New Phytologist

Research

Is RNA editing truly absent in the complex thalloid liverworts (Marchantiopsida)? Evidence of extensive RNA editing from *Cyathodium cavernarum*

Chao Shen[1], Hao Xu[1], Wen-Zhuan Huang[1], Qiong Zhao[1] and Rui-Liang Zhu[1,2]

[1]Bryology Laboratory, School of Life Sciences, East China Normal University, Shanghai, 200241, China; [2]Tiantong National Station of Forest Ecosystem, Shanghai Key Lab for Urban Ecological Processes and Eco-Restoration, East China Normal University, Shanghai, 200241, China

Authors for correspondence:
Rui-Liang Zhu
Email: rlzhu@bio.ecnu.edu.cn

Qiong Zhao
Email: qzhao@bio.ecnu.edu.cn

Received: *9 January 2024*
Accepted: *20 March 2024*

New Phytologist (2024) **242:** 2817–2831
doi: 10.1111/nph.19750

Key words: Bryophytes, chloroplasts, *Cyathodium*, DYW domain, evolution, mitochondria, pentatricopeptide repeat proteins, RNA editing.

Summary

- RNA editing is a crucial modification in plants' organellar transcripts that converts cytidine to uridine (C-to-U; and sometimes uridine to cytidine) in RNA molecules. This post-transcriptional process is controlled by the PLS-class protein with a DYW domain, which belongs to the pentatricopeptide repeat (PPR) protein family. RNA editing is widespread in land plants; however, complex thalloid liverworts (Marchantiopsida) are the only group reported to lack both RNA editing and DYW-PPR protein.
- The liverwort *Cyathodium cavernarum* (Marchantiopsida, Cyathodiaceae), typically found in cave habitats, was newly found to have 129 C-to-U RNA editing sites in its chloroplast and 172 sites in its mitochondria.
- The *Cyathodium* genus, specifically *C. cavernarum*, has a large number of PPR editing factor genes, including 251 DYW-type PPR proteins. These DYW-type PPR proteins may be responsible for C-to-U RNA editing in *C. cavernarum*.
- *Cyathodium cavernarum* possesses both PPR DYW proteins and RNA editing. Our analysis suggests that the remarkable RNA editing capability of *C. cavernarum* may have been acquired alongside the emergence of DYW-type PPR editing factors. These findings provide insight into the evolutionary pattern of RNA editing in land plants.

Introduction

RNA editing is an important post-transcriptional modification to maintain essential functions at the RNA level (Small *et al.*, 2020; Knoop, 2023). Diverse RNA editing systems were found in viruses, protozoa, metazoans, fungi, and land plants (Takenaka *et al.*, 2013; Xin *et al.*, 2023; Zhang *et al.*, 2023). In land plants, RNA editing is suggested to be a general correction mechanism that restores conserved amino acids whose codons have been changed by mutations and plays crucial roles in organelle biogenesis and plant physiology (Small *et al.*, 2020). Recent progresses have demonstrated that the pentatricopeptide repeat (PPR) protein family, especially the PLS-class protein that harbors the DYW domain on the C terminus, contributes to this post-transcriptional process (Small *et al.*, 2023). Two types of RNA editing transitions occur in plant organellar transcripts: the more common conversion of cytidine to uridine (C-to-U) and the less frequent conversion of uridine to cytidine (U-to-C) (Gray, 2012; Takenaka *et al.*, 2013; Small *et al.*, 2020). Currently, the latter is known only from hornworts, lycophytes, and ferns (Takenaka *et al.*, 2013; Gerke *et al.*, 2020). The complex thalloid liverworts (Marchantiopsida), one of the three major extant lineages of liverworts (Bowman *et al.*, 2022), are the only land plant group that is reported to lack RNA editing, whereas the remaining major lineages of liverworts (Haplomitriopsida and Jungermanniopsida) harbor RNA editing capability but exhibit remarkable variations in the abundance of RNA editing (Malek *et al.*, 1996; Freyer *et al.*, 1997; Steinhauser *et al.*, 1999; Groth-Malonek *et al.*, 2007; Rüdinger *et al.*, 2008, 2012; Dong *et al.*, 2019). The absence of RNA editing in the Marchantiopsida was hypothesized to be a secondary loss (Takenaka *et al.*, 2013; Knoop, 2023).

Marchantiopsida is the second-largest class of Marchantiophyta, with 330–340 species in 35 genera in 18 families belonging to three orders (Söderström *et al.*, 2016; Villarreal *et al.*, 2016; Xiang *et al.*, 2022). *Cyathodium* Kunze ex Lehm., the only genus of Cyathodaceae in the Marchantiopsida with only 12 species, is known for its luminescent nature, typical cave habitats, and the presence of operculum (a lid-like structure on the sporophyte capsule), which are unique characteristics among liverworts (Fig. 1; Srivastava & Dixit, 1996; Bischler-Causse *et al.*, 2005; Salazar Allen & Korpelainen, 2006; Duckett & Ligrone, 2006). Recent phylogenetic studies have revealed *Cyathodium* is a relatively recent offshoot within the Marchantiopsida, with a notably high substitution rate and a long branch in both nuclear and organellar gene markers (Villarreal

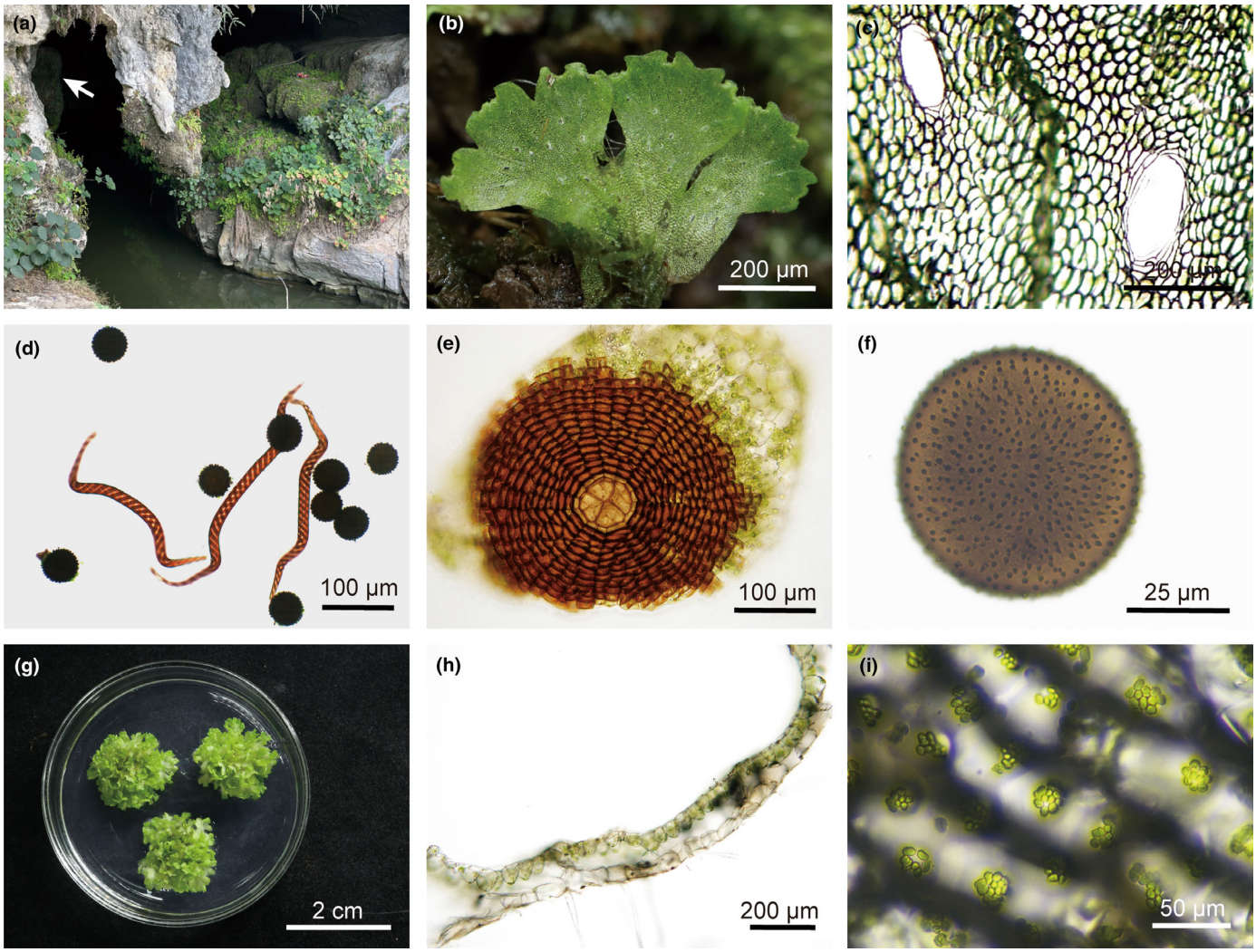

Fig. 1 *Cyathodium cavernarum* Kunze ex Lehm. (a) Cave habitat with populations on rock in cave (white arrow). (b) Habit. (c) Dorsal view of thallus showing simple air pores. (d) Elaters and spores. (e) Apex of capsule showing operculum. (f) Spore. (g) Aseptic populations in Petri dish. (h) Transverse section of thallus. (i) Chloroplasts in epidermal cells. Bars: (b, c, h) 200 μm; (d, e) 100 μm; (f) 25 μm; (g) 2 cm; (i) 50 μm.

et al., 2016; Xiang *et al.*, 2022; Bechteler *et al.*, 2023) or the second split within Marchantiales using coalescence approach (Bechteler *et al.*, 2023: appendix S3). Furthermore, the GC content, which has been found to have a positive relationship with RNA editing abundance (Schallenberg-Rüdinger & Knoop, 2016; Dong *et al.*, 2019), was significantly higher in *Cyathodium* chloroplasts compared with chloroplasts in other species of Marchantiopsida (Xiang *et al.*, 2022). These initial investigations suggest that the exceptional lineage of the Marchantiopsida may undergo RNA editing.

Is RNA editing truly absent in the Marchantiopsida, especially in *Cyathodium*? To investigate whether RNA editing occurs in the Marchantiopsida, specifically in *Cyathodium*, we conducted a comprehensive study here. First, we established a sterile culture system for *Cyathodium cavernarum* Kunze ex Lehm. (Fig. 1), which is the type species of *Cyathodium*. Then, we sequenced and assembled its chloroplast and mitochondrial genomes from axenic samples. By analyzing the transcriptomic data, we identified RNA editing events and a remarkable expansion of the DYW-type PPR proteins in this intriguing plant. Our findings present compelling evidence of RNA editing in *C. cavernarum*, which contradicts the previous belief that complex thalloid liverworts lack this mechanism. Two competing hypotheses to explain the presence of RNA editing in *Cyathodium* are proposed.

Materials and Methods

Plant culture

Plants bearing mature spore capsules of *C. cavernarum* were collected from a natural population in a cave in Guilin City, Guangxi, China on 20 August 2019 (Fig. 1). The sporophytes of *C. cavernarum* were rinsed with distilled water at least five times, sterilized in 75% alcohol solution for 5 s, and subsequently rinsed with distilled water three times. Spore suspension was further sterilized in 0.05% sodium hypochlorite for several

seconds. Disinfected spores were spread onto the Knop agar medium for germination and growth in Petri dishes in an incubator (Percival I-36VL) under 16 h : 8 h, 22 ± 1°C : 18 ± 1°C, light : dark. The well-grown thalli were selected for DNA and RNA extractions. The genome skimming data and transcriptomes of two other species of *Cyathodium* (*C. aureonitens* (Griff.) Mitt. and *C. tuberosum* Kashyap) were generated from the field materials collected from Yunnan, China (Supporting Information Notes S1). The voucher specimens (Zhu *et al.* 20220827-7 for *C. aureonitens*, Shen 20190820-5 for *C. cavernarum*, and Zhu *et al.* 20220829-8 for *C. tuberosum*) were deposited in the Herbarium of East China Normal University (HNSU).

DNA extraction and sequencing

Total genomic DNA was extracted using the modified CTAB method (Allen *et al.*, 2006). The DNA library with an insert size of 350 bp was constructed using the NEBNext® library kit according to the standard protocol provided by Illumina and was sequenced on HiSeq Xten PE150 sequencing platform at GrandOmics Wuhan, China.

RNA extraction and sequencing

Total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). RNA was processed using the RiboMinus Plant Kit for RNA-Seq (Thermo Fisher Scientific) to deplete ribosomal RNAs (rRNAs). Subsequently, the depleted RNA was used to prepare a stranded RNA-sequencing library (TruSeq mRNA library kit). The paired-end reads (2 × 150 bp) were generated on the Illumina HiSeq Xten platform at Novogene, Beijing, China.

Assembly and annotation of organelle genomes

GetOrganelle v.1.7.5.1 (Jin *et al.*, 2020) was used to assemble the plastid and mitochondrial genome (cpDNA and mtDNA) of *C. cavernarum* with default settings. The assembled graph was visualized in Bandage v.0.9.0 (Wick *et al.*, 2015). The assembly of cpDNA revealed a complete architecture represented by a standard graph (Fig. S1), similar to that of land plants. However, the mtDNA graph was more complex, consisting of four connections mediated by short repeats (SRs), specifically repeat9, repeat10, repeat11, and repeat12 (Fig. S2). Except for repeat12, the remaining SRs can be grouped into two conditions. Therefore, a maximum of eight potential architectures may occur in the mitochondrial genome of *C. cavernarum* (Fig. S2). With the prior settings where repeat10 and repeat12 were untied in the putative circular mtDNA, four configurations were identified (Fig. S3). The configuration of circle1 was selected for RNA editing analyses. Whole-genome alignment of these four configurations was performed in Mauve (Fig. S4; Darling *et al.*, 2004), and the genome recombination of *C. cavernarum* mtDNA was detailed in Notes S2.

The cpDNA and mtDNA were annotated using Cpgavas2 (Shi *et al.*, 2019) and GeSeq (Tillich *et al.*, 2017), respectively. The resulting annotations of organelle genomes were manually checked and edited in Geneious Primer v.2022.0.1 (Kearse *et al.*, 2012) with the organelle genomes (cpDNA: NC_037507; mtDNA: NC_037508) of *Marchantia polymorpha* L. as reference.

Determination of RNA editing event

RNA reads of *C. cavernarum* were mapped against the organelle genomes using tophat2 (Kim *et al.*, 2013) with default settings, and then, the bam results were sorted by Samtools (Li *et al.*, 2009). The final bam files together with their corresponding genome sequences were imported into Geneious Primer v.2022.0.1 (Kearse *et al.*, 2012). The 'Find Variations' function implemented in Geneious Primer v.2022.0.1 (Kearse *et al.*, 2012) was employed to search for RNA editing sites in protein-coding transcripts with the following thresholds: minimum coverage = 3, minimum variant frequency = 0.1, maximum variant *P*-value = 10^{-6}, and minimum strand-bias *P*-value = 10^{-5} when exceeding 65% bias (Wu & Chaw, 2022). DNA reads were also used to map against the corresponding organelle genome and identify genomic variations in order to verify the edited sites.

Validation for C-to-U RNA editing sites in *Cyathodium*

Total RNA was extracted using the TRIzol reagent (Thermo Fisher Scientific), and first-strand cDNA was synthesized using a HiScript III 1st strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, Jiangsu, China). We chose six RNA editing sites in five chloroplasts (*atpI*, *ndhJ*, *petD*, *psaA*, and *psaB*) and seven sites in four mitochondrial (*atp1*, *cob*, *cox3*, and *nad2*) transcripts for PCR validation. The primers were designed in Geneious Primer v.2022.0.1 (Kearse *et al.*, 2012), and specific cDNA fragments containing RNA editing sites were amplified and sequenced directly. The edited uridine was compared with its template base in the genome. The primer sequences are listed in Table S1.

Comparison of homologous sites in representative Marchantiopsida organelle genomes

C-to-U RNA editing has been explained as a mechanism to restore T-to-C mutations at the DNA level, to preserve the conserved function of proteins (Freyer *et al.*, 1997). However, whether RNA editing in *Cyathodium* follows such a rule is unknown. Here, we employed published organelle genomic datasets of nine Marchantiopsida species (Table S2) to compare the genomic DNA bases of C-to-U RNA editing sites in *C. cavernarum* and their homologous sites in other species of Marchantiopsida. To do this, we extracted 48 and 27 RNA editing-affected protein-coding genes (CDS) from cpDNA and mtDNA, respectively. The CDS sequences were aligned by Mafft (Katoh & Standley, 2013) and visualized in Geneious Primer v.2022.0.1 (Kearse *et al.*, 2012). The synonymous RNA editing sites and ndhFeU2024SL, which is likely a recent lineage-specific insertion of *C. cavernarum*, were ignored in this analysis,

Table 1 DNA base information and quantity statistics of homologous sites of RNA editing sites in organelle genomes of *Cyathodium cavernarum* and nine Marchantiopsida species without RNA editing.

Base to be edited in *C. cavernarum*	Homologous base in nine Marchantiopsida without RNA editing	Count	Location
C	T	114	CpDNA
C	C	3	CpDNA
CC	CT	1	CpDNA
C	A/T	1	CpDNA
C	T	149	MtDNA
CC	TT	7	MtDNA
C	C	7	MtDNA
CC	CT	1	MtDNA

and thus, 119 sites in cpDNA and 164 sites in mtDNA of nonsynonymous RNA editing were included. Then, we compared the bases between the sites to be edited in *C. cavernarum* and their homologous sites in other Marchantiopsida species, and then compared the amino acids derived from C-to-U RNA editing in *C. cavernarum* and the corresponding amino acids on homologous sites in other Marchantiopsida species. The summary of the comparison is listed in Table 1.

Comparison of RNA editing sites in other lineages of bryophytes

According to the phylogenetic positions and data availability, eight other bryophytes (one Haplomitriopsida liverwort, four Jungermanniopsida liverworts (two simple thalloid and two leafy liverworts), two mosses and one hornwort; Table S2) were chosen to investigate the presence of shared RNA editing sites between *C. cavernarum* and these representative bryophytes using the multiple alignment method, for the 301 RNA editing sites identified in *C. cavernarum*. These datasets, containing organelle genomes and their respective RNA editing sites, were obtained from previous studies (Rüdinger *et al.*, 2009; Ichinose *et al.*, 2014; Dong *et al.*, 2019; Gerke *et al.*, 2020; Hu *et al.*, 2023).

Identification of PPR DYW proteins as candidate RNA editing factors

An online PPR finder tool (https://ppr.plantenergy.uwa.edu.au/fasta/) (Cheng *et al.*, 2016) was used to identify the candidate RNA editing factors in the genome assembly of *C. cavernarum* (unpublished data) based on the reassessment of PPR motifs. The numbers of different types of PPR proteins were counted based on the category of motif on the C terminus. Other published genomic and transcriptomic data of Marchantiopsida were also selected to identify PPR proteins in the same manner (Table S3).

We aligned the protein sequences of *C. cavernarum* DYW domains that are longer than 120 amino acids using the *hmmalign* function in the HMMER package (Potter *et al.*, 2018) with the profile HMM in Gutmann *et al.* (2020). We then filtered the domains that lack the cytidine deaminase signature ($HxEx_{25}CxxC$). The conservation plot of the 245 DYW domains in *C. cavernarum* was drawn using the WEBLOGO service (Crooks *et al.*, 2004). In bryophytes, only the DYW domains of the model plant *Physcomitrium patens* (Hedw.) Mitt. have been experimentally investigated and confirmed their functions in RNA editing (Rüdinger *et al.*, 2011; Yang *et al.*, 2023). Therefore, we compared the sequence similarity between *C. cavernarum* and *P. patens*.

To trace the evolution of the DYW domain in *C. cavernarum*, two other DYW domain datasets were used: (1) the DYW domain matrix developed from the OneKP dataset where the DYW : KP variant sequences were eliminated (Gutmann *et al.*, 2020); (2) the DYW domain matrix derived from Haplomitriopsida representing the first branching clade in liverworts, which were not sampled in the OneKP dataset. The DYW sequences of the latter dataset were obtained from the previous study (Dong *et al.*, 2022). These two DYW domain matrix were combined with the *C. cavernarum* DYW domain matrix using the 'Consensus Align' function in GENEIOUS PRIMER v.2022.0.1 (Kearse *et al.*, 2012). Subsequently, an alignment containing 13 977 sequences was used to construct an approximate maximum-likelihood tree using FASTTREE 2 (Price *et al.*, 2010) with the JTT model. The resulting tree was visualized using TVBOT online (Xie *et al.*, 2023).

Results

The plastome

The chloroplast genome of *C. cavernarum* follows the typical quadripartite structure found in land plants (Fig. 2). It consists of a large single-copy (LSC, 79683 bp) region and a small single-copy (SSC, 19479 bp) region, separated by a pair of inverted repeats (IRs, 9795 bp × 2), resulting in a circular molecule with a total length of 118 752 bp. The *C. cavernarum* cpDNA contains the expected gene complements found in other taxa of Marchantiopsida. A total of 135 genes were annotated, including 89 protein-coding genes, 38 tRNA genes, and 8 rRNA genes (Fig. 2). However, *C. cavernarum* lacks introns in *pafI* (*ycf3*) and *rpl16* genes, unlike other liverworts (Dong *et al.*, 2021; Xiang *et al.*, 2022). The *ffs* gene, an essential signal recognition particle pathway component located between *petN* and *trnC* in bryophytes (Träger *et al.*, 2012), was also found in *C. cavernarum* cpDNA.

The mitochondria and potential rearrangement

The mtDNA assembly graph produced by GETORGANELLE v.1.7.5.1 (Jin *et al.*, 2020) displays a complex and interconnected structure. It is comprised of eight contigs and four pairs of SRs, with lengths ranging from 160 to 281 bp (Fig. S2a; Table S4). These SRs potentially facilitate the mitochondrial genome recombination (detailed in Notes S2).

After simplifying the graph using BANDAGE v.0.9.0, four configurations were identified (Fig. S3). The configuration named

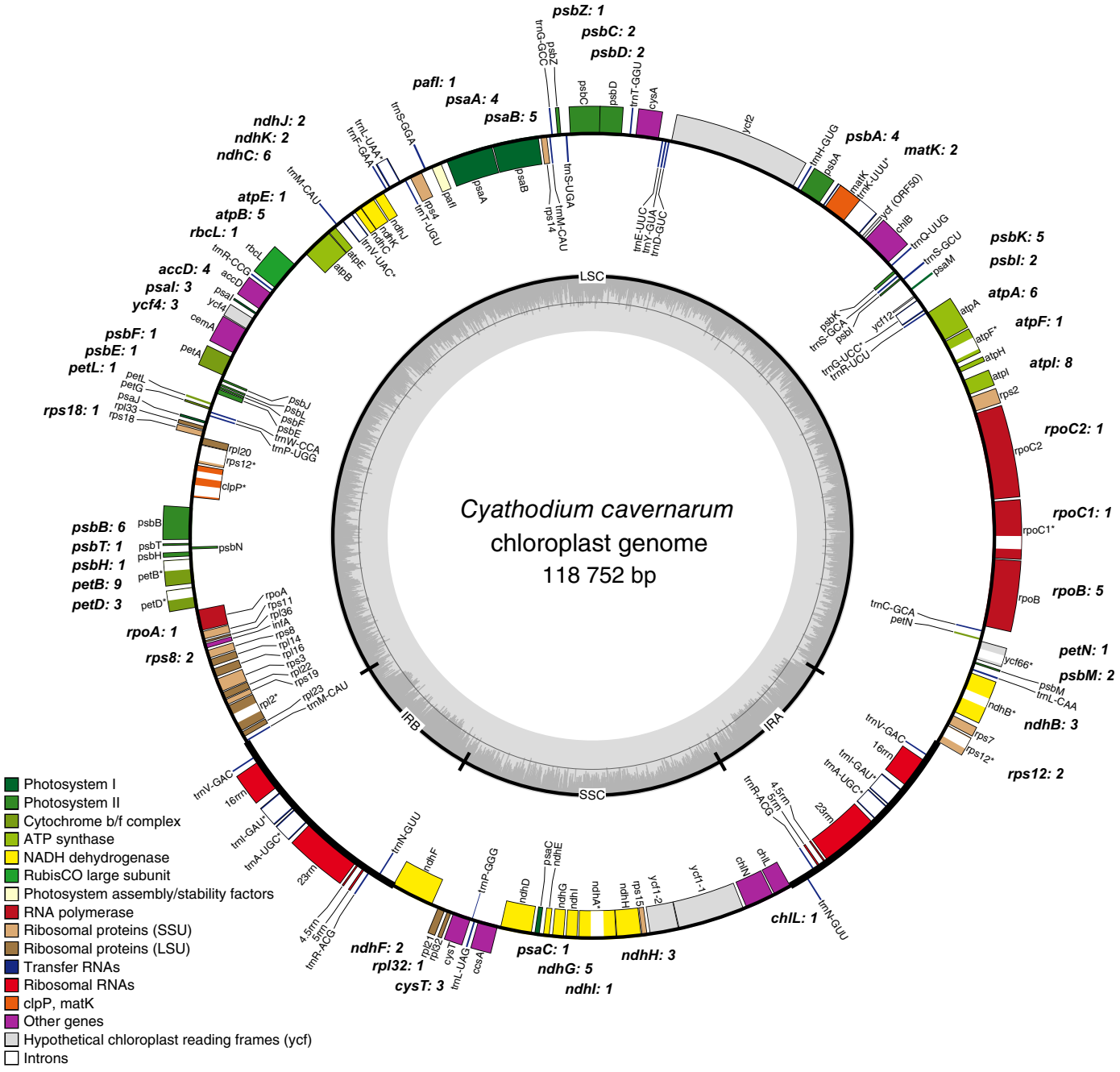

Fig. 2 Chloroplast genome of *Cyathodium cavernarum* with RNA editing site. The map of chloroplast DNA (cpDNA) was generated using OGDRAW (Lohse *et al*., 2013). Gene categories are indicated in the key. The numbers given for protein-coding sequences indicate the count of cytidine to uridine (C-to-U) RNA editing sites within this gene coding region.

'circle1' exhibits the same gene order as the published mtDNA of *M. polymorpha* (NC_037508) and was used for RNA editing analyses. The mtDNA of *C. cavernarum* forms a circular molecule with a total length of 175 523 bp and is the smallest among the published species of Marchantiopsida owing to the lack of a long noncoding region. It contains a conserved gene content observed in liverworts, including a pseudogene, *nad7*. In total, 73 genes were annotated, consisting of 40 protein-coding genes, 2 pseudogenes, 28 tRNA genes, and 3 rRNA genes (Fig. 3). The gene contents of mtDNA are distributed across different contigs, as listed in Table S4.

RNA editing events in chloroplasts

After analyzing the stranded transcriptome, we ultimately identified 129 sites of C-to-U RNA editing in the protein-coding region of *C. cavernarum* chloroplast with a coverage threshold of 3 (Table S5). We followed the nomenclature proposed by Rüdinger

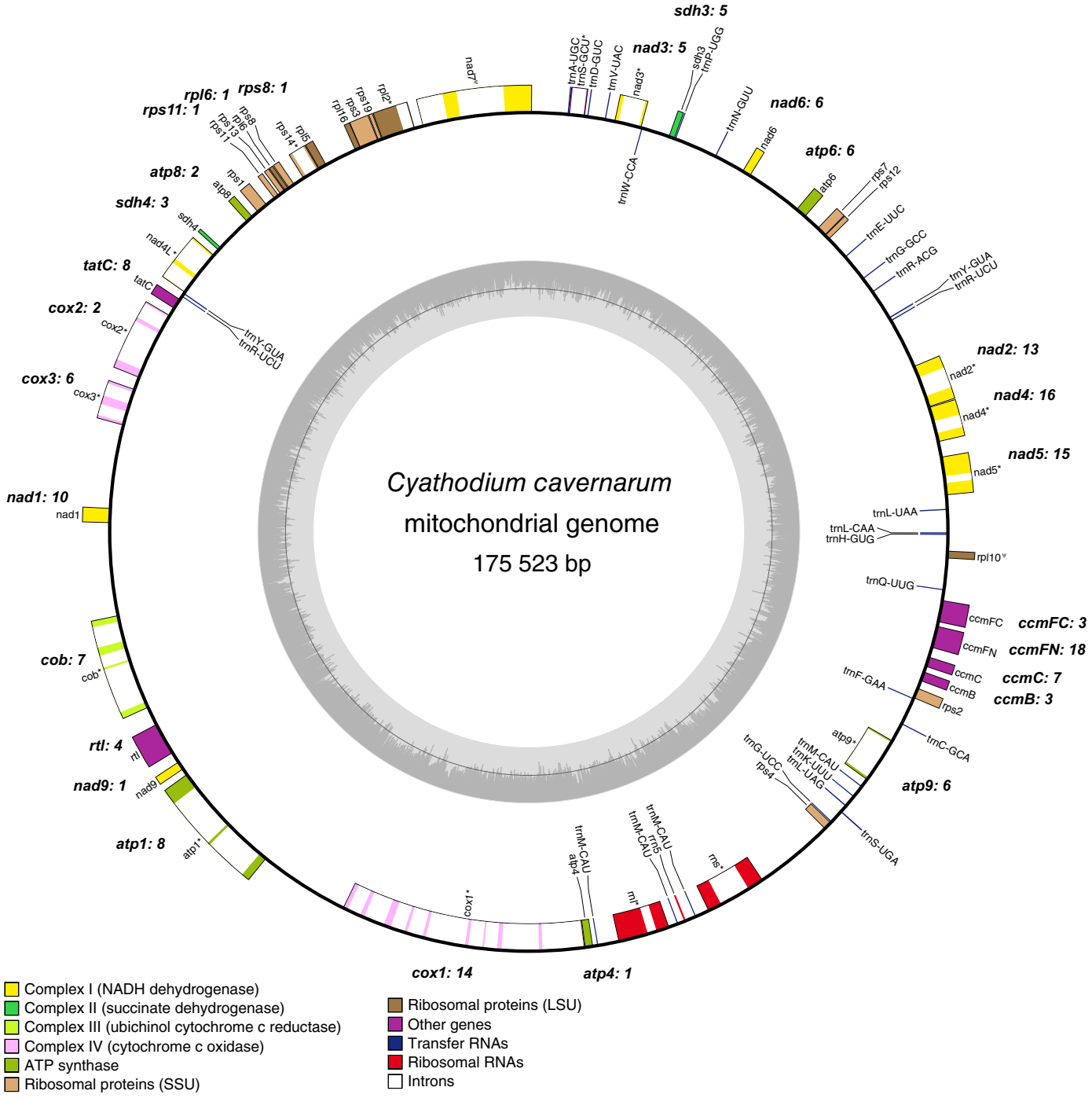

Fig. 3 Mitochondrial genome of *Cyathodium cavernarum* with RNA editing sites. The map of mitochondrial DNA (mtDNA) was created using OGDRAW (Lohse *et al*., 2013). Gene categories are shown in the key. Pseudogenes are denoted by ψ. The numbers given for protein-coding sequences indicate the count of cytidine to uridine (C-to-U) RNA editing sites within this gene coding region.

et al., (2009) to designate RNA editing sites. For example, in the case of 'psbMeU74PL', 'psbM' represents the affected gene, 'eU74' indicates the conversion of cytidine (C) to uridine (U) at position 74 in that transcript, and 'PL' signifies a change in the amino acid from proline (P) to leucine (L) at the mature transcript.

The identified RNA editing sites are distributed among 48 protein-coding genes (Fig. 2), with gene *petB* harboring the largest number of nine edited sites. No RNA editing site was found in the remaining 41 genes. However, the reads overlap and coverage on several long transcripts, such as *ycf2* and *rpoC2*, are very low (Table S6). Therefore, our determination of the number of RNA editing sites in *C. cavernarum* chloroplast may have been underestimated, and the true number may exceed 129. In the present study, we also identified 174 and 116 RNA editing sites

in the chloroplast genomes of two other *Cyathodium* species (*C. aureonitens* and *C. tuberosum*), which suggests that the most recent common ancestor (MRCA) of *Cyathodium* may possess the RNA editing capability (detailed in Notes S1; Fig. S5).

The editing events mainly occur at the second codon position (106 sites, 82.2%), followed by the first codon position (13 sites, 10.1%; Fig. S6; Table S5), resulting in significant changes to the amino acids. Changes at the third codon position (nine sites, 7.0%) are synonymous. In addition, this study also discovered a relatively rare editing event – atpEeU176TI (see Table S5), where editing occurred at both the second and third positions within the same codon. In total, three stop codons and two translation start codons (Fig. 4a) are created by C-to-U RNA editing. Apart from the synonymous edited events and stop codon-created sites, we discovered 10 different formats of amino acid changes in *C. cavernarum* chloroplast (Fig. 4a). The most frequent changes are serine to leucine (S to L), serine to phenylalanine (S to F), and proline to leucine (P to L), with 48, 25, and 30 occurrences,

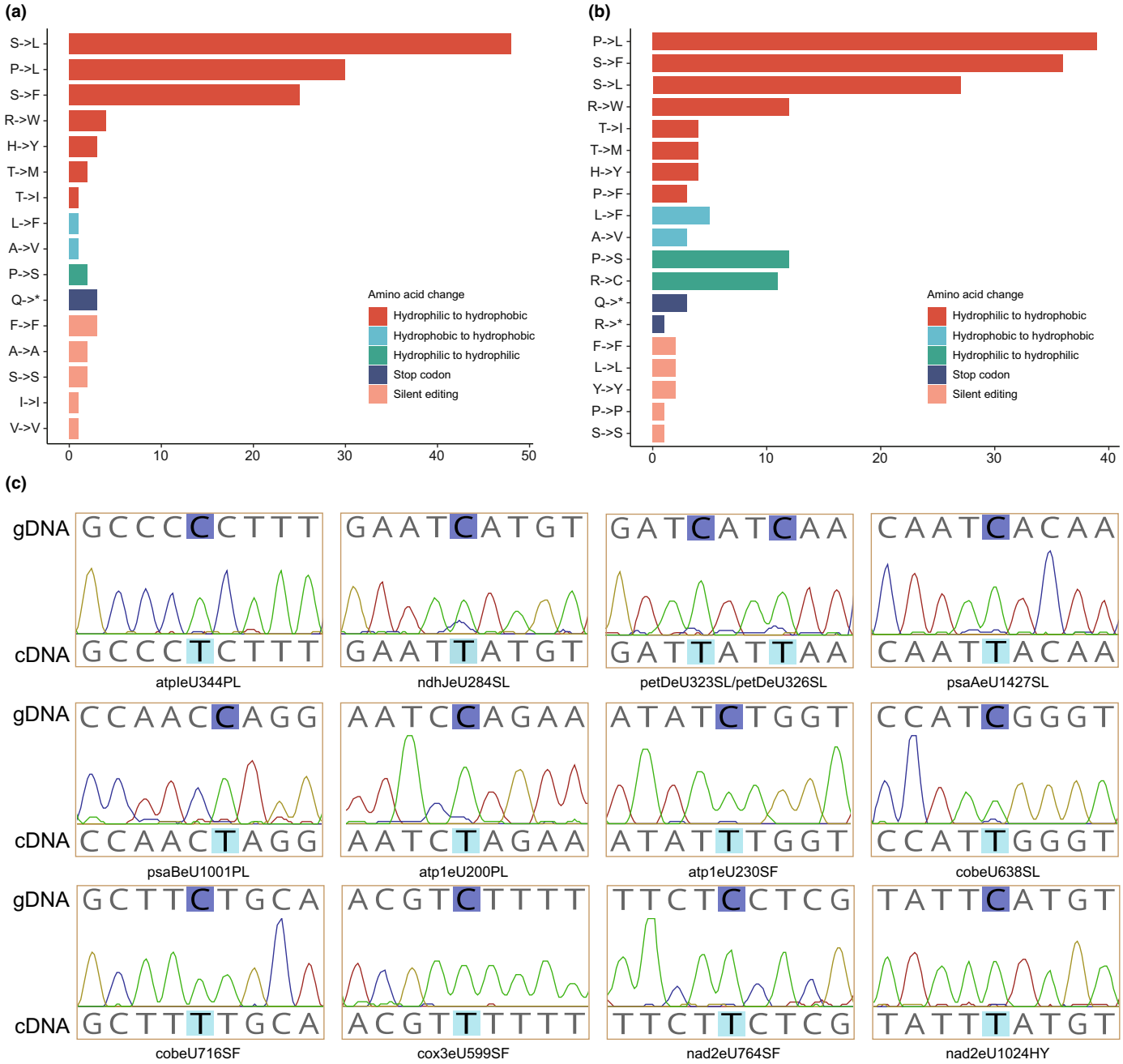

Fig. 4 Distribution of amino acid changes resulting from cytidine to uridine (C-to-U) RNA editing in *Cyathodium cavernarum*. (a) Amino acid changes in chloroplast DNA (cpDNA). (b) Amino acid changes in mitochondrial DNA (mtDNA). (c) Sanger sequencing chromatogram displaying 13 RNA editing sites in *C. cavernarum*. In each plot, the top section represents the bases of DNA, while the bottom section represents the bases of transcripts. Bases of C on DNA labeled with purple, and changed bases of T on transcripts labeled with light blue.

respectively. These three formats of amino acid change account for 85.8% of the nonsynonymous editing events. Additionally, RNA editing tends to increase or maintain the hydrophobicity of proteins (Fig. 4a), with the exception of only two edited sites with a proline to serine (P to S) amino acid change.

RNA editing events in mitochondria

We identified 172 sites of C-to-U RNA editing in the protein-coding region of mitochondria using the same thresholds as in chloroplast (Table S5). The number of edited sites in mtDNA is higher than in cpDNA, showing a similar pattern of RNA editing abundance between cpDNA and mtDNA as observed in most land plants (Edera *et al.*, 2018; Dong *et al.*, 2019; Huang *et al.*, 2022; Wu & Chaw, 2022).

The identified RNA editing events occur in 27 out of 40 protein-coding genes in the mitochondrial genome (Fig. 3). Similar low overlapping and coverage of transcriptomic reads was observed as in cpDNA (Table S6), hence, our determination of C-to-U RNA editing in mtDNA may be also underestimated. Of the mt-RNA editing events, 107 sites (62.2%) occur at the second codon position, followed by 50 sites (29.1%) at the first position, and seven sites (4.1%) at the third position (Fig. S6; Table S5). Notably, we detected eight events (4.7%) where two specific sites were successively edited within one codon (Fig. S6; Table S5), with five occurring at the first and second codon positions, and three at the second and third positions. Among these events, eight sites (4.7%) were found to be synonymous (Fig. 4b), including one first-position edited site (sdh3eU199LL) and all seven third-position edited sites. The remaining 164 sites out of the 172 edited sites at the first and second positions are nonsynonymous. RNA editing resulted in the creation of four stop codons and one translation start codon (Fig. 4b).

The most frequent amino acid changes observed in mtDNA are S to F, S to L, and P to L, with 36, 27, and 39 occurrences, respectively, accounting for a total of 62.2% of the nonsynonymous RNA editing sites in *C. cavernarum* mtDNA (Fig. 4b). Additionally, two amino acid changes, proline to phenylalanine (P to F) and arginine to cysteine (R to C), were observed in mtDNA but not in cpDNA (Fig. 4a,b; Table S5). These changes result from successive editing at the first and second codon positions of CCU, and editing at the first position of CGU, respectively. Furthermore, the nonsynonymous C-to-U RNA editing in mtDNA has a preference for increasing or maintaining the hydrophobicity of proteins (Fig. 4b), with the exception of 23 edited sites with amino acid changes from P to S and R to C.

The validation of C-to-U RNA editing by PCR sequencing

Here, we selected six sites in cpDNA and seven sites in mtDNA for PCR validation. The Sanger sequencing results indicate that the bases of RNA editing sites are Ts on the cDNA of these 13 C-to-U RNA editing sites in *C. cavernarum*, while the bases are Cs on the genomic DNA (Fig. 4c). These results provide strong evidence supporting the presence of C-to-U RNA editing in *C. cavernarum*.

Homologous sites in other non-RNA editing species of Marchantiopsida

We compared the bases between the sites to be edited in *C. cavernarum* and their homologous sites in nine representative Marchantiopsida species. For nonsynonymous C-to-U RNA editing sites in *C. cavernarum*, the bases of their homologous sites in all nine non-*Cyathodium* species are Ts for 114 (95.8%) of 119 sites in cpDNA and 156 (95.1%) of 164 sites in mtDNA (Fig. S7; Table 1). At the amino acid level, most cases (110 sites in cpDNA and 151 sites in mtDNA) show the same amino acid as the one derived from C-to-U RNA editing in *C. cavernarum* and the one encoded in the homologous sites in other nine Marchantiopsida species (Fig. S7).

A total of 110 sites in cpDNA and 150 sites in mtDNA follow a set of rules: (1) in *C. cavernarum*, the base of editable site in genomic DNA is a C, which is altered to a U in transcripts through the editing process, and the corresponding base in genomic DNA of non-RNA editing species of Marchantiopsida is a T; (2) the amino acid derived from RNA editing in *C. cavernarum* is the same as that encoded in the homologous site in non-RNA editing species of Marchantiopsida. These findings suggest that C-to-U RNA editing in *C. cavernarum* likely restores T-to-C mutations at the first and second codon positions, thereby increasing the conservation of the amino acid. Furthermore, the absence of RNA editing in non-*Cyathodium* Marchantiopsida species may be attributed to the fixation of C-to-T mutations in their genomic DNA.

The shared RNA editing sites in other lineages of bryophytes

All 301 identified RNA editing sites in *C. cavernarum* were examined for the presence of shared sites with the eight representative bryophytes. It shows that *C. cavernarum* shares RNA editing sites with each of the representative bryophytes (Fig. 5). In total, *C. cavernarum* shares 137 RNA editing sites with at least one of the tested bryophytes, with 49 sites located in the cpDNA and 88 sites in the mtDNA (Fig. 5; Table S7). The remaining 164 RNA editing sites in *C. cavernarum* could be considered putatively unique.

Expansion of PLS-DYW protein genes

A total of 497 PPR protein genes were identified in the *C. cavernarum* genome (unpublished data) in this study (Fig. 6a; Table S8), containing 67 in the P-class and 430 in the PLS-class, based on different motifs of PPR protein. Additionally, the PLS-class includes 76 PLS-type proteins, 19 E1-type proteins, 61 E2-type proteins, 22 E+-type proteins, 1 SS-type protein, and 251 DYW-type proteins (Fig. 6a; Table S3). In other non-*Cyathodium* Marchantiopsida species, the numbers of P-class proteins were comparable to those in *C. cavernarum*. By contrast, no DYW-type protein was found in any of the species and the remaining subtypes of PLS-class proteins were rare, limited to PLS-type and E1-type proteins (Fig. 6a; Table S3). These results suggest a significant expansion of PLS-class protein genes, especially the DYW-type proteins in *Cyathodium*.

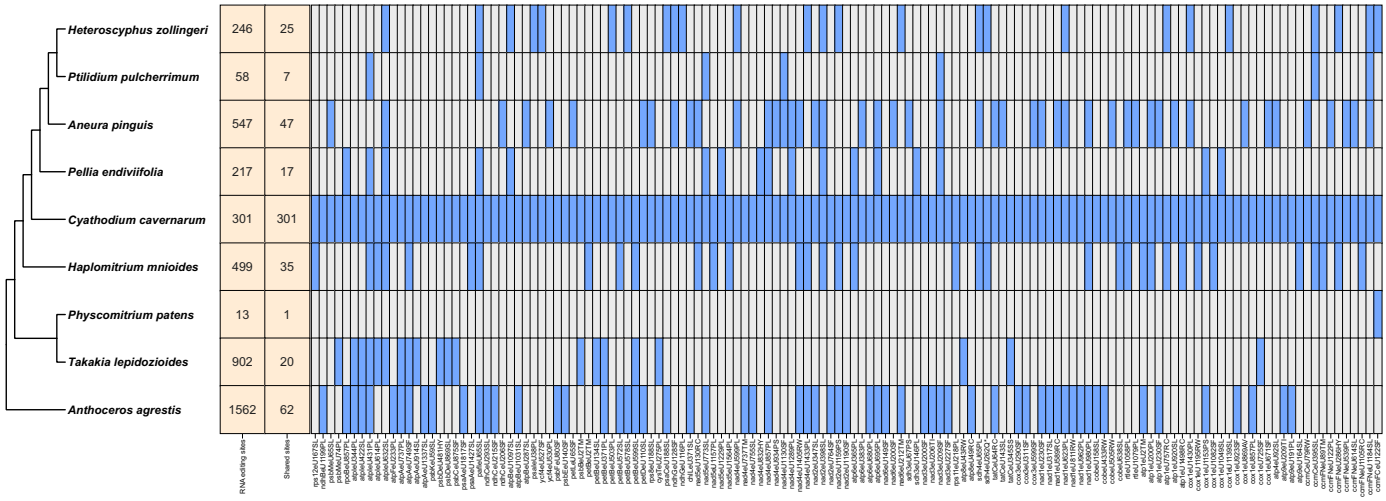

Fig. 5 Presence of shared RNA editing sites between *Cyathodium cavernarum* and tested bryophyte species. The numbers in the first column following the species names indicate the abundance of cytidine to uridine (C-to-U) RNA editing for that species (Rüdinger *et al*., 2009; Ichinose *et al*., 2014; Dong *et al*., 2019; Gerke *et al*., 2020; Hu *et al*., 2023). The numbers in the second column indicate the number of shared RNA editing sites between *C. cavernarum* and that species. The blue block indicates a shared common RNA editing site between *C. cavernarum* and each of the representative bryophytes.

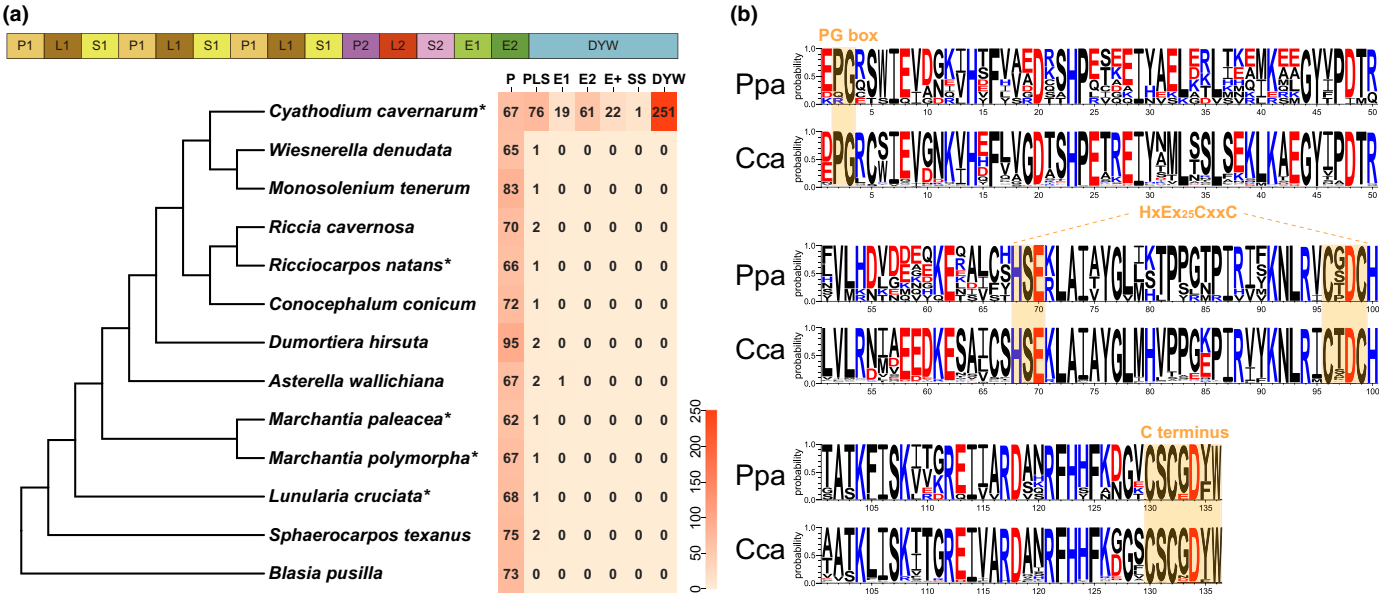

Fig. 6 Features of pentatricopeptide repeat (PPR) proteins in *Cyathodium cavernarum*. (a) Distribution of PPR protein genes in the class Marchantiopsida. The topology was derived from Xiang *et al*. (2022). The heatmap indicates the abundance of different types of PPR proteins from low to high, with the color red varying from light to deep. An asterisk following the species name indicates a genomic dataset, otherwise a transcriptome. The schematic structure at the top refers to a standard DYW-type PPR protein architecture in *C. cavernarum*. (b) Sequence conservation of DYW domains between *Physcomitrium patens* (Ppa) and *C. cavernarum* (Cca). Three crucial characteristics of the DYW domain were labeled with shadowed orange blocks.

The DYW domain in *C. cavernarum* exhibits a protein sequence structure similar to that found in other land plants (Cheng *et al*., 2016; Gutmann *et al*., 2020), which includes the PG box, $HxEx_{25}CxxC$ deaminase signature, and residues of the C-terminus DYW. Here, we compared the sequence similarity of DYW domains between *C. cavernarum* and the model moss *P. patens* (Fig. 6b), whose DYW-type PPR proteins have already been functionally characterized in C-to-U RNA editing (Ichinose *et al*., 2013; Schallenberg-Rüdinger *et al*., 2013). The three crucial characteristics (PG box, HxEx25CxxC, and C terminus) in the DYW domain (Gutmann *et al*., 2020; Takenaka *et al*., 2021) between *C. cavernarum* and *P. patens* are highly conserved (Fig. 6b), suggesting that these DYW sequences potentially contribute to the C-to-U RNA editing in *C. cavernarum*.

Additionally, 244 of the DYW-type PPR editing proteins have full PPR motifs (Table S8), indicating that the *cis* interaction process, which is governed by the PPR motifs of editing factors, is likely dominant in RNA editing of *C. cavernarum*.

Given the complexity of alignments and phylogenetic inferences caused by the modularity and highly repetitive nature of PPR sequences, we aimed to focus on tracing the evolutionary trajectory of the DYW domain, because this DYW domain has been well recognized as a deaminase enzyme involved in C-to-U RNA editing. The phylogenetic analysis yielded a topology similar to that of Gutmann *et al.* (2020) (Fig. 7a). In the present analysis, all the DYW domain sequences of *C. cavernarum* are clustered together, forming a single clade with a high bootstrap value of 0.956. This clade is nested within the moss DYW domain sequences and is most closely related to a group comprising 13 DYW domain sequences of Bryopsida with moderate support (Fig. 7b). These results suggest that the expansion of the DYW domain in *C. cavernarum* is clade-specific.

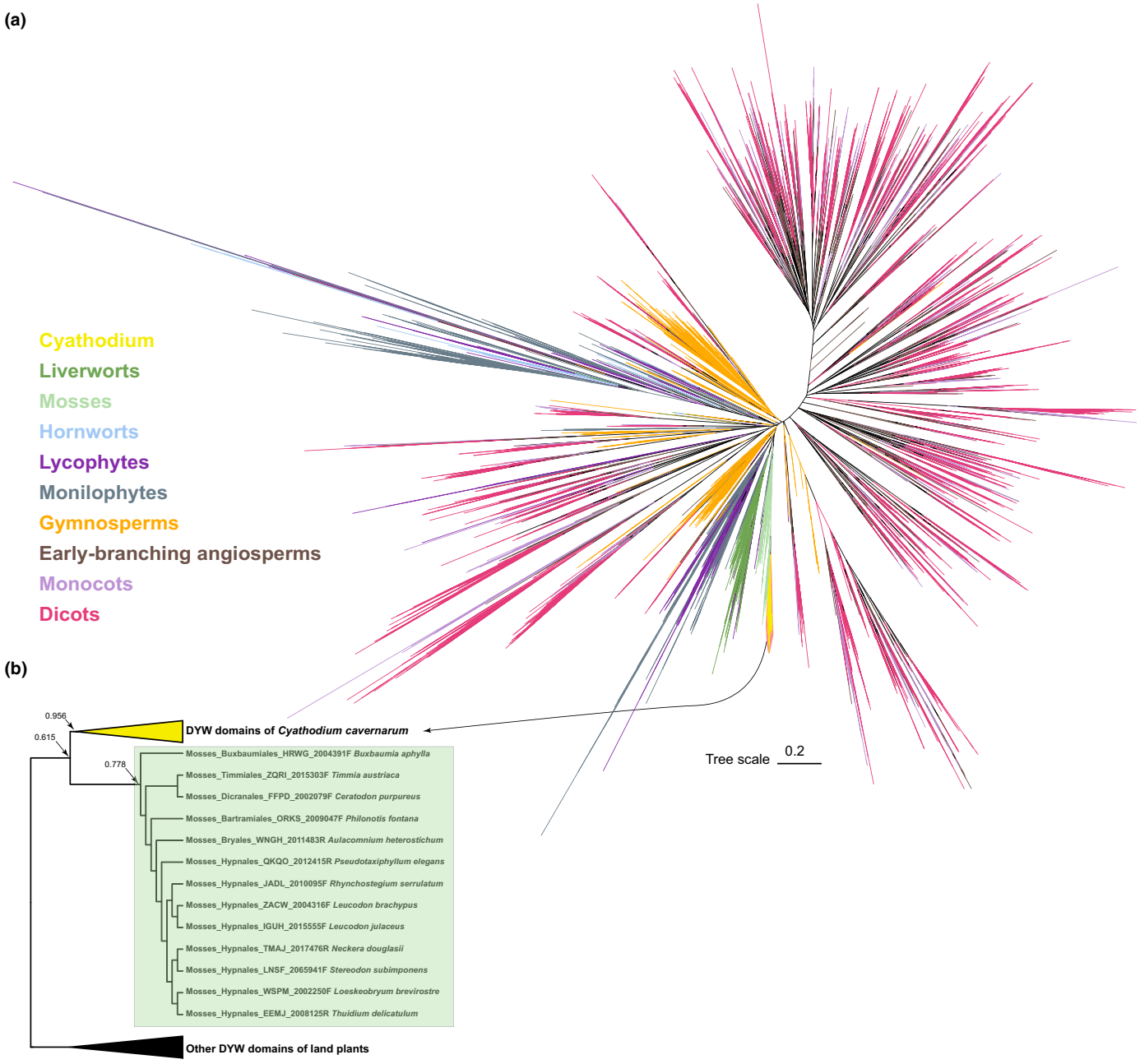

Fig. 7 Maximum-likelihood tree of DYW domain sequences of land plants reconstructed by FastTree 2. (a) 13 977 DYW sequences longer than 120 amino acids were included. The tree was unrooted. The branch colors of the tree are color-coded based on the clade of origin, as indicated by the colored labels. (b) A cladogram showing DYW domains of *Cyathodium cavernarum* and their most closely related clade comprising 13 moss DYW domains. The 13 moss DYW domains are marked with light green blocks. DYW domains of *C. cavernarum* are collapsed into a yellow triangle and the remaining DWW domains of land plants are collapsed into a black triangle. The decimals are support values of nodes pointed by arrows.

Discussion

Hypotheses on the presence of RNA editing in *Cyathodium*

Complex thalloid liverworts (Marchantiopsida) have been thought to be the only land plant group that lacks RNA editing among land plants (Rüdinger *et al.*, 2008; Dong *et al.*, 2019; Knoop, 2023). The absence of RNA editing in the Marchantiopsida was hypothesized to be a secondary loss (Takenaka *et al.*, 2013; Knoop, 2023). The presence of RNA editing in *Cyathodium* complicates our understanding of the evolutionary pattern of RNA editing in Marchantiopsida. Although most studies revealed *Cyathodium* as a relatively recent lineage in Marchantiopsida, sister to Corsiniaceae (Villarreal *et al.*, 2016; Flores *et al.*, 2021; Xiang *et al.*, 2022), Bechteler *et al.* (2023) suggested a different phylogenetic position, placing it as the second split within Marchantiales. However, regardless of the inconsistent opinions on the phylogenetic position of *Cyathodium* within the Marchantiopsida, we can propose two hypotheses. Naturally, one hypothesis is that the presence of RNA editing in *Cyathodium* may be the result of a secondary acquisition event within the evolutionary history of Marchantiopsida. This scenario, where the loss of RNA editing capability at an ancient node is followed by an acquirement in a more recently diverged clade, is reported for the first time and is likely to be unique across the broad evolutionary timescale of land plants. An alternative hypothesis suggests that the RNA editing capability of *Cyathodium* may derive from the uninterrupted inheritance of the MRCA of Marchantiopsida, while other lineages within Marchantiopsida may independently lose RNA editing ability during evolution. The presence of shared RNA editing sites between *Cyathodium* and the bryophyte species that do possess RNA editing seems to support this idea (Fig. 5). The shared common RNA editing sites could be the outcome of natural evolutionary processes in distantly related groups, or parallel evolution in closely related groups. This is probably due to the significant divergence and variability of RNA editing across and within different levels of terrestrial plant groups (He *et al.*, 2016; Knie *et al.*, 2016; Dong *et al.*, 2019; Fan *et al.*, 2019). Currently, we do not have enough evidence to definitively support any one hypothesis about the evolution of RNA editing in Marchantiopsida.

The C-to-U RNA editing events in *C. cavernarum* exhibit similarities to those observed in liverworts and other land plants (Edera *et al.*, 2018; Dong *et al.*, 2019; Wu & Chaw, 2022). These events are characterized by the following features: (1) the average number of RNA editing sites per gene in mtDNA is typically higher than in cpDNA. (2) The editing events mainly occur at the second codon position and then at the first position, resulting in changes to the amino acid. The synonymous RNA editing at the third position, which appears to be 'evolutionary noise', is rare, but it can play important roles in regulating gene expression, protein folding, and other aspects of protein functions (Bentolila *et al.*, 2008; Sloan *et al.*, 2010; Sloan & Taylor, 2010; Duan *et al.*, 2023). (3) Amino acid changes caused by nonsynonymous C-to-U RNA editing tend to increase or maintain the hydrophobicity of proteins. (4) The most notable feature is that most C-to-U RNA editing sites in *C. cavernarum* seem to correct unfavorable T-to-C mutations when compared with other species of Marchantiopsida without RNA editing.

In simpler terms, RNA editing in *C. cavernarum* likely serves to restore ancestral states for alleles and amino acids. The majority of nonsynonymous RNA editing cases, specifically 110 out of 119 sites in cpDNA and 150 out of 164 sites in mtDNA, align perfectly with the restorative hypothesis. This hypothesis has been used to explain the biological significance of nonsynonymous editing in vascular plants (Jiang & Zhang, 2019; Duan *et al.*, 2023). Hence, these nonsynonymous RNA editing sites in *C. cavernarum* can be considered to be evolutionarily adaptive. Now, the question arises: what drives this evolutionary adaptation? Naturally, the primary focus lies on the distinctive characteristics of *Cyathodium* species, which is their capability to adapt to low-light conditions and even cave habitats. However, currently, there is no direct evidence linking the significance of RNA editing to low-light adaptation in *C. cavernarum*. This is an important aspect that should be explored in future research.

The significant expansion of PPR editing factors likely contributing to RNA editing in *C. cavernarum*

In land plants, PLS-class PPR proteins, specifically DYW-type RNA editing factors, are the principal enzyme factors executing the RNA editing events (Yagi *et al.*, 2013; Cheng *et al.*, 2016; Knoop, 2023; Small *et al.*, 2023). The upstream arrays of PPR motifs play a crucial role in binding specifically to transcripts, with one PPR motif per ribonucleotide. This is what determines which cytidines are targeted for editing (Shen *et al.*, 2016; Yan *et al.*, 2019; Gerke *et al.*, 2020; Small *et al.*, 2023). The C-terminal DYW domain has been identified as a cytidine deaminase, containing a highly conserved signature $HxEx_{25}CxxC$ motif, which catalyzes cytidine deamination in the C-to-U RNA editing process (Oldenkott *et al.*, 2019; Takenaka *et al.*, 2021). The DYW domains in *C. cavernarum* exhibit a conserved sequence structure compared with that of validated editing factors in *P. patens* (Fig. 6b), and thus, they are largely the candidates of cytidine deaminases responsible for C-to-U RNA editing events in *C. cavernarum*. Moreover, it has been extensively documented that there is a direct correlation between the quantity of nuclear DYW-type protein genes and the abundance of organellar RNA editing (Rüdinger *et al.*, 2012; Schallenberg-Rüdinger & Knoop, 2016). It is reasonable to assume that the 251 DYW-type RNA editing factors (244 DYW-type proteins have full PPR motifs) in the nuclear genome may account for the 301 C-to-U RNA editing sites in *C. cavernarum* organelles. The number of RNA editing events slightly surpasses the number of DYW-type editing factors, possibly because one editing factor can target multiple editable cytidines (Rüdinger *et al.*, 2011; Hein *et al.*, 2020).

The loss of a specific RNA editing site can be explained by the fixation of C-to-T mutations, which may be induced by gene conversion with reverse-transcribed mRNA (i.e. retroprocessing) at potential editable sites on a genomic level (Freyer *et al.*, 1997; Sloan *et al.*, 2010; Grewe *et al.*, 2011). In non-*Cyathodium*

Marchantiopsida species, the predominant bases at potential editable sites are Ts (Table 1) and the absence of RNA editing capability in these species corresponds to the lack of DYW-type protein genes, crucial for C-to-U RNA editing (Fig. 6a) (Bowman *et al.*, 2017; Dong *et al.*, 2019). Instead, a comparative analysis of homologous sites in the Marchantiopsida suggests that RNA editing in *C. cavernarum* probably serves to restore T-to-C mutations in DNA and maintain protein conservation. The C-to-U RNA editing is recognized as an innovative adaptive molecular trait exhibited by early land plants in response to increased mutational stress during their transition from aquatic to terrestrial environments (Fujii & Small, 2011; Takenaka *et al.*, 2013). RNA editing in *Cyathodium* can also be regarded as a toolkit that resists mutational stress caused by environmental changes through the expansion of DYW-type PPR editing factors. However, C*yathodium* species usually occupy sheltered niches, in some cases dark caves, and are not easily exposed to strong UV radiation that can cause mutational stress. Therefore, further research is needed to investigate the specific acquisition and adaptive mechanisms of C-to-U RNA editing in *Cyathodium*.

The emergence of the DYW domain in *C. cavernarum* is obviously clade-specific (Fig. 7a); however, its origin remains uncertain. One posited scenario is that the appearance of the first DYW domain in *C. cavernarum* is the result of new-gene formation within its nuclear genome. Alternatively, the DYW domains in *C. cavernarum* are most closely related to the clade comprising 13 DYW domains of Bryopsida with moderate support (Fig. 7b), indicating that horizontal gene transfer (HGT) could potentially explain the origin of the first DYW domain in *C. cavernarum*. If so, the RNA editing capability in *Cyathodium* may derive from the DYW-type PPR protein genes of HGT, which is a crucial clue to trace the possibility of the secondary acquisition of RNA editing in *Cyathodium*. Indeed, there appear to be no discernible geographical or ecological relationships between these moss species and *Cyathodium*. Whether the potential moss species showing the closest relationship to *Cyathodium*'s DYW domain is not sampled in our dataset, or whether the ancestors of Bryopsida and *Cyathodium* have ever interacted to transfer the DNA in ancient times remains a question. There are some potential limitations to using the DYW domain as a stand-in for the entire DYW-type PPR protein, which may not fully capture its true evolutionary history (Cheng *et al.*, 2016; Gutmann *et al.*, 2020). The PPR protein genes are known for their modularity and repetition, which paint a complex picture of their evolution (Cheng *et al.*, 2016; Gutmann *et al.*, 2020; Knoop, 2023). DYW-type proteins, as a subset of PPR proteins, pose their own evolutionary puzzles. This is especially true when considering DYW-type proteins in *Cyathodium*, which bind to and catalyze deamination of a wide range of RNA transcript targets. There also seems to be a pattern of parallel evolution between *C. cavernarum* and other bryophytes in terms of shared RNA editing sites. However, the specifics of the DYW-type PPR proteins underlying this pattern are still unclear. To shed light on these issues, more comprehensive bioinformatic analyses and experimental investigations using the culture system and the genetic transformation system of *C. cavernarum* will be vital in answering these questions.

Cyathodium cavernarum, an excellent choice for studying RNA editing in land plants

One of the primary theories regarding the origin of RNA editing in land plants is that it evolved as a result of their long-term interaction with the challenges posed by the environment (Fujii & Small, 2011; Takenaka *et al.*, 2013). This mechanism of RNA editing is believed to have emerged through a series of evolutionary events influenced by natural selection, allowing land plants to overcome the constraints of their terrestrial habitat (Takenaka *et al.*, 2013; Small *et al.*, 2020; Knoop, 2023). Nonetheless, the process by which plants acquire these RNA editing abilities remains unknown.

The understanding of the molecular mechanism of RNA editing and the factors that affect it in land plants is primarily based on discoveries made in angiosperm plants, especially model plants and crops (Lurin *et al.*, 2004; Kotera *et al.*, 2005; Guillaumot *et al.*, 2017; Yan *et al.*, 2017; Yang *et al.*, 2020, 2022; Zu *et al.*, 2023). Specifically, a complex editosome consisting of PPR editing factors and other co-factors such as MORF/RIP, ORRM, OZ1, and PPO1 performs the process of RNA editing in those plants (Sun *et al.*, 2016; Gutmann *et al.*, 2017; Yan *et al.*, 2018). Among nonseed plants, *Physcomitrium patens* is the primary system for studying plant RNA editing. Experimental evidence has shown that individual single DYW-PPR proteins in *P. patens* can specifically target and catalyze RNA deamination (Rüdinger *et al.*, 2009, 2011; Ichinose *et al.*, 2013, 2014; Oldenkott *et al.*, 2020; Knoop, 2023; Yang *et al.*, 2023). However, no research has been conducted on the RNA editing mechanism in liverworts, likely because the liverwort model *M. polymorpha* lacks the RNA editing capability (Bowman *et al.*, 2022).

Some *Cyathodium* species flourish in lighter though still continuously shaded habitats, such as along banks and tracks in the pantropics (*C. acrotrichum* Schiffn.) (Singh & Singh, 2007) and under dripping recesses in Southern Italy (*C. foetidissimum* Schiffn.) (Duckett & Ligrone, 2006) as opposed to the usual restriction of *C. cavernarum* to caves. It would now be of interest to investigate the extent of RNA editing, not only across the genus but also in deep shade ferns, *Selaginella*, and mosses like *Schistostega pennata* (Hedw.) F.Weber & D.Mohr and *Eucladium verticillatum* (With.) Bruch & Schimp. (Atherton *et al.*, 2010). *Cyathodium* is so far the only reported Marchantiopsida genus with RNA editing. *Cyathodium cavernarum* possesses a large number of RNA editing sites and DYW-type PPR factors. It may be an ideal candidate model plant for further research on RNA editing in land plants.

Acknowledgements

We are grateful to Dr Volker Knoop (University of Bonn) for the discussion on the evolution of RNA editing, Dr Bernard Gutmann (Kyushu University) for advice on the phylogenetic tree analysis of DYW domains, Yu-Mei Wei and Qi-Ming Tang (Guangxi Institute of Botany, Chinese Academy of Sciences), Chang Cao, Zi-Yin Cao, Zhi-Fang Zhao, Lei Shu, and Hao Li (East China Normal University) for their help in the field collections, sterile culture, and data analyses. This work was supported by the National Natural Science Foundation of China (nos.

31970215 and 32370218) and East China Normal University (the Research Funds of Happiness Flower ECNU).

Competing interests

None declared.

Author contributions

R-LZ and QZ conceived the study; CS performed experiments and analyzed the data; HX and W-ZH analyzed the data; R-LZ, QZ and CS interpreted the results and wrote the manuscript draft. All authors read and approved the final manuscript.

ORCID

Wen-Zhuan Huang https://orcid.org/0000-0001-5871-5699
Chao Shen https://orcid.org/0000-0002-7553-7457
Hao Xu https://orcid.org/0000-0003-2521-1368
Qiong Zhao https://orcid.org/0000-0003-4557-3139
Rui-Liang Zhu https://orcid.org/0000-0002-0163-8255

Data availability

The organelle genome assembly and annotation of *C. cavernarum* were deposited in GenBank under accession no.: PP078732 (mitochondrial genome) and PP078733 (chloroplast genome). The raw data of the transcriptome for *C. cavernarum* were deposited in the National Genomics Data Center (NGDC) under Genome Sequence Archive (GSA) accession no.: CRA014222 (https://bigd.big.ac.cn/gsa/browse/CRA014222). The PPR protein sequences of *C. cavernarum* genome were deposited in Figshare (doi: 10.6084/m9.figshare.25256221.v1). The alignment and phylogenetic tree of DYW domain were deposited in Figshare (doi: 10.6084/m9.figshare.25266757.v1). The chloroplast genomes with RNA editing information of *C. aureonitens* and *C. tuberosum* were deposited in Figshare (doi: 10.6084/m9.figshare.25256275.v1). The raw data of transcriptomes of *C. aureonitens* and *C. tuberosum* were deposited in the National Genomics Data Center, China (NGDC) under GSA accession no.: CRA010517 (https://bigd.big.ac.cn/gsa/browse/CRA010517).

References

Allen GC, Flores-Vergara MA, Krasynanski S, Kumar S, Thompson WF. 2006. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. *Nature Protocols* **1**: 2320–2325.

Atherton I, Bosanquet S, Lawley M, eds. 2010. *Mosses and liverworts of Britain and Ireland: a field guide*. London, UK: British Bryological Society.

Bechteler J, Peñaloza-Bojacá G, Bell D, Gordon Burleigh J, McDaniel SF, Christine Davis E, Sessa EB, Bippus A, Christine Cargill D, Chantanoarrapint S *et al.* 2023. Comprehensive phylogenomic time tree of bryophytes reveals deep relationships and uncovers gene incongruences in the last 500 million years of diversification. *American Journal of Botany* **110**: e16249.

Bentolila S, Elliott LE, Hanson MR. 2008. Genetic architecture of mitochondrial editing in *Arabidopsis thaliana*. *Genetics* **178**: 1693–1708.

Bischler-Causse H, Gradstein SR, Jovet-Ast S, Long DG, Salazar Allen N. 2005. Marchantiidae. *Flora Neotropica* **97**: 1–262.

Bowman JL, Arteaga-Vazquez M, Berger F, Briginshaw LN, Carella P, Aguilar-Cruz A, Davies KM, Dierschke T, Dolan L, Dorantes-Acosta AE *et al.* 2022. The renaissance and enlightenment of *Marchantia* as a model system. *Plant Cell* **34**: 3512–3542.

Bowman JL, Kohchi T, Yamato KT, Jenkins J, Shu S, Ishizaki K, Yamaoka S, Nishihama R, Nakamura Y, Berger F *et al.* 2017. Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell* **171**: 287–304.e15.

Cheng S, Gutmann B, Zhong X, Ye Y, Fisher MF, Bai F, Castleden I, Song Y, Song B, Huang J *et al.* 2016. Redefining the structural motifs that determine RNA binding and RNA editing by pentatricopeptide repeat proteins in land plants. *The Plant Journal* **85**: 532–547.

Crooks GE, Hon G, Chandonia J-M, Brenner SE. 2004. WebLogo: a sequence logo generator. *Genome Research* **14**: 1188–1190.

Darling ACE, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Research* **14**: 1394–1403.

Dong S, Yu J, Zhang L, Goffinet B, Liu Y. 2022. Phylotranscriptomics of liverworts: revisiting the backbone phylogeny and ancestral gene duplications. *Annals of Botany* **130**: 951–964.

Dong S, Zhang S, Zhang L, Wu H, Goffinet B, Liu Y. 2021. Plastid genomes and phylogenomics of liverworts (Marchantiophyta): conserved genome structure but highest relative plastid substitution rate in land plants. *Molecular Phylogenetics and Evolution* **161**: 107171.

Dong S, Zhao C, Zhang S, Wu H, Mu W, Wei T, Li N, Wan T, Liu H, Cui J *et al.* 2019. The amount of RNA editing sites in liverwort organellar genes is correlated with GC content and nuclear PPR protein diversity. *Genome Biology and Evolution* **11**: 3233–3239.

Duan Y, Cai W, Li H. 2023. Chloroplast C-to-U RNA editing in vascular plants is adaptive due to its restorative effect: testing the restorative hypothesis. *RNA* **29**: 141–152.

Duckett JG, Ligrone R. 2006. *Cyathodium* Kunze (Cyathodiaceae: Marchantiales), a tropical liverwort genus and family new to Europe, in Southern Italy. *Journal of Bryology* **28**: 88–96.

Edera AA, Gandini CL, Sanchez-Puerta MV. 2018. Towards a comprehensive picture of C-to-U RNA editing sites in angiosperm mitochondria. *Plant Molecular Biology* **97**: 215–231.

Fan W, Guo W, Funk L, Mower JP, Zhu A. 2019. Complete loss of RNA editing from the plastid genome and most highly expressed mitochondrial genes of *Welwitschia mirabilis*. *Science China Life Sciences* **62**: 498–506.

Flores JR, Bippus AC, Suárez GM, Hyvönen J. 2021. Defying death: incorporating fossils into the phylogeny of the complex thalloid liverworts (Marchantiidae, Marchantiophyta) confirms high order clades but reveals discrepancies in family-level relationships. *Cladistics* **37**: 231–247.

Freyer R, Kiefer-Meyer M-C, Kössel H. 1997. Occurrence of plastid RNA editing in all major lineages of land plants. *Proceedings of the National Academy of Sciences, USA* **94**: 6285–6290.

Fujii S, Small I. 2011. The evolution of RNA editing and pentatricopeptide repeat genes. *New Phytologist* **191**: 37–47.

Gerke P, Szövényi P, Neubauer A, Lenz H, Gutmann B, McDowell R, Small I, Schallenberg-Rüdinger M, Knoop V. 2020. Towards a plant model for enigmatic U-to-C RNA editing: the organelle genomes, transcriptomes, editomes and candidate RNA editing factors in the hornwort *Anthoceros agrestis*. *New Phytologist* **225**: 1974–1992.

Gray MW. 2012. Evolutionary origin of RNA editing. *Biochemistry* **51**: 5235–5242.

Grewe F, Herres S, Viehöver P, Polsakiewicz M, Weisshaar B, Knoop V. 2011. A unique transcriptome: 1782 positions of RNA editing alter 1406 codon identities in mitochondrial mRNAs of the lycophyte *Isoetes engelmannii*. *Nucleic Acids Research* **39**: 2890–2902.

Groth-Malonek M, Wahrmund U, Polsakiewicz M, Knoop V. 2007. Evolution of a pseudogene: exclusive survival of a functional mitochondrial *nad7* gene supports *Haplomitrium* as the earliest liverwort lineage and proposes a secondary loss of RNA editing in Marchantiidae. *Molecular Biology and Evolution* **24**: 1068–1074.

Guillaumot D, Lopez-Obando M, Baudry K, Avon A, Rigaill G, Falcon de Longevialle A, Broche B, Takenaka M, Berthomé R, De Jaeger G *et al.* 2017.

Two interacting PPR proteins are major *Arabidopsis* editing factors in plastid and mitochondria. *Proceedings of the National Academy of Sciences, USA* **114**: 8877–8882.

Gutmann B, Royan S, Schallenberg-Rüdinger M, Lenz H, Castleden IR, McDowell R, Vacher MA, Tonti-Filippini J, Bond CS, Knoop V *et al.* 2020. The expansion and diversification of pentatricopeptide repeat RNA-editing factors in plants. *Molecular Plant* **13**: 215–230.

Gutmann B, Royan S, Small I. 2017. Protein complexes implicated in RNA editing in plant organelles. *Molecular Plant* **10**: 1255–1257.

He P, Huang S, Xiao G, Zhang Y, Yu J. 2016. Abundant RNA editing sites of chloroplast protein-coding genes in *Ginkgo biloba* and an evolutionary pattern analysis. *BMC Plant Biology* **16**: 257.

Hein A, Brenner S, Polsakiewicz M, Knoop V. 2020. The dual-targeted RNA editing factor AEF1 is universally conserved among angiosperms and reveals only minor adaptations upon loss of its chloroplast or its mitochondrial target. *Plant Molecular Biology* **102**: 185–198.

Hu R, Li X, Hu Y, Zhang R, Lv Q, Zhang M, Sheng X, Zhao F, Chen Z, Ding Y *et al.* 2023. Adaptive evolution of the enigmatic *Takakia* now facing climate change in Tibet. *Cell* **186**: 3558–3576.

Huang KY, Kan SL, Shen TT, Gong P, Feng YY, Du H, Zhao YP, Wan T, Wang XQ, Ran JH. 2022. A comprehensive evolutionary study of chloroplast RNA editing in gymnosperms: a novel type of G-to-A RNA editing is common in gymnosperms. *International Journal of Molecular Sciences* **23**: 10844.

Ichinose M, Sugita C, Yagi Y, Nakamura T, Sugita M. 2013. Two DYW subclass PPR proteins are involved in RNA editing of *ccmFc* and *atp9* transcripts in the moss *Physcomitrella patens*: first complete set of PPR editing factors in plant mitochondria. *Plant and Cell Physiology* **54**: 1907–1916.

Ichinose M, Uchida M, Sugita M. 2014. Identification of a pentatricopeptide repeat RNA editing factor in *Physcomitrella patens* chloroplasts. *FEBS Letters* **588**: 4060–4064.

Jiang D, Zhang J. 2019. The preponderance of nonsynonymous A-to-I RNA editing in coleoids is nonadaptive. *Nature Communications* **10**: 5411.

Jin JJ, Yu WB, Yang JB, Song Y, dePamphilis CW, Yi TS, Li DZ. 2020. GetOrganelle: a fast and versatile toolkit for accurate *de novo* assembly of organelle genomes. *Genome Biology* **21**: 241.

Katoh K, Standley DM. 2013. Mafft multiple sequence alignment software v.7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C *et al.* 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**: 1647–1649.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology* **14**: R36.

Knie N, Grewe F, Fischer S, Knoop V. 2016. Reverse U-to-C editing exceeds C-to-U RNA editing in some ferns – a monilophyte-wide comparison of chloroplast and mitochondrial RNA editing suggests independent evolution of the two processes in both organelles. *BMC Evolutionary Biology* **16**: 134.

Knoop V. 2023. C-to-U and U-to-C: RNA editing in plant organelles and beyond. *Journal of Experimental Botany* **74**: 2273–2294.

Kotera E, Tasaka M, Shikanai T. 2005. A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* **433**: 326–330.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* **25**: 2078–2079.

Lohse M, Drechsel O, Kahlau S, Bock R. 2013. OrganellarGenomeDRAW – a suite of tools for generating physical maps of plastid and mitochondrial genomes and visualizing expression data sets. *Nucleic Acids Research* **41**: W575–W581.

Lurin C, Andreés C, Aubourg S, Bellaoui M, Bitton F, Bruyère C, Caboche M, Debast C, Gualberto J, Hoffmann B *et al.* 2004. Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* **16**: 2089–2103.

Malek O, Lättig K, Hiesel R, Brennicke A, Knoop V. 1996. RNA editing in bryophytes and a molecular phylogeny of land plants. *EMBO Journal* **15**: 1403–1411.

Oldenkott B, Burger M, Hein A-C, Jörg A, Senkler J, Braun H-P, Knoop V, Takenaka M, Schallenberg-Rüdinger M. 2020. One C-to-U RNA editing site and two independently evolved editing factors: Testing reciprocal complementation with DYW-type PPR proteins from the moss *Physcomitrium* (*Physcomitrella*) *patens* and the flowering plants *Macadamia integrifolia* and *Arabidopsis*. *Plant Cell* **32**: 2997–3018.

Oldenkott B, Yang Y, Lesch E, Knoop V, Schallenberg-Rüdinger M. 2019. Plant-type pentatricopeptide repeat proteins with a DYW domain drive C-to-U RNA editing in *Escherichia coli*. *Communications Biology* **2**: 85.

Potter SC, Luciani A, Eddy SR, Park Y, Lopez R, Finn RD. 2018. Hmmer web server: 2018 update. *Nucleic Acids Research* **46**: W200–W204.

Price MN, Dehal PS, Arkin AP. 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLoS ONE* **5**: e9490.

Rüdinger M, Funk HT, Rensing SA, Maier UG, Knoop V. 2009. RNA editing: only eleven sites are present in the *Physcomitrella patens* mitochondrial transcriptome and a universal nomenclature proposal. *Molecular Genetics and Genomics* **281**: 473–481.

Rüdinger M, Polsakiewicz M, Knoop V. 2008. Organellar RNA editing and plant-specific extensions of pentatricopeptide repeat proteins in Jungermanniid but not in Marchantiid liverworts. *Molecular Biology and Evolution* **25**: 1405–1414.

Rüdinger M, Szövényi P, Rensing SA, Knoop V. 2011. Assigning DYW-type PPR proteins to RNA editing sites in the funariid mosses *Physcomitrella patens* and *Funaria hygrometrica*: DYW type PPR proteins and moss RNA editing. *The Plant Journal* **67**: 370–380.

Rüdinger M, Volkmar U, Lenz H, Groth-Malonek M, Knoop V. 2012. Nuclear DYW-type PPR gene families diversify with increasing RNA editing frequencies in liverwort and moss mitochondria. *Journal of Molecular Evolution* **74**: 37–51.

Salazar Allen N, Korpelainen H. 2006. Notes on neotropical *Cyathodium*. *Cryptogamie Bryologie* **27**: 85–96.

Schallenberg-Rüdinger M, Kindgren P, Zehrmann A, Small I, Knoop V. 2013. A DYW-protein knockout in *Physcomitrella* affects two closely spaced mitochondrial editing sites and causes a severe developmental phenotype. *The Plant Journal* **76**: 420–432.

Schallenberg-Rüdinger M, Knoop V. 2016. Coevolution of organelle RNA editing and nuclear specificity factors in early land plants. In: Rensing SA, ed. *Genomes and evolution of charophytes, bryophytes and ferns*. Amsterdam, the Netherlands: Elsevier Academic Press, 37–93.

Shen C, Zhang D, Guan Z, Liu Y, Yang Z, Yang Y, Wang X, Wang Q, Zhang Q, Fan S *et al.* 2016. Structural basis for specific single-stranded RNA recognition by designer pentatricopeptide repeat proteins. *Nature Communications* **7**: 11285.

Shi L, Chen H, Jiang M, Wang L, Wu X, Huang L, Liu C. 2019. CPGAVAS2, an integrated plastome sequence annotator and analyzer. *Nucleic Acids Research* **47**: W65–W73.

Singh DK, Singh D. 2007. Rediscovery of *Cyathodium acrotrichum* Schiffn. (Hepaticae: Cyathodiaceae)–A long-lost liverwort often treated as doubtful taxon. *Current Science* **92**: 1354–1356.

Sloan DB, MacQueen AH, Alverson AJ, Palmer JD, Taylor DR. 2010. Extensive loss of RNA editing sites in rapidly evolving silene mitochondrial genomes: selection vs. retroprocessing as the driving force. *Genetics* **185**: 1369–1380.

Sloan DB, Taylor DR. 2010. Testing for selection on synonymous sites in plant mitochondrial DNA: the role of codon bias and RNA editing. *Journal of Molecular Evolution* **70**: 479–491.

Small I, Melonek J, Bohne A-V, Nickelsen J, Schmitz-Linneweber C. 2023. Plant organellar RNA maturation. *Plant Cell* **35**: 1727–1751.

Small I, Schallenberg-Rüdinger M, Takenaka M, Mireau H, Ostersetzer-Biran O. 2020. Plant organellar RNA editing: what 30 years of research has revealed. *The Plant Journal* **101**: 1040–1056.

Söderström L, Hagborg A, von Konrat M, Bartholomew-Began S, Bell D, Briscoe L, Brown E, Cargill DC, da Costa DP, Crandall-Stotler BJ *et al.* 2016. World checklist of hornworts and liverworts. *PhytoKeys* **59**: 1–828.

Srivastava S, Dixit R. 1996. The genus *Cyathodium* Kunze. *The Journal of the Hattori Botanical Laboratory* **80**: 149–215.

Steinhauser S, Beckert S, Capesius I, Malek O, Knoop V. 1999. Plant mitochondrial RNA editing. *Journal of Molecular Evolution* **48**: 303–312.

Sun T, Bentolila S, Hanson MR. 2016. The unexpected diversity of plant organelle RNA editosomes. *Trends in Plant Science* **21**: 962–973.

Takenaka M, Takenaka S, Barthel T, Frink B, Haag S, Verbitskiy D, Oldenkott B, Schallenberg-Rüdinger M, Feiler CG, Weiss MS *et al.* 2021. DYW domain structures imply an unusual regulation principle in plant organellar RNA editing catalysis. *Nature Catalysis* **4**: 510–522.

Takenaka M, Zehrmann A, Verbitskiy D, Härtel B, Brennicke A. 2013. RNA editing in plants and its evolution. *Annual Review of Genetics* **47**: 335–352.

Tillich M, Lehwark P, Pellizzer T, Ulbricht-Jones ES, Fischer A, Bock R, Greiner S. 2017. GeSeq – versatile and accurate annotation of organelle genomes. *Nucleic Acids Research* **45**: W6–W11.

Träger C, Rosenblad MA, Ziehe D, Garcia-Petit C, Schrader L, Kock K, Vera Richter C, Klinkert B, Narberhaus F, Herrmann C *et al.* 2012. Evolution from the prokaryotic to the higher plant chloroplast signal recognition particle: the signal recognition particle RNA is conserved in plastids of a wide range of photosynthetic organisms. *Plant Cell* **24**: 4819–4836.

Villarreal JC, Crandall-Stotler BJ, Hart ML, Long DG, Forrest LL. 2016. Divergence times and the evolution of morphological complexity in an early land plant lineage (Marchantiopsida) with a slow molecular rate. *New Phytologist* **209**: 1734–1746.

Wick RR, Schultz MB, Zobel J, Holt KE. 2015. Bandage: interactive visualization of *de novo* genome assemblies. *Bioinformatics* **31**: 3350–3352.

Wu C, Chaw S. 2022. Evolution of mitochondrial RNA editing in extant gymnosperms. *The Plant Journal* **111**: 1676–1687.

Xiang YL, Jin XJ, Shen C, Cheng XF, Shu L, Zhu RL. 2022. New insights into the phylogeny of the complex thalloid liverworts (Marchantiopsida) based on chloroplast genomes. *Cladistics* **38**: 649–662.

Xie J, Chen Y, Cai G, Cai R, Hu Z, Wang H. 2023. Tree Visualization By One Table (tvBOT): a web application for visualizing, modifying and annotating phylogenetic trees. *Nucleic Acids Research* **51**: W587–W592.

Xin K, Zhang Y, Fan L, Qi Z, Feng C, Wang Q, Jiang C, Xu JR, Liu H. 2023. Experimental evidence for the functional importance and adaptive advantage of A-to-I RNA editing in fungi. *Proceedings of the National Academy of Sciences, USA* **120**: e2219029120.

Yagi Y, Hayashi S, Kobayashi K, Hirayama T, Nakamura T. 2013. Elucidation of the RNA recognition code for pentatricopeptide repeat roteins involved in organelle RNA editing in plants. *PLoS ONE* **8**: e57286.

Yan J, Yao Y, Hong S, Yang Y, Shen C, Zhang Q, Zhang D, Zou T, Yin P. 2019. Delineation of pentatricopeptide repeat codes for target RNA prediction. *Nucleic Acids Research* **47**: 3728–3738.

Yan J, Zhang Q, Guan Z, Wang Q, Li L, Ruan F, Lin R, Zou T, Yin P. 2017. MORF9 increases the RNA-binding activity of PLS-type pentatricopeptide repeat protein in plastid RNA editing. *Nature Plants* **3**: 17037.

Yan J, Zhang Q, Yin P. 2018. RNA editing machinery in plant organelles. *Science China Life Sciences* **61**: 162–169.

Yang Y, Liu X, Wang K, Li J, Zhu G, Ren S, Deng Z, Zhu B, Fu D, Qu G *et al.* 2020. Molecular and functional diversity of organelle RNA editing mediated by RNA recognition motif-containing protein ORRM4 in tomato. *New Phytologist* **228**: 570–585.

Yang Y, Ritzenhofen K, Otrzonsek J, Xie J, Schallenberg-Rüdinger M, Knoop V. 2023. Beyond a PPR-RNA recognition code: Many aspects matter for the multi-targeting properties of RNA editing factor PPR56. *PLoS Genetics* **19**: e1010733.

Yang YZ, Liu XY, Tang JJ, Wang Y, Xu C, Tan BC. 2022. GRP23 plays a core role in E-type editosomes via interacting with MORFs and atypical PPR-DYWs in *Arabidopsis* mitochondria. *Proceedings of the National Academy of Sciences, USA* **119**: e2210978119.

Zhang P, Zhu Y, Guo Q, Li J, Zhan X, Yu H, Xie N, Tan H, Lundholm N, Garcia-Cuetos L *et al.* 2023. On the origin and evolution of RNA editing in metazoans. *Cell Reports* **42**: 112112.

Zu X, Luo L, Wang Z, Gong J, Yang C, Wang Y, Xu C, Qiao X, Deng X, Song X *et al.* 2023. A mitochondrial pentatricopeptide repeat protein enhances cold tolerance by modulating mitochondrial superoxide in rice. *Nature Communications* **14**: 6789.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The assembly graph of *Cyathodium cavernarum* cpDNA generated by GetOrganelle v.1.7.5.1.

Fig. S2 The assembly graphs of *Cyathodium cavernarum* mtDNA generated by GetOrganelle v.1.7.5.1.

Fig. S3 The graphs of *Cyathodium cavernarum* mtDNA in the configurations of circle1, circle2, circle3 and circle4.

Fig. S4 Whole-genomic synteny of different configurations of *Cyathodium cavernarum* mtDNA by Mauve.

Fig. S5 Whole-genome alignment of three chloroplast genomes of *Cyathodium*.

Fig. S6 Distribution of RNA editing sites based on codon position in *Cyathodium cavernarum*.

Fig. S7 The bases of edited sites in *Cyathodium cavernarum* and the bases of their homologous sites in other nine species of Marchantiopsida without RNA editing.

Notes S1 RNA editing in the chloroplasts of two other *Cyathodium* species.

Notes S2 Potential genome recombination of *Cyathodium cavernarum* mtDNA.

Table S1 Primer sequences of PCR validation for C-to-U RNA editing in *Cyathodium cavernarum*.

Table S2 The selected species of bryophytes with their organelle genomes for comparison.

Table S3 The distribution of PPR protein genes in Marchantiopsida and dataset resource.

Table S4 The length, depth, contained genes, and gene orders of each assembled contig of mtDNA in *Cyathodium cavernarum*.

Table S5 The details of C-to-U RNA editing sites in *Cyathodium cavernarum*.

Table S6 Expression level of protein-coding genes in *Cyathodium cavernarum* organelles.

Table S7 The shared RNA editing sites between *Cyathodium cavernarum* and representatives of bryophytes.

Table S8 The PPR motif arrangement of PPR proteins in *Cyathodium cavernarum*.

Please note: Wiley is not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.