The Cycas genome and the early evolution of seed plants

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Cycads are often referred to as ‘living fossils’; they originated in the mid-Permian and dominated terrestrial ecosystems during the Mesozoic, a period called the ‘age of cycads and dinosaurs’. Although the major cycad lineages are ancient, modern cycad species emerged from several relatively recent diversifications. Cycads are long-lived woody plants that, unlike other extant gymnosperms, bear frond-like leaves clustered at the tip of the stem. Extant cycads comprise 10 genera and approximately 360 species, two-thirds of which are on the International Union for Conservation of Nature Red List of threatened species. All living cycad species are dioecious, with individual plants developing either male or female cones (except in Cycas, which produces a loose cluster of megasporophylls rather than a true female cone; Fig. 1a). Unlike other extant seed plants, cycads and Ginkgo retain flagellated sperm, an ancestral trait shared with bryophytes, lycophytes and ferns. Cycads exhibit other special features, such as the accumulation of toxins that deter herbivores in seeds and vegetative tissues. They also produce coralloid roots that host symbiotic cyanobacteria, making them the only gymnosperms associated with nitrogen-fixing symbionts. The origin of the seed marked one of the most important events of plant evolution. As one of the four extant gymnosperm groups (cycads, Ginkgo, conifers and gnetophytes), cycads hold an important evolutionary position for understanding the origin and early evolution of seed plants. We therefore generated a high-quality genome assembly for a species of Cycas to explore fundamental questions in seed plant evolution, including the phylogenetic position of cycads, the occurrence of ancient whole-genome duplications (WGDs), innovation in gene function and the evolution of sex determination.

A chromosome-scale genome assembly

Here, we report a high-quality, chromosome-level genome assembly of Cycas panzhihuaensis based on sequencing of the haploid megagametophyte using a combination of MGI-SEQ short-read, Oxford Nanopore long-read and Hi-C sequencing methods (Supplementary Note 2). The genome comprises 10.5 Gb assembled in 5,123 contigs (N50 = 12 Mb), with 95.3% of these contigs anchored to the largest 11 pseudomolecules, corresponding to the 11 chromosomes (n = 11) of the C. panzhihuaensis karyotype (Supplementary Note 3 and Extended Data Fig. 1). The annotated genome describes
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32,353 protein-coding genes and is mostly composed of repetitive elements adding up to 7.8 Gb (Supplementary Note 4). Based on BUSCO estimation, the gene space completeness of the C. panzhihuanaensis genome assembly is 91.6% (Supplementary Note 4).

Compared with other gymnosperms, the size of the Cycas genome is similar to that of Ginkgo (10.6 Gb)11,14 and intermediate between the relatively compact genome of Gnetum (4.1 Gb)15 and the very large genomes of conifers (for example, ~20-Gb genomes of Picea and Pinus)16–18. As in other gymnosperm genomes, a large portion (76.14%) of the C. panzhihuanaensis genome consists of ancient repetitive elements (Supplementary Note 4). In addition, the genome contains almost equal proportions of copia and gypsy long terminal repeat (LTR) elements, in contrast to other gymnosperm genomes, in which gypsy repeats are more frequent19,20 (Supplementary Note 6). Among all sequenced plant genomes, C. panzhihuanaensis has the longest average introns (~30.8 kb) and genes (~121.3 kb) (Extended Data Fig. 2a), surpassing those of Ginkgo11. In comparison with Ginkgo, in which LTRs dominate intron content, the introns of C. panzhihuanaensis contain a large portion of unknown sequences (Extended Data Fig. 2b). The longest gene, CYCAS_013063, encoding a kinesin-like protein KIF3A, covers 2.1 Mb in the C. panzhihuanaensis genome; the longest intron is approximately 1.5 Mb and was detected in CYCAS_030563, a gene that encodes a photosystem II CP43 reaction centre protein. Both genes are expressed, as evidenced by our long-read transcriptome data.

Phylogeny of cycads and seed plants

The C. panzhihuanaensis genome provides an opportunity to revisit the long-standing debate on the evolutionary relationships among living seed plants. On the basis of molecular phylogenetic analyses, extant gymnosperms are resolved as a monophyletic group, but the branching order among their major lineages has remained controversial19–24. Our phylogenetic analyses of separate nuclear (Fig. 1b, Extended Data Fig. 3 and Supplementary Note 5) and plastid datasets strongly support cycads plus Ginkgo as sister to the remaining extant gymnosperms, in agreement with several other analyses25–27, whereas mitochondrial data resolve cycads alone in that position (Fig. 1c). This conflict arising from the mitochondrial data cannot be explained by the presence of extensive RNA editing sites in the mitochondrial data (Fig. 1c), which in some cases has been reported to bias phylogenetic inferences28,29 and instead may be best explained by incomplete lineage sorting, which is supported by our PhyloNet27 and coalescent analyses of nuclear genes (Supplementary Note 5).

The extant diversity of cycads was previously considered to have arisen synchronously within the past 9–50 million years (Myr)29. Our inferences, based on 1,170 low-copy nuclear genes sampled for 339 cycad species and 6 fossil calibrations30 corroborate recent broad analyses of gymnosperm comparisons indicating that extant species-rich cycad genera emerged from rapid radiations ranging from 11 to 20 Myr ago, which may have been a consequence of dramatic Miocene global temperature changes30,31. Notably, major temperate and tropical radiations in several major clades of flowering plants have been shown to be associated with Miocene cooling in the past 15 Myr (refs. 20–21).

Cycas is an ancient polyploid

WGD is a major driving force in the evolution of land plants and has dramatically promoted the diversification of flowering plants32,33. Sympatric substitutions within synonymous sites (Ks) analysis of duplicate genes34 revealed a clear peak at similar Ks values (~0.85, range 0.5–1.2) for both Cycas and Ginkgo, suggestive of an ancient WGD possibly shared by these two lineages (Supplementary Note 7)34. However, the precise evolutionary position of this WGD event remains ambiguous. Our phylogenomic analyses based on 15 genomes and 1 transcriptome revealed 2,469 gymnosperm-wide duplications in 9,545 gene families and indicate that this WGD event dates to the most recent common ancestor (MRCA) of extant gymnosperms (Fig. 2a), supporting recent findings based on transcriptome data35. We also identified 69 ancient syntenic genomic segments that further support a gymnosperm-wide WGD (Extended Data Fig. 3, Supplementary Fig. 23 and Supplementary Tables 24 and 25). Furthermore, a mixed dataset with increased sampling—29 genomes and 61 transcripts—also yielded the same result (Fig. 2a and Extended Data Fig. 4). This gymnosperm-wide WGD, here named omega (ω), is independent of the WGD preceding the split between gymnosperms and angiosperms36 and may have contributed to the subsequent evolution of gymnosperm-specific genes involved in plant hormone signal transduction, biosynthesis of secondary metabolites, plant–pathogen interaction and terpenoid biosynthesis (Supplementary Note 7).

Ancestral gene innovation in the origin of the seed

The origin of seed plants is marked by the emergence of key traits including the seed, pollen and secondary growth of xylem and phloem37. Reconstruction of the evolution of gene families across the seed plant tree of life revealed that 663 orthogroups were gained and 368 expanded in the MRCA of extant seed plants compared with non-seed plants (Fig. 2b, node 1). Among these, 106 of the new orthogroups and 55 of the expanded orthogroups are associated with seed development in Arabidopsis38, including the regulation of development during early embryogenesis, seed dormancy and germination, and seed coat formation, as well as in immunity and stress response of the seed (Supplementary Note 6).

Genes of the LAFL family are well-known as core regulatory genes of seed development, including LEAFY COTYLEDON1 (LEC1), ABSCISIC ACID INSENSITIVE3 (ABI3), LEAFY COTYLEDON2 (LEC2) and FUSCA3 (FUS3), which encode master transcriptional regulators, interacting to form complex that control embryo...
development and maturation\textsuperscript{38}. \textit{LEC1} genes are found only in vascular plants, but \textit{ABI3} is widely distributed in embryophytes (Supplementary Note 10.6). \textit{Cycas} and \textit{Ginkgo} each contain a small number of \textit{LEC1} (two and three in each, respectively) and \textit{ABI3} (one in each) genes, whereas \textit{C. panzhihuaensis} encodes a burst of \textit{FUS3} (ten) and \textit{LEC2} (seven) genes in the form of tandem repeats. \textit{FUS3} and \textit{LEC2} are shared by all living seed plants; the \textit{Cycas} and other gymnosperm genomes contain genes composing a new clade of B3 domain proteins, that is, the \textit{FUS3/LEC2}-like clade, which is sister to the clade of \textit{FUS3} and \textit{LEC2} (Extended Data Fig. 5). The \textit{FUS3/LEC2}-like families are unique to gymnosperms, show significant expression after pollination in \textit{C. panzhihuaensis} (Extended Data Fig. 5c) and may play specific roles in initiating embryogenesis in gymnosperms.

\textbf{Regulation of seed development in Cycas}

To better understand the dynamic changes in gene regulation and regulatory programmes during ovule pollination and fertilization, we performed a weighted correlation network analysis (WGCNA) and identified 11 co-expression modules at different developmental stages of the \textit{C. panzhihuaensis} ovule and seed (Fig. 3a). The modules are enriched in seed nutrition metabolic processes (M2, M6 and M8), membrane biosynthesis (M9, which may relate to the development of the integument) and genes synthesizing callose, a major component of the pollen tube (M4) (Supplementary Note 10). A survey of phytohormones showed that salicylic acid and jasmonic acid, which are both involved in pathogen resistance, were produced at higher levels in unpollinated ovules versus post-pollinated ovules (Fig. 3b), and genes involved in the biosynthesis of these two
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ovule in Cycas of abscisic acid and expressed the genes related to cell wall orga-
ning, underwent obvious expansions in the MRCA of extant seed
features are regulated in cycads relative to other gymnosperms will

Secondary growth and cell wall synthesis

Secondary growth is also a major innovation of seed plants, and it has been recognized from fossils of now-extinct progymno-

The evolution of pollen, pollen tube and sperm

Another major innovation during seed plant evolution is the pro-
duction of pollen and the pollen tube. We found that many genes

phytohormones were also more highly expressed in unpollinated
ovules, indicating the higher demand for these hormones as agents
of pathogen resistance in the unpollinated ovule. Gibberellin, which
is reported to regulate integument development in the ovules of
flowering plants, accumulated in the late stage of the pollinated
ovule in Cycas. We also found gene families related to integument
development (for example, those involved in cutin, suberine and
wax biosynthesis), with increased expression levels at the late stage
of the pollinated ovule. Fertilized ovules accumulated a high level
of abscisic acid and expressed the genes related to cell wall orga-

PHYLOGENETIC ANALYSIS OF EXPANSIONS AND CONTRACTIONS OF THE CUPIN PROTEIN FAMILY

Among genes related to seed development, the most notable is
the cupin protein family, expanded in Cycas panzhihuaensis compared
with all other green plants. Phylogenetic analysis revealed that the
cupin family can be subdivided into two groups: the germin-like
and seed storage protein (SSP)-encoding genes. Surprisingly, we
identified a new type of gene encoding vicilin-like storage proteins
in Cycas panzhihuaensis; this type appears to be homologous to the
vicilin-like antimicrobial peptides (v-AMP) and is organized as a
tandem gene array in the Cupin family can be subdivided into two
groups: the germin-like and seed storage protein (SSP)-encoding genes.
In Cycas panzhihuaensis, we found a new type of gene encoding vicilin-like storage proteins in C. panzhihuaensis; this type appears to be homologous to the vicilin-like antimicrobial peptides (v-AMP) and is organized as a tandem gene array in the C. panzhihuaensis genome (Fig. 3c). These v-AMP homologues are mostly expressed in C. panzhihuaensis at the late stage of pollinated ovules and fertilized ovules, with expression gradually decreasing during embryogenesis, suggesting the potentially important role of v-AMP genes in seed development (Fig. 3d and Supplementary Note 10.6).

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Fig. 2 | Ancient polyploidy events and evolution of gene families in seed plants. a, Inference of the number of gene families with duplicated genes surviving after WGD events mapped on a phylogenetic tree depicting the relationships among 16 vascular plants included in this study. The number of gene families with retained gene duplicates reconciled on a particular branch of the species tree are shown above the branch across the phylogeny (Methods). Numbers in square brackets denote the number of gene families with duplicated genes also supported by synteny evidence. b, Evolutionary analyses and phylogenetic profiles depicting the gains (light green), losses (light red), expansions (light yellow) and contractions (light blue) of orthogroups, according to the reconstruction of the ancestral gene content at key nodes and the dynamic changes of the lineage-specific gene characteristics.
regulating pollen and pollen tube development (pollen maturation, pollen tube growth, pollen tube perception and prevention of multiple-pollen tube attraction) were gained (or the respective gene family expanded) in the MRCA of extant seed plants (Fig. 2b), as might be predicted for these features. For instance, those genes encoding egg cell-secreted proteins that prevent attraction of multiple pollen tubes originated in the MRCA of living seed plants. The *Ole e 1*-like gene families, which encode proteins that accumulate in the pollen tube cell wall and play a role in pollen germination and pollen tube growth, are remarkably expanded in the MRCA of extant seed plants compared with non-seed plants, and the MRCA of living seed plants compared with non-seed plants, and the evolution of seed storage proteins.

**Fig. 3** | Gene expression and phytohormone synthesis at different developmental stages of the seed of *Cycas* and the evolution of seed storage proteins. a, Heatmap showing relative expression of genes in 11 co-expression modules by WGCNA across 4 developmental stages of the seed: S1, unpollinated ovule; S2, early stage of pollinated ovule; S3, late stage of pollinated ovule; and S4, fertilized ovule. b, Quantification of eight plant phytohormone amounts in the same four developmental stages of the *Cycas* seed as above. The grey histogram represents the amount of hormone (n = 2 biologically independent experiments) and the error bar represents the standard error. c, Phylogeny of SSps in some representative species in land plants. The SSps analysed include germin-like protein (GLP), legumin-like SSP (l-SSp), vicilin-like SSP (v-SSp) and v-AMP. A maximum likelihood tree with 500 bootstrap replicates was constructed using RAxML. Bootstrap values (≥50%) for each major clade (highlighted in colour) and the relationships among them are provided. The *Cycas* sequences are highlighted in red. d, Expression levels of SSP in different tissues of *C. parviflora*.
Cobra-like protein localizes at the tip of the pollen tube membrane and plays an important role in pollen tube growth and guidance (Supplementary Note 11).

All seed plants produce pollen and deliver their sperm through the growth of a pollen tube, whereas all non-seed land plants (that is, bryophytes, lycophytes and ferns) rely on free-swimming motile sperm for sexual reproduction, as do the ancestors of land plants (Extended Data Fig. 7a,b). The exceptions among seed plants are cycads and Ginkgo, both of which have pollen grains that release motile spermatozoids that, following pollination, swim the remaining minute distance within the ovule to fertilize the egg (Supplementary Video 1). Sperm motility is conferred by a flagellar apparatus, and most genes related to its assembly occur in the C. panzhihuaensis genome. Ginkgo also retains flagellar genes, although fewer, and most notably lacks those encoding radial spoke proteins (RSP) (that is, RSP2, RSP3, RSP9 and RSP11; Extended Data Fig. 7c). By contrast, Gnetum, conifers and angiosperms, which develop non-flagellated spermatozoa, lost many flagellar genes.

**Fig. 4 | Identification of male-specific chromosomal region in Cycas.**

- **a**, Manhattan plot of GWAS analysis of sex differentiation in 31 male and 31 female Cycas samples. The red horizontal dashed line represents the Bonferroni-corrected threshold for genome-wide significance (α = 0.05). P values were calculated from a mixed linear model association of SNPs. Association analyses were performed once with a population of 31 male and 31 female individuals. **b**, Ratio of FST and difference of pooled heterozygosity (∆H) within a 100-kb sliding window between the female and male sequences. Colour represents values from low (blue) to high (red). **c**, Genome alignment of the MSY scaffolds with the corresponding female-specific region on chromosome 8. Scaffolds are separated by grey dashed lines. Red lines represent alignments >5 kb on the forward strand, and blue lines represent those on the reverse strand. Pink boxes in **a–c** represent the most differentiated regions between the sex chromosomes. **d**, Photographs of microsporophyll and megasporophyll of C. panzhihuaensis. Bar, 1 cm. **e**, Sex-specific expression of MADS-Y (CYCAS_034085) and CYCAS_010388 in male and female reproductive organs. Microsporophyll tissues were collected before meiosis (BFm), during prophase (Prophase), after meiosis (AFm) and before pollination (BFp); female tissues were collected at 0, 7, 11 and 21 days post-pollination. **f**, Phylogeny of MADS-Y homologues across land plants. Genes from MSY and autosomes are marked on the right, and those from Selaginella and Physcomitrium are used as outgroups. Numbers above branches represent bootstrap scores from IQ-TREE. **g**, Molecular genotyping of male and female cycad samples from Cycas deboeoeu, Macrozamia lucida and Zamia furfuracea using primers specific to homologues of MADS-Y and CYCAS_010388.
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association studies (GWAS) analysis of sex as a binary phenotype
mechanism of sex determination, we carried out genome-wide

Cycas
Sex chromosomes and sex determination in
late to non-flagellate sperm cells.

unique accessory structures that maintain the structural integrity
structural genes (Supplementary Note 12). Outer dense fibres are
are unique accessory structures that maintain the structural integrity
flagella and are vital for flagellar function [55]. Outer dense fibres exist in C. panzhihuaensis and Gingko biloba, as well as all non-seed
land plants, but are absent in Gnetum, conifers and angiosperms, all of which have non-motile sperm (Extended Data Fig. 7c).
The shift from swimming to non-motile sperm is a major innovation in
land plant evolution, and C. panzhihuaensis and G. biloba exhibit an
ancestral genetic content that is part of the shift from producing flagel-
late to non-flagellate sperm cells.

Sex chromosomes and sex determination in Cycas
Heteromorphic chromosomes have been reported to be associated
with sex determination in Cycas[44]. To reveal the underlying genetic
mechanism of sex determination, we carried out genome-wide
association studies (GWAS) analysis of sex as a binary phenotype
for C. panzhihuaensis and identified the most significant association
signals on chromosome 8, spanning the first 124 Mb on the refer-
ence female genome (Fig. 4a). This sex-associated region is also the
most differentiated between male and female Cycas genomes, with
the largest fixation index (FST; Supplementary Fig. 37) and the most
differentiated nucleotide diversity (σ2) and heterozygosity ratios
characterizing the window between 18 and 50 Mb on chromosome
8 (Fig. 4b and Supplementary Note 13). These results confirm that
Cycas possesses an XY sex determination system positioned on
chromosome 8.

Assembling the male-specific region of the Y chromosome
(MSY) based on Nanopore long-read and Hi-C data resulted in
45.5 Mb of sequence distributed over 43 scaffolds, most of which
aligned to the sex-differentiation region on chromosome 8 (Fig. 4c
and Supplementary Fig. 38). The assembled MSY had an almost
80-Mb difference in length from the corresponding region on the
X chromosome, which agrees with the heteromorphy of the Cycas
sex chromosomes. We annotated 624 putative protein-coding
genes within the MSY, 11 of which were highly expressed (transcripts
per million (TPM) > 1) in the microsporophylls. The most
highly expressed gene in the MSY and also the most differentially
regulated gene between the two sexes is CYCAS_034085 (Fig.
4d,e and Extended Data Fig. 8), which encodes a GGM13-like
MADS-box transcription factor (TF), belonging to a lineage sister
to the angiosperm AP3/PI clade that plays crucial roles in
flower development. Its closest homologue, CYCAS_010388, was
identified on autosomal chromosome 2. In contrast to CYCAS_034085,
CYCAS_010388 was much more highly expressed in the ovule than
in the microsporophyll (Fig. 4e). A male-specific polymerase chain
reaction (PCR) product of CYCAS_034085 was amplified from all
tested male cycad samples, but was not detected in female samples,
whereas a CYCAS_010388-specific PCR product was amplified
in both males and females (Fig. 4g and Supplementary Fig. 39b).
Because of the presence in MSY and its exclusive expression pattern
in males, we named CYCAS_034085 as MADS-Y, a potential
sex determination gene.

Fig. 5 | Origin of a Cycas insecticidal protein. a, Phylogenetic analysis of the TcdA/TcdB pore-forming domain containing proteins shows that the genes
encoding four cytotoxin proteins of Cycas were likely acquired from fungi through an ancient horizontal gene transfer event. The maximum likelihood
tree was generated by RAxML with the PROTCLATGTr model and 1,000 bootstrap replicates. The numbers above the branches are bootstrap support
values. b, The expression level of four cytotoxin proteins in different tissues of
C. panzhihuaensis was generated by rAxML with the prOTCATGTr model and 1,000 bootstrap replicates. The numbers above the branches are bootstrap support
values. c, The expression level of four cytotoxin proteins in different tissues of
C. panzhihuaensis was likely acquired from fungi through an ancient horizontal gene transfer event. The maximum likelihood
tree was generated by RAxML with the PROTCLATGTr model and 1,000 bootstrap replicates. The numbers above the branches are bootstrap support
values. d, The expression level of four cytotoxin proteins in different tissues of
C. panzhihuaensis was generated by rAxML with the prOTCATGTr model and 1,000 bootstrap replicates. The numbers above the branches are bootstrap support
values. e, f, Morphologies of Plutella xylostella (e) and Helicoverpa armigera (f) after receiving PBS and cytotoxin treatments.
The reduced size of MSY compared with the X chromosome indicates that the Y chromosome of Cycles, unlike that reported for some angiosperms, underwent severe degeneration and gene loss. The most divergent 32-Mb region (between the 18 and 50 Mb locations) between the X and Y chromosomes probably represents an ancient evolutionary segment in the Cycles sex chromosomes. The broad association of the MADS-Y homologue with sex in cycads indicates a conserved sex determination system within this ancient lineage (Fig. 4f and Supplementary Fig. 39). Moreover, the presence of GbMADS4, a homologue of the Cycles MADS-Y, in Ginkgo male-specific contigs suggests that the same mechanism for sex determination might have originated before the split of cycads and Ginkgo, thus representing an ancient system of sex determination in seed plants.

**Evolution of disease and herbivore resistance genes**

All three types of immune receptors—CC-NBS-LRR (CNL), TIR-NBS-LRR (TNL) and RPW8-NBS-LRR (RNL)—show patterns of expansion in C. panzhihuaensis and other gymnosperms, compared with non-seed plants (Supplementary Note 14). CNLs are expanded widely in both gymnosperms and angiosperms, whereas the TNL family tends to have been more expanded in gymnosperms than in most angiosperms, indicating different evolutionary patterns of plant resistance (R) genes in these two lineages. Our data suggest that RNL genes occur widely in gymnosperms. The RNL family plays a critical role in downstream resistance signal transduction in angiosperms, and the broad occurrence of the RNL family in gymnosperms suggests that this signalling pathway may have been established no later than the origin of seed plants. Gene families encoding resistance-related proteins are greatly expanded in C. panzhihuaensis and other gymnosperm genomes compared with non-seed plants (Supplementary Note 14). For example, genes encoding endochitinases and chitinases as defences against chitin-containing fungal pathogens are expanded as tandem repeats in the C. panzhihuaensis and most gymnosperm genomes compared with other land plants.

Cycads comprise many more living species than Ginkgo, which was once diverse in the Mesozoic but includes only one extant species. One possible explanation is that cycads may have acquired enhanced resistance to pathogens and herbivores through encoding diversified resistance-related genes and the biosynthesis of diversified secondary compounds. Indeed, comparisons of the Cycles and Ginkgo genomes reveal many Cycles-specific orthogroups enriched in pathogen interaction pathways (Supplementary Note 14), and C. panzhihuaensis also shows remarkable expansions in plant immunity and stress response gene families compared with Ginkgo, including genes that encode programmed cell death, abiotic stress response, serine protease inhibitors against pests and ginkgolobin with antibacterial and antifungal activities (Supplementary Note 14).

Terpenoids are a diverse group of secondary metabolites encoded by terpene synthase (TPS) genes. Several TPS subfamilies (TPS-a to TPS-h1) are known in plants, among which the TPS-d family is unique to gymnosperms, and three of the four types of TPS-d were found in C. panzhihuaensis, with remarkable expansions of TPS-d2 compared with Ginkgo and most other gymnosperms (Supplementary Note 15). In addition, we identified a novel TPS subfamily in Cycles, with three copies in C. panzhihuaensis and eight copies in Cycles debaensis (Extended Data Fig. 9a). The gene expression levels of all TPS genes across different C. panzhihuaensis tissues (Extended Data Fig. 9b) reveal that many TPS genes are mainly expressed in the root (especially primary root and cortical root), microsporophyll and pollen sac, late stage of the pollinated ovule and fertilized ovule. The three Cycles-specific TPS genes were mainly expressed in the root and male cone, but one of them (CYCAS_009486) is particularly highly expressed in the megagametophyte and in the post-pollination and fertilized ovule.

Cycles obtained a cytotoxin defence gene via horizontal gene transfer

Genes of fungal or bacterial origin are rare in seed plants. However, we identified a gene family in the C. panzhihuaensis genome that appears to have been acquired from a microbial organism and that codes for a Pseudomonas fluorescens insecticidal toxin (fitD). The acquired genes are flanked by vertically inherited plant sequences. We further confirmed that the relevant assembled regions were free of bacterial contamination. Transcriptomes and PCR amplification from genomic DNA indicated that these genes occur in many Cycles species (Supplementary Note 16). The fitD gene family comprises four gene copies in the C. panzhihuaensis genome and three copies in the C. debaensis genome (Supplementary Table 51); each copy encodes a protein that is similar to the fit toxin and the ‘makes caterpillars floppy’ (mcf) toxin of the bacterium Photorhabdus luminescens, a lethal pathogen of insects. Both fit and mcf toxins are known for their insecticidal properties, and fit- or mcf-producing bacteria are often used in pest biocontrol. Phylogenetic analyses suggest that the fitD genes might have been acquired from fungi and then expanded before the divergence of C. panzhihuaensis and C. debaensis (Fig. 5a). The fitD family genes are mainly expressed in roots, reproductive tissues such as male cones, unpollinated or early stages of pollinated ovules and embryos (Fig. 5b). Injection of the synthesized C. panzhihuaensis fitD protein resulted in significantly higher mortality in larvae of both the diamondback moth (Plutella xylostella) and cotton bollworm (Helicoverpa armigera) (Fig. 5c,d). The acquisition of the fitD gene family may have provided an important defence for Cycles against insect pests.

**Conclusions**

The high-quality genome sequence for Cycles, the last major lineage of seed plants for which a high-quality genome assembly was lacking, closes an important gap in our understanding of genome structure and evolution in seed plants. This genome enables comparative genomics and phylogenomic analyses to unravel the genetic control of important traits in cycads and other gymnosperms, including a WGD shared by gymnosperms, a sex determination mechanism that appears to be shared by cycads and Ginkgo, and critical gene innovations including those that enable seed and pollen tube formation, as well as chemical defence.

**Methods**

**Plant materials.** Fresh megagametophytes of Cycles panzhihuaensis, cultivated in the garden of the Kunming Institute of Botany, Chinese Academy of Sciences, were collected for genome sequencing. The plant was originally transplanted from the Pudu River, Luquan county, Yunnan, China (25°57′33.2584″N, 102°43′41.5848″E) and the voucher specimen (collection number: PZHF03) has been deposited in the Herbarium of the Kunming Institute of Botany (KUN). For transcriptome sequencing, we sampled 12 different types of organs and tissues from C. panzhihuaensis, including megagametophyte, pollen sac, microsporophylls, apical meristem of stem, cortex of stem, pith of stem, cambium of stem, mature leaf, young leaf, primary root, precoraloid roots and coralloid roots (Supplementary Table 2). Ovule material was collected from two artificially pollinated individuals, and we divided the development stages into four: unpollinated ovule (before the artificial pollination), early stage of pollinated ovule (21 d after the artificial pollination), late stage of pollinated ovule (88 d after the artificial pollination) and fertilized ovule or seed (119 d after the artificial pollination) (Supplementary Tables 2 and 13). In addition, stem and root tissues of C. panzhihuaensis were used to generate full-length transcriptomes (Supplementary Table 2). For phylogenomic analyses, we newly generated transcriptomes of 47 gymnosperm genomes (Supplementary Tables 2 and 13). We also sequenced transcriptomes of 339 cycad species (Supplementary Tables 2 and 14). For population resequencing, fresh leaf samples were collected for 31 male and 31 female plants that were randomly sampled in the Cycles panzhihuaensis National Natural Reserve in Sichuan, China, where there is a population of approximately 38,000 C. panzhihuaensis individuals (Supplementary Table 4).

**DNA and RNA sequencing.** For genome sequencing, the genomic DNA was extracted by the QIAHEN Genomic kit followed the manufacturer’s instructions. Nanodrop and Qubit (Invitrogen) were used to quantify the DNA and RNA sequencing.

For genome sequencing, the genomic DNA was extracted by the QIAHEN Genomic kit followed the manufacturer’s instructions. Nanodrop and Qubit (Invitrogen) were used to quantify the DNA and RNA sequencing. For genome sequencing, the genomic DNA was extracted by the QIAHEN Genomic kit followed the manufacturer’s instructions. Nanodrop and Qubit (Invitrogen) were used to quantify the DNA and RNA sequencing. For genome sequencing, the genomic DNA was extracted by the QIAHEN Genomic kit followed the manufacturer’s instructions. Nanodrop and Qubit (Invitrogen) were used to quantify the DNA and RNA sequencing. For genome sequencing, the genomic DNA was extracted by the QIAHEN Genomic kit followed the manufacturer’s instructions. Nanodrop and Qubit (Invitrogen) were used to quantify the DNA and RNA sequencing. For genome sequencing, the genomic DNA was extracted by the QIAHEN Genomic kit followed the manufacturer’s instructions. Nanodrop and Qubit (Invitrogen) were used to quantify the DNA and RNA sequencing.
A Nanopore PromethION sequencer. The rest of the DNA was used to generate short-read sequences using an MGI-SEQ platform, with 150-bp read length and 300–500 DNA-fragment insert size. Hi-C libraries were created from fresh megagametophyte protoplasts using a previously published method. Briefly, the tissue was fixed in formaldehyde, lysed and the cross-linked DNA was digested overnight with HindIII. Sticky ends were biotinylated and proximity-ligated to generate chimeric junctions, which were subsequently physically sheared to 500–700 bp in size. The initial cross-linked long-distance physical interactions were then represented by characteristic, which were processed into paired-end sequencing libraries. Paired-end reads were produced on both the MGI-SEQ and Illumina HiSeq X platforms. See Supplementary Note 3 for details on transcriptome, organelle genome and small RNA sequencing.

**Genome assembly.** About 1.01 Gb (~100×) Nanopore long-read data were used for genome assembly using NextDenovo (https://github.com/Nextomics/NextDenovo) with default parameters (read_cutoff=1k, seed_cutoff=12k, minimap2._options_cns=-x ava-ont -k17 -w17). To further enhance assembly contiguity, about 456 Gb of Hi-C data were used to execute Hi-C chromosome conformation in conjunction with 3D-dna algorithm. The accuracy of Hi-C based chromosomal assembly was assessed using Juicerbox’s chromatin contact matrix.

**Repeat annotation.** We identified tandem repeats and transposable elements throughout the genome. Tandem repeats were predicted using Tandem Repeat Finder (v.4.47)77 with the following parameters: Mismatch = 2, Mismatch Score = 5, Delta = 7, PM = 80 and Period = 2,000, and to optimize the opportunity of identifying transposable elements, a combination of de novo and homology-based approaches was performed following the Repeat Library Construction-Advanced pipeline (http://weatherby.genetics.utah.edu/MAKER/wiki/index.php/Repeat_Library_Construction-Advanced). RepeatMasker70 and RepeatProteinMask71 were used to search for known repeat sequences; MITEFinder72 and LTR_FINDER73 were then used to search the repeats de novo. The MITE, LTR and consensus repetitive libraries generated by RepeatModeler were combined and further used as the input data for RepeatMasker.

**LTR identification and estimation of LTR insertion times.** All the candidate LTR elements were first identified using LTR_FINDER and LTR_retreiver. LTR_STRUC74 was then used to extract the complete 5' - and 3' -ends of the LTR elements. RepeatClassifier was then used to classify the candidate LTR. Distmat from the EMBoss (v.6.5.7) package was then used to calculate the K value of the retrotransposon. 5' - and 3' -LTR sequences. Finally, the insertion time (T) of LTRs was calculated using the formula \( T = \frac{1}{r} \), where \( r \) is the average substitution rate of 2,2×10^-8 substitutions per year per synonymous site.

**Gene annotation and functional annotation.** Three types of evidence were used to predict protein-coding genes in the *C. panzhihuaensis* genome. For protein evidence, Genewise was used to predict gene models based on Cytops transcripts downloaded from the UniProt protein database and other proteins collected from representative plant species. Next, Hisat77 was used to map the transcriptome to the genome, and then StringTie78 was used to predict transcriptome-based gene models. Next, a custom training hint parameter was used to predict ab initio-based gene models in AUGUSTUS79. The gene evidence was finally combined and integrated by EVidenceModeler80. To maximize the opportunity of identifying high-confidence genes, we further filtered the genes that were not expressed in the full-length transcriptome or did not match to functional annotation results. For functional annotation, the gene models were blasted against the UniProt, TREMBL, KEGG, KOG and NR databases. The domain and gene ontology of the gene models were identified by InterProScan+ (using data from Pfam, PRINTS, SMART, ProDom and PROSITE).

**Identification of key candidate functional genes.** Based on the following criteria, all candidate genes were screened: first, candidate gene sequences were detected by BLASTx search with a cut-off E value of 10^-4, and second, features of candidate genes should be similar to the online functional annotation or UniProt functional annotation as the query genes. With regard to the identification of flagellar genes, 58 flagellar-related genes were collected from previous studies100. The Reciprocal Best Blast hit method was employed to identify flagella-related genes. For seed-related genes, we searched the genes against both the known seed database (seedgenes.org) and previous studies. We firstly used an e value (1×10^-10) as a cut-off to filter candidates and then filtered the candidates with functional annotation. Regarding the identification of TFs, we used the HMMER search method. HMMER domain structure models were downloaded from the Pfam website (https://pfam.xfam.org), for each TF as present in the TAPscan v2 database for TFs (https://plantcode.online.uni-marburg.de/tapscan/). Preliminary TF candidate genes were collected for each species (<1×10^-10) by searching the Hidden Markov Model profile. Parts of genes were then filtered if they were not the homologues according to their functional annotation of SwissProt (<1×10^-10).

In the end, we filtered genes containing a wrong domain under the TAPscan v2 transcription factor database domain rules. Phylogenetic tree analysis was used to verify the majority of TFs and transcriptional regulators. Details about phylogenetic tree reconstruction for each TF can be found in the figure captions.

**Phylogenetic reconstruction and divergence-time estimation.** Nuclear phylogenetic reconstruction. The downloaded genome sequences and the newly generated genome sequences of *C. panzhihuaensis* and the newly orthologous groups using OrthoFinder101 with default settings. The software ProteinCutter102 was used to select, which exonic gene families for phylogenetic reconstruction with default parameters. TranslatorX103 was used to build gene alignments for codon (nt), codon 1st + 2nd (mt2) and amino acid (aa) sequences (command: perl translatox_vlocal.pl -i gene.fa -o gene.out -P f - t F - W 1 - c 1 -g "b1l-b2l-b3l-b4l-b5l-b6l""). IQ-TREE 2 (ref. 104) was used to infer the maximum likelihood trees with an initial partition scheme of codon positions combined to predict gene families, tree search, and ultrafast bootstrap. ASTRAL was used to summarize the coalescent species tree and the quartet supports with default settings (-t 8). ASTRAL uses the quartet trees of the maximum likelihood phylogenies of each gene to produce the topology of the species tree while quartet supports (bar charts) show the percentage of quartets that agree with a specific branch in the species tree. STAG (https://github.com/davidemms/STAG) was then used to construct the species tree with default settings using low-copy genes (one to four copies). The software PHYPARTS was used to infer and visualize the gene tree conflicts on the species tree topology with default settings. The software DISCOVISTA was used to summarize the conflicts among different analytical methods and datasets, and several global phylogenetic relationships.

**Molecular dating and diversification analysis.** The transcriptome sequencing reads from 339 cycad species were generated in the current study. Clean reads were assembled with TRINITY105, and the longest transcripts were selected and translated with TRANSDECORDER (https://github.com/TransDecoder). OrthoFinder was then used to construct ortofamilies for all the cycad species using Ginkgo as the outgroup. The software KinFin70 was used to select the mostly single–copy genes for phylogenetic reconstruction with default settings. TranslatorX103, IQ-TREE 2 (ref. 104) and ASTRAL were used to align the sequences and to infer the species tree for cycads as aforementioned. The software SORTADATE was used to select genes with mostly concordant evolutionary histories for dating analyses using MCMCtree within the software PAML 4 (ref. 106). Rate priors and time priors were set following the method of Morris et al.107. A total of 27 fossils were used to calibrate the chronogram of seed plants, and six fossils for the chronogram of cycads. The diversification pattern for cycads were analysed with Bayesian analysis of mixed evolutionary mixture (www.bannm-project.org) following Condamine et al.108.

See Supplementary Note 5 for details on organellar phylogenetic reconstruction, evaluation of the impact of RNA editing and investigation of cyto-nuclear incongruences.

**Identification of whole-genome duplication.** An integrated phylogenomic approach and a method to analyse synteny as described previously109 were used to identify the WGD events in seed plant evolution. The protein-coding sequences of 15 completely sequenced genomes and 1 transcriptome, representing seven gymnosperms (*C. panzhihuaensis*, *Encephalartos longifolius*, *G. brabhamii*, *G. grandiflorum*, *Picea abies* and *S. cucullata*), three angiosperms (*Arabidopsis thaliana*, *Amborella trichopoda*, *Cinnamomum microphyllum*, *Lividodendron chinense*, *Nymphaea colorata* and *Orzya sativa*) and three other vascular plant outgroups (*Azolla filiculoides*, *Salvinia cucullata* and *Selaginella moellendorffii*) were classified into putative gene families/subfamilies by OrthoFinder101, and then scored for gene duplications across global gene families. For the phylogenetic analysis of gene families, amino acid sequences of each gene family were first aligned with MAFFT110, the program PAL2NAL111 was then used to construct their corresponding nucleotide sequence alignments. We used trimAl111 to remove poorly aligned portions of alignments using the ‘automated1’ option, which implements a heuristic algorithm to optimize the process for maximum likelihood to the collected seed plants with default settings using RAxML112 with the GTRGAMMA model and bootstrap support was estimated based on 100 replicates. Following Wu et al.113, we applied two basic requirements for the determination of a reliable duplication event: (1) at least one common species’ genes are present in two child branches; and (2) the bootstrap values of the parental node and one of the child nodes are both ≥50%. After scoring gene duplications in a large-scale analysis on gene families, we were able to confidently identify the nodes with concentrated gene duplications across the phylogeny, which possibly support the WGD events. Furthermore, because syntenic information is the most solid evidence for WGD, and the legacy of syntenic blocks may be found if the concentrated gene duplications are indeed derived from WGD events, we also identified into whether the intra- and intergeneric syntenic blocks exist. The intra- and intergeneric syntenic analyses were conducted using MCScanX114, with the default settings.

In addition, the Nee–Gojobori method115 as implemented in the PAML package’s ynj00 program116 was used to estimate synonymous substitutions per synonymous site (Ks) for pairwise comparisons of paralogous genes located on syntenic blocks. To search for genome-wide duplications, we used DupGen, which was developed as an R package to search for genome-wide duplications.
Identification of the sex-differentiation region. To identify the sex-differentiation region in the Cycas genome, a GWAS approach was adopted on sequence variations from 31 male and 31 female individuals with sex treated as a binary phenotype. Briefly, raw reads were filtered by Trimmomatic (v.0.38) (ILLUMINACLIP:adapter.2:30:10 HEADCROP:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:5:15 MINLEN:140) and read alignment and single-nucleotide polymorphism (SNP) calling were performed using the Sentieon pipeline\(^{10}\), SNPs were filtered using the following criteria: (1) SNPs were filtered by GATK VariantFiltrations with ‘QD < 2.0 || FS > 60.0 || MQ < 40.0 || QD > 3.0 || MQRankSum > 12.5 || ReadPosRankSum > 8.0; and indels with ‘QD < 2.0 || FS > 200.0 || MQ > 10.0 || MQRankSum > 12.5 || ReadPosRankSum > 8.0.’; (2) total depth <80 or >1,300; (3) variants with more than two alleles; (4) variants with a missing rate >10% or minor allele frequencies <0.1 were removed; and (5) a linkage disequilibrium pruning with PLINK (v.1.9) using a window size of 10 kb with a step size of one SNP and \( r^2 \) threshold of 0.5, resulting a 4.65-million pruned SNP set for association analysis of sex differentiation. GWAS analysis of sex differentiation was performed on the linkage disequilibrium-pruned SNP set using the EMMAX program\(^{10}\) (beta-07Mar2010 version). The BN kinship matrix and the first five components calculated from the principal component analysis\(^{104}\) (v.1.91.4beta3) were included as random effects. Genetic differentiation (\( F_{ST} \)) and nucleotide diversity (\( \pi \)) were calculated within a non-overlapping 100-kb window using VCFtools (v.1.13). See Supplementary Note 13 for details on assembly of Cycas male-specific regions, phylogenetic analysis of MADS-Y and CYCAS_010388 homologues, and genotyping of cycad male and female samples.

Analysis of the differentially expressed genes. Transcriptome sequencing reads were trimmed using Trimmomatic\(^{106}\) program (ILLUMINACLIP:adapter.2:30:10 HEADCROP:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:5:15 MINLEN:140) and mapped against \( C.\) \( panzhihuaensis \) annotated gene models using bowtie2 (with sensitive mode and default alignment parameters) by retaining the best alignments. TPM were calculated using the eXpress program, which was incorporated in the Trinity\(^{89}\) package. Furthermore, differentially expressed genes with a differential expression level of false discovery rate \(< 0.01\) and at least a twofold expression change were identified using DESeq2 \(^{107}\). To identify the co-expressed genes during the seed development, we used the R package \( \text{pcGraph}^{29} \) to identify statistical associations between the expression levels, we normalized the expression results. For each gene, the \( \chi^2 \) association test was performed using a self-constructed database that was built using the reference standards. To accomplish quantitative analysis, different concentrations of standard were utilized.

Data availability

The genome and transcriptome data, genome assemblies and annotations can be found at https://db.cngb.org/cycadlist/projects/CNP0001756 and the CNGB data center (https://db.cngb.org/) under project number CNP0001756. Source data are provided with this paper.

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Detection of metabolites and phytohormones. The plant tissues were collected and stored in liquid nitrogen, then transferred to freezer at −80°C. For detection of metabolites, tissue samples were preliminarily disposed using 2-phenylethylamine (4 ppm) methanol. Samples and glass beads were then put into a tissue grinder for 90 s at 55 Hz, followed by centrifugation at 13,780g at 4°C for 10 min, taking the supernatant and filtering through a 0.22-μm membrane, and transferring the filtrate into the detection bottle before liquid chromatography mass spectrometry analysis. The sample extracts were the analyzed using the ultra-high-performance liquid chromatography system. Vanquish (ThermoFisher Scientific) and Q Exactive HF-X (ThermoFisher Scientific). For the quantitative detection of phytohormones (auxin, cytokinins, ethylene, abscisic acid, jasmonic acid, gibberellin, salicylic acid and brassinolide), tissue samples of primary root, precoralloid roots and coralloid roots, unpollinated ovule, early stage of pollinated ovule, late stage of pollinated ovule, fertilized ovule and mature embryo were collected. Vanquish (ThermoFisher Scientific) and the Q Exactive HF-X (ThermoFisher Scientific) were used for the detection of various phytohormones. The qualitative study was carried out using a self-constructed database that was built using the reference standards. To accomplish quantitative analysis, different concentrations of standard were utilized.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Author contributions

S.Z., H.L., X.G. and Y.L. led and managed the project. S.Z., H.L. and Yang Liu conceived the study. Yang Liu, S.W., L.L., S.D., T.W., J.M. and S. Wu wrote the manuscript. S.D., Y.G., X.F., A.J.L., Y.Y., X.G., D.L., N.L., H.W. and L.T. prepared materials. S.W., L.L., T.Y., Yang Liu, J.R., J.W., S. Zaman, J.-Y.X., J.C., Z.-Q.S., C.S., S.H., Na Li, M.L., G.F., H. Wang, J.Y., M. Lisby, S.K.S., W.M., Y.F. and Z.Z. performed bioinformatic analyses. J.H., J.M., G.C. and P.L. performed horizontal gene transfer analysis. T.W., S.L., X.W. and X.L. performed SDR analysis. S.D., Yang Liu, Y.G., J.L., Y.Y. and Jianquan Liu performed gene family clustering and comparative phylogenomics. S. Wu, Y.V.d.P., J.Y., Z.-J.L. and Z.L. performed WGD analysis. S.S., Y.V.d.P., D.E.S., B.G., X.-Q.W., J.H., E.C.S., E.W. and M. Lisby contributed substantially to revisions. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Genome features of *C. panzhihuaensis*. Outer ring: The 11 chromosomes are labeled from Chr1 to Chr11. Inner rings 1-4 (from outside to inside): Repeat elements number shown in light purple. GC content colored indicated in light blue (y-axis min-max: 0.27–0.48). Expressed base percentage colored in light blue (y-axis min-max: 0–0.20). Gene numbers colored in light orange (y-axis min-max: 0–30). The sliding window of the inner rings 1-4 is 1 Mb. The inner ring 5 indicates the miRNA location over the genome. The blue lines inside represent the syntenic regions in *Cycas*. 
Extended Data Fig. 2 | Comparative analysis of *C. panzhihuaensis*. Extended Data Fig. 2. Comparative analysis of *C. panzhihuaensis*. (a) Comparison of the longest 10% of introns and gene in the representative land plants. The minimum, first quartile (Q1), median, third quartile (Q3), and maximum value was indicated in the box-plot by order after excluding the outliers. (b) Comparison of components of intron across the selected plants.
Extended Data Fig. 3 | The chronogram of 90 vascular plant species inferred with MCMCTree based on 100 nuclear single copy genes with concordant evolutionary histories. 25 fossil calibrations and 2 secondary calibrations were used. Individual gene trees (1,569 NT tree) were mapped on the nuclear coalescent tree with Phyparts. The pie charts at each node show the proportion of genes in concordance (blue), conflict (green = a single dominant alternative; red = all other conflicting trees), and without enough information (gray). Quartet support for six internal branches I, II, III, IV, V, VI were indicated on the left panel as barcharts. Image courtesy of Zanqian Li and Xiaolian Zeng.
Extended Data Fig. 4 | Ancestral polyploidy events in extant gymnosperms. Example showing both the phylogenomic and syntenic evidence supporting an ancestral polyploidy event in extant gymnosperms. Four pairs of paralogous genes in OG0000093, OG0000255, OG00000276 and OG0000316 were duplicated before the divergence of gymnosperms and after the split of angiosperms and gymnosperms based on phylogenetic trees. These pairs of duplicated genes are located on the same syntenic block identified in the C. panzhihuaensis genome. The abbreviated name given before the protein ID represents species name: CYCAS: Cycas panzhihuaensis, Gb: Ginkgo biloba, ELO: Encephalartos longifolius, SEGI: Sequoiadendron giganteum, GMON: Gnetum montanum, PICABI: Picea abies, PITA: Pinus taeda.
Extended Data Fig. 5 | The phylogeny of LAFL(NF-YB, ABI3, FUS3, and LEC2) transcriptional regulators. (a) Phylogenetic tree of the NF-YB. The tree was constructed using the maximum likelihood method with 500 bootstrap replicates. The bootstrap values are shown on the branches. (b) Phylogenetic tree of the B3 domain containing the gene family of *C. panzhihuaensis*. Bootstrap values are shown on the branches. (c) Transcript expression level is indicated by TPM during seed development. The phylogenetic trees were built using RAxML (estimating branch support values by bootstrap iterations with 500 replicates) with PROTGAMMAGTrX amino acid substitution model. The abbreviated name given before the protein ID represents species name: CYCAS: *Cycas panzhihuaensis*, Gb: *Ginkgo biloba*, SEG1: *Sequoiadendron giganteum*, GMON: *Gnetum montanum*, PICABI: *Picea abies*, PITA: *Pinus taeda*, ATH: *Arabidopsis thaliana*, DEBAO: *Cycas debaoensis*, AMTR: *Amborella trichopoda*, OS: *Oryza sativa*, AFIL1: *Azolla filiculoides*, SACU: *Salvinia cucullata*, SELMO: *Selaginella moellendorffii*, PPATEH: *Physcomitrella patens*, MARPO: *Marchantia polymorpha*.
Extended Data Fig. 6 | Phylogenetic tree of CESA/CSL gene families. (a) Phylogenetic trees of CESA and CSL gene families. (b) Phylogenetic tree of CSLB and CSLH genes. (c) The phylogenetic tree of CSLE and CSLG genes. The CSLE/G from gymnosperm are the ancestral form of the angiosperm CSLE and CSLG. The phylogenetic trees were generated using RAxML with PROTCATGTR model and 500 bootstrap replicates. Bootstrap values $\geq 50\%$ are shown. The abbreviated name given before the protein ID represents species name: CYCAS: Cycas panzhihuaensis, Gb: Ginkgo biloba, SEGi: Sequoiadendron giganteum, GMON: Gnetum montanum, PICABI: Picea abies, PITA: Pinus taeda, ATH, Arabidopsis thaliana, DEBAO: Cycas debaoensis, AMTr: Amborella trichopoda, OS: Oryza sativa.
Extended Data Fig. 7 | The Evolution of flagella related genes in embrophyta. (a) Sketch of the Cycas sperm. (b) Schematic diagram of flagellum loss events in green lineage. (c) Distribution of outer dense fiber protein and other key flagellar proteins across representative embrophyta.
Extended Data Fig. 8 | The phylogeny and expression level of TPS. (a) Phylogenetic tree of the TPS gene family. The tree was constructed using RAxML (the maximum-likelihood method) with PROTCATGTR amino acid substitution model and 500 bootstrap replicates. The bootstrap values ≥ 50% are shown in the central branches. The red colors in the tree represent the cycas genes. (b) Heatmap of TPS gene family in different tissues of C. panzhihuaensis. The * denotes the C. panzhihuaensis specific TPS genes.
Extended Data Fig. 9 | Two MADS-box transcription factor genes differentially expressed in reproductive organs of *C. panzhihuaensis*. (a) Heatmap of 1,971 genes differentially expressed in males and females’ organs. Arrows indicate CYCAS_034085 on the MSY and CYCAS_010388 on chromosome 2. (b) Expression of CYCAS_034085 on MSY and CYCAS_010388 on chromosome 2 in male microsporophyll and in the ovule.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

The long read data were generated by the Nanopore PromethION sequencer, and the short read data were sequenced by the MGI-SEQ or Illumina HiSeq X platform, with 150-bp read length and 300-500 insert size.

Data analysis

The softwares used in this study are listed as follows:

- wtdbg2 version 2.5 (https://github.com/ruanjue/wtdbg2)
- miniasm version 0.3 (https://github.com/h3/miniasm)
- NextDenovo version 2.3.0 (https://github.com/Nextomics/NextDenovo)
- NextPolish version 1.2.4 (https://github.com/Nextomics/NextPolish)
- 3d-dna version 201008 (https://github.com/aidenlab/3d-dna)
- Juicebox version 1.13.01 (https://github.com/aidenlab/juicebox)
- MITE-hunter version 11-2011 (http://target.plantcollaborative.org/mite_hunter.html)
- LTRharvest version 1.5.10 (http://genometools.org/)
- BUSCO version 4.0.2 (https://busco.ezlab.org/)
- RepeatModeler version 2.0.1 (http://repeatmasker.org/RepeatModeler/)
- RepeatMasker version 4.0.7 (https://www.repeatmasker.org/)
- LTRdigest version 1.5.10 (https://genometools.org/)
- Genewise version 2 (https://www.ebi.ac.uk/Tools/psa/genewise/)
- Hisat version 2.2.1 (http://ccb.jhu.edu/software/htslib/index.shtml)
- Stringtie version 1.3.3b (https://ccb.jhu.edu/software/strangtie/)
- AUGUSTUS version 3.2.3 (https://bioinf.uni-greifswald.de/augustus/)
- EVIDencEModeler version 1.1.1 (https://evidencemodeler.github.io/)
- InterProScan version 5.30-69.0 (https://www.ebi.ac.uk/interpro/search/sequence/)
- miRDeep2 version 0.1.3 (https://github.com/rajewsky-lab/mirdeep2)
Data

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- Accession codes, unique identifiers, or web links for publicly available datasets
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The genome and transcriptome data, genome assemblies, and annotations can be found at https://db.cngb.org/codeplot/datasets/public_data?id=PwRGHPSqSGjE. The raw genomic, transcriptomic and HiC data generated in this study were deposited in the NCBI Sequence Read Archive [SRA, BioProject PRJNA734434], and the CNGB data center (https://db.cngb.org/) under project number CNP0001756.

Some known functional protein databases we used: Uniprot database (version 2021_01), KEGG (version 93.0), NCBI NR (version 20201015), KOG (version 20090331).

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
For genome sequencing, for each experiment, megagametophytes from a individual of Cyclus panzhihuaensis was used. For genome and transcriptome short-read sequencing for phylogeny studies, one sample from a same individual for each taxon was used.

Data exclusions
The sequence reads with low quality are more likely derived from sequencing errors, and were thus excluded. To reduce the effect of sequencing error on assembly, we performed the quality control of raw data using Trimmmomatic (v. 0.38).

Replication
Since this is a genome sequencing project, no replication was applied for our genome sequencing experiment. For RNA-seq in gene expression studies and metabolite measurements, three or two biological replicates were applied.

Randomization
Since this is a genome sequencing project. The data were generated from a single individual, no randomizations were required.

Blinding
Since this is a genome sequencing project. The data were generated from a single individual, no blinding experiment was performed.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

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Methods

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- [ ] Involved in the study
  - [ ] ChiP-seq
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