 1371.
Replicate 3: sample peak particle number = 1450, standard1 peak particle number = 1036.

Regnellidium diphyllum:
Replicate 1: sample peak particle number = 1222, standard1 peak particle number = 1737.
Replicate 2: sample peak particle number = 1180, standard1 peak particle number = 1613.
Replicate 3: sample peak particle number = 1137, standard1 peak particle number = 1759.

Marsilea minuta:
Replicate 1: sample peak particle number = 1892, standard1 peak particle number = 1118.
Replicate 2: sample peak particle number = 1850, standard1 peak particle number = 1209.
Replicate 3: sample peak particle number = 1892, standard1 peak particle number = 1227.

Salvinia cucullata:
Replicate 1: sample peak particle number = 1084, standard1 peak particle number = 1484, standard2 peak particle number = 1170.
Replicate 2: sample peak particle number = 1129, standard1 peak particle number = 1552, standard2 peak particle number = 1253.
Replicate 3: sample peak particle number = 1229, standard1 peak particle number = 1584, standard2 peak particle number = 1500.

Gating strategy

For particle acquisition, we set a threshold of FL2-H = 52 for the samples of Pilularia americana, Regnellidium diphyllum, and Marsilea minuta. For Salvinia cucullata, a threshold of FL2-H = 100 is applied.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.