Mitochondrial genome from Andreaea wangiana reveals structural conservatism and a trend of size reduction in mosses

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Mitochondrial genome from *Andreaea wangiana* reveals structural conservatism and a trend of size reduction in mosses

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Abstract. To further reveal the structural characteristics of moss mitochondrial genomes from the still unexplored orders, we sequenced and assembled the mitogenome from the granite moss *Andreaea wangiana* (Andreaeaceae, Andreaeales). The newly generated genome consisted of 117,857 base pairs with an average GC content of 42%. The gene contents and gene order were found to be identical to those previously published from mosses, reconfirming the hypothesis of structural conservatism in this lineage. Comparison of the newly generated mitogenome with those published suggested an evolutionary trend towards size reduction of mitogenomes across the tree of mosses. The pattern was largely caused by hierarchical loss of introns and/or shortening of intergenic spacers. We found evidence to support a positive correlation between GC content and genome size in bryophytes. Furthermore, we identified 233 putative RNA editing sites for all protein-coding genes and 60 simple sequence repeats in this mitogenome. By reporting the complete mitogenome from an important early diverging lineage of mosses, this study provided valuable data for further studies to explore the mechanisms maintaining the stability of genome structure during nearly 400 Ma of independent adaptation to changing terrestrial environments. The study further identified a few highly variable regions that could be used as DNA markers to clarify the genetic diversity of granite moss populations.

Keywords. Andreaeaceae, reduction of mitogenome size, RNA editing sites, Setaphyta, simple repeat sequence, structural conservatism.

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Our understanding of the evolutionary dynamics of mitochondrial genomes in the tree of land plants has been greatly improved as a consequence of a number of phylogenomic/genomic studies (e.g., Dong et al. 2019; Guo et al. 2016a,b; Hecht et al. 2011; Liu et al. 2011, 2014a,b; Palmer et al. 2000; Xue et al. 2010; Zervas et al. 2019). The diversity of mitogenome sizes across the phylogeny of land plants is arguably one of the most intriguing and long-standing topics (Alverson et al. 2010; Liu et al. 2014b; Schneider & Ebert 2004). Compared to the remarkable variations of mitogenome size observed in seed plants—ranging from 220 kb to over 2.7 Mb (Chang et al. 2011; Rodríquez-Moreno et al. 2011), length variation appears to be limited in the three bryophyte lineages with the hornworts showing a range from ~185 kb to ~242 kb, liverworts from ~143 kb to ~187 kb and mosses from ~100 kb to ~141 kb (**Supplementary Table S1**). These results could be considered in the context of the putative monophyly of bryophytes or the Setaphyta hypothesis (de Sousa et al. 2019; Morris et al. 2018; Puttick et al. 2018) as evidence for structural conservatism of mitochondrial genomes during the phylogenetic history of these plants. Several factors have been proposed to influence the plastome or mitogenome size, such as duplication of genes, expansion or loss

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of introns, replication of repetitive DNA, a diverse set of foreign sequences through horizontal or intracellular transfer, resource allocation, and a trade-off between GC content and genome size (Alverson et al. 2010; Goremykin et al. 2012; Liu et al. 2012a; Rice et al. 2013; Veleba et al. 2014). Nevertheless, the processes maintaining the stability of bryophyte mitogenomes in terms of the structural conservation and limited size variations remain unclear (Liu et al. 2011, 2014b; Wang et al. 2009). Resolving these mysteries first requires extensive sampling of mitogenome sequences from all major clades of bryophytes. However, neither the taxon sampling density nor the lineage sampling of these plants is currently sufficient to sustain testing these hypotheses.

Mosses, with a worldwide distribution and ca. 13,000 species, 122 families and 32 orders, are arguably the most successful bryophyte lineage (Goffinet & Buck 2019). Using a genome-wide dataset, this group was well supported as sister to liverworts (de Sousa et al. 2019; Morries et al. 2018; Puttick et al. 2018) rather than as sister to the remaining land plants excluding liverworts (Lemieux et al. 2016; Ruhfel et al. 2014; Zhong et al. 2013). An increasing number of moss mitogenomes has been published (as of Sept. 2019, a total of 42 moss mitogenomes covering 17 families and 13 orders have been published; see **Supplementary Table S1**). These data do not only provide useful genetic characters to resolve the phylogenetic relationships of these taxa, but also greatly improve our understating of the mitogenome evolution in mosses. Specifically, the improved sampling enhances our ability to test three key hypotheses, which expect that (1) bryophyte species share a similar gene assembly of mitochondrial genomes, (2) the size variation is correlated with the length change of non-coding regions, and (3) the most recently diverging lineages likely have the most reduced mitogenomes (Liu et al. 2014b). Evidently, the generality of these hypotheses needs to be further confirmed, because insufficient lineage and species sampling used in previous studies hampered the reliability of published inferences. To provide additional evidence to support the above three hypotheses, we here report for the first time the mitochondrial genome sequences from an Andreaeaceae species—*Andreaea wangiana* Chen in Chen & Wan. Comparative analyses of this newly generated genome and all available moss mitogenomes previously published enabled to test the three key hypotheses and explore the dynamics of mitogenome evolution in mosses.

As the only family of the order Andreaeales, the granite moss family Andreaeaceae contain about 110 species (according to the plant list, Version 1.1, http://www.theplantlist.org/) in two genera, *Andreaea* and *Acroschisma* (Goffinet & Buck 2019). Species in this family prefer rocky habitats and occur widely in temperate montane and arctic-alpine regions (Crosby et al. 1999). They are characterized by a unique combination of morphological characters, including sporophytes placed terminally on an elongate gametophytic stalk (= pseudopodium), absence of a seta, capsule opening by usually four lateral longitudinal valves, and absence of operculum and peristome (Zander, 2007). In recent phylogenetic and phylogenomic studies, Andreaeaceae was consistently supported as sister to the monotypic genus *Andreaeobryum* (Andreaeobryaceae, Andreaeobryales), and the combined clade was in turn supported as sister to the remaining mosses following the separation of Takakiales and Sphagnales (Chang & Graham 2011; Cox et al. 2004; Liu et al. 2019). In this study, we used genome skimming sequencing to obtain the mitochondrial genome sequence of *A. wangiana*, and performed comparative analyses to explore the pattern of mitogenome evolution across mosses, with particular focus on gene assembly and size. In addition, we estimated the putative RNA editing sites and identified simple sequence repeats (SSRs), which carry the potential to be DNA markers for further studies of population genetics.

Material and Methods

The *Andreaea wangiana* plants were collected by Dr. Wen-Zhang Ma and Dr. James R. Shevock in the Shangri-La area (Along Lang-Du road, Ge-Zan Xiang, Diqing Tibetan Autonomous Prefecture, Yunnan province, China, 28°9′19″N, 99°54′30″E). The voucher specimens have been deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Science (KUN) (*W.-Z. Ma & J. R. Shevock 16-8029*). Total genomic DNA was extracted from 10–50 μg of gametophyte material using the modified CTAB method (Forrest et al. 2011). The DNA quantity and quality were assessed using the

Qubit fluorometer system with a Quant-iT™ dsDNA BR Assay (Invitrogen, San Diego, CA, U.S.A.) and NanoDrop Spectrophotometer 2000C (NanoDrop Technologies, Wilmington, DE, U.S.A.), respectively. Approximately 1 μg high quality genomic DNA was sheared ultrasonically using a Covaris S220 sonicator (Covaris, Woburn, MA, U.S.A.). A genome library was constructed using Illumina Nextera XT DNA library preparation based on c. 500 bp long DNA-fragments obtained by shearing the obtained genomic DNA following the manufacturer's manual (Illumina, San Diego, CA, U.S.A.). By generating 90 bp long paired-end sequences using an Illumina HiSeq 2000 at BGI-Shenzhen, about 2 Gb of sequences were accumulated. The raw sequence data were assembled using CLC Genomic workbench (https://www.qiagenbioinformatics.com/) involving quality control of the raw sequences with the NGS QC Tool Kit (https://www.qiagenbioinformatics.com/) with cut-off values for read length and PHRED quality scores set as recommended in Yang et al. (2014). Subsequently, we obtained a total of 509,879 contigs. By blasting, the whole mitogenome was found in a large contig, and then assembled and annotated using published genomes as reference in Geneious v11.1.5 (http://www.geneious.com/). The genome map was generated using OGDRAW (Lohse et al. 2013). The newly generated mitochondrial genome was deposited in GenBank (accession number: MN056355).

A comparative analysis was performed with a dataset consisting of 43 moss mitogenomes. Besides the newly generated one, the dataset included 42 moss mitogenomes previously published (**Supplementary Table S1**). The whole dataset was aligned using the plugin 'Mauve ' in Geneious v11.1.5. (https://www.geneious.com/). Special attention was given to genome size, GC content and gene composition. We tested the hypothesized correlation between GC content and genome size using linear regression analyses for all bryophytes and for each of the three bryophyte lineages separately, namely hornworts, liverworts and mosses. The nonadjacent repeated sequence for the newly generated mitogenome was estimated using REPuter (Kurtz et al. 2001), and putative RNA editing sites (C-to-U and U-to-C editing sites) of the 40 protein-coding regions were identified using PREPACT 3.0 (Lenz et al. 2018) with the BLASTX prediction and 0.001 e-value cut-off, and *Physcomitrella patens* mitogenome was used as a reference. Simple sequence repeats were detected using GMATo v1.2 (Wang et al. 2013). The concept of SSRs follows Gandhi et al. (2010).

Results

The mitochondrial genome of *Andreaea wangiana* had a total length of 117,857 bp (**Fig. 1**, **Supplementary Table S1**), including coding regions with a total length of 39,279 bp, introns with 36,491 bp, and intergenic spacers with 42,083 bp. We identified 67 genes, including three rRNAs, 24 tRNAs and 40 protein-coding genes. The average GC content of the complete genome was 42%. The comparative analyses revealed that the newly generated genome had nearly identical gene content and order with those previously published for mosses. The mitogenome of *A. wangiana* had the second largest sequence, exceeded only by that of the peat moss *Sphagnum palustre* that had a length of 141,276 bp (**Fig. 2**, **Supplementary Table S1**). Genomes of a similar size as *A. wangiana* were found in Polytrichales mosses *Atrichum angustatum* (Brid.) Bruch et Schimp. (115,146 bp) and *Polytrichum commune* Hedw. (114,831 bp), whereas all other mosses had genomes below 109,586 bp and 100,342 bp. Early diverging lineages represented by the peat moss *Sphagnum* and the granite moss *Andreaea* exhibited relatively larger mitogenomes than derived mosses, which is concordant with the loss of some introns and reduction of intergenic spacers during the phylogenetic history of mosses (**Supplementary Table S1**).

A positive correlation between GC content and genome size was found if all bryophyte lineages were analyzed together ($R^2 = 0.657$ $p < 0.001$, **Fig. 3**). However, this correlation was rejected in separated analyses for each lineage, including hornworts ($R^2 = 0.001$, $p = 0.968$), liverworts ($R^2 = 0.107$, $p = 0.185$), and mosses ($R^2 = 0.002$, $p = 0.759$). Using *Physcomitrella patens* (Hedw.) Bruch & Schimp. as reference, 233 (including 161 C-to-U and 72 U-to-C editing sites) putative editing sites were detected for 40 protein-coding genes in the *Andreaea* mitogenome (**Supplementary Table S2**). In total, the mitogenome of this moss contained 60 SSRs loci, including 37 SSRs in intergenic regions and 23 SSRs in genes (**Supplementary Table S3**). Most of SSRs referred to mono- and dinucleotides (29 and 25 loci, respectively). 91.67% of detected SSRs were composed only of A/T bases. The total length of the SSR

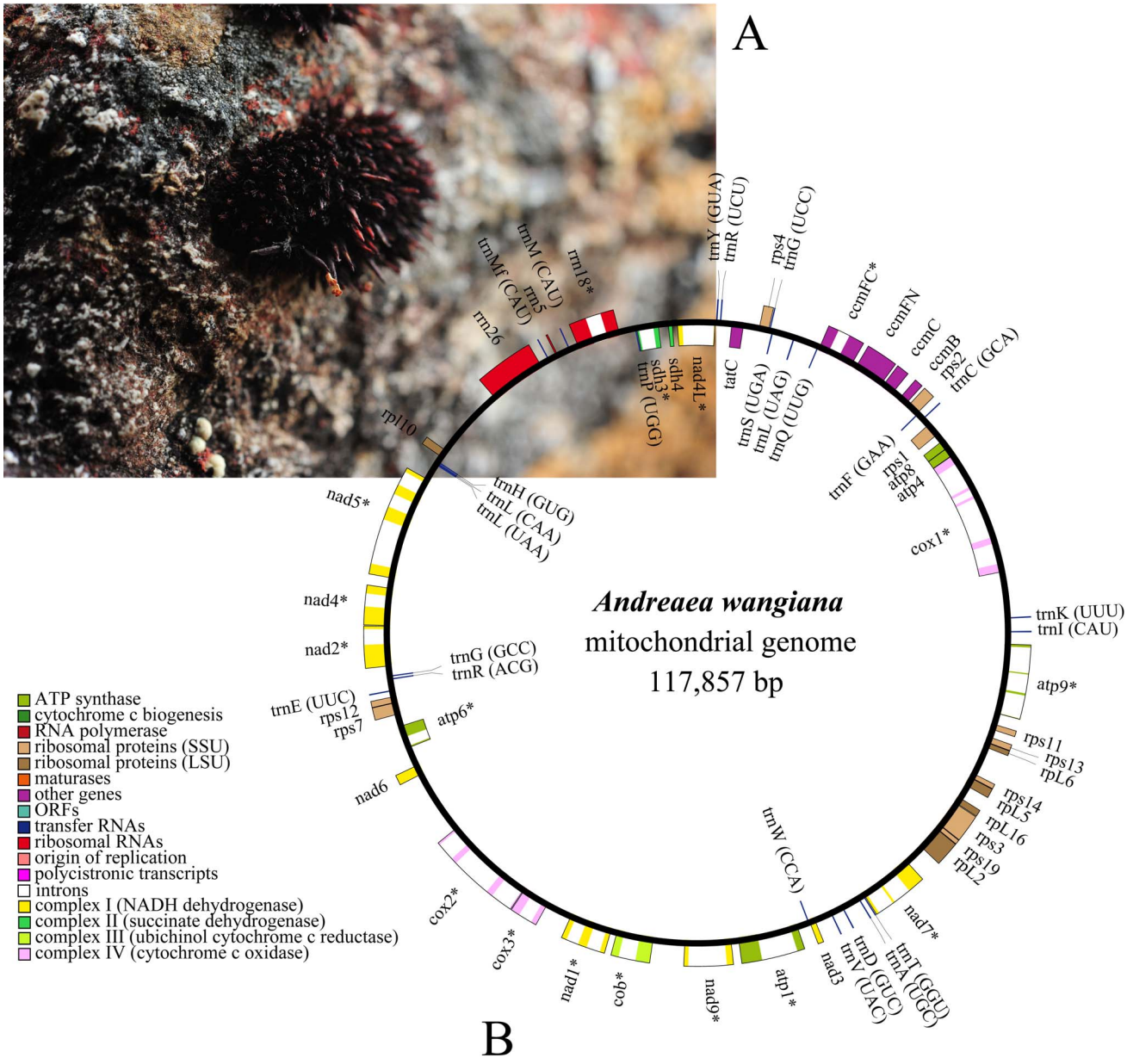

Figure 1. A. *Andreaea wangiana* (Andreaceae) forming dark brown and reddish cushions. Photographs by Wen-Zhang Ma. **B.** Circular visualization mitochondrial genome of *Andreaea wangiana*. Genes (exons are shown as closed boxes) shown on the outside of the circle are transcribed clockwise, whereas on the inside are transcribed counter-clockwise. Genes with group I or II introns (open boxes) are labeled with asterisks.

loci was 714 bp, accounting for approximately 0.61% of the whole genome.

Discussion

The mitogenomes of mosses available to this point provide support for the hypothesis that the mitogenomes are relatively stable in terms of gene assembly in this group (Liu et al. 2011, 2014b; Wang et al. 2009), with the exception that *nad7* was lost in *Buxbaumia aphylla* Hedw., *Mielichhoferia elongata* (Hoppe & Hornsch.) Nees & Hornsch and *Tetraphis pellucida* Hedw., and *rpl*10 pseudogenized in *Ptychomnion cygnisetum* (Müll. Hal.) Kindb (Bell et al. 2014; Goryunov et al. 2018; Liu et al. 2014b). This hypothesis obtained further support by our newly generated mitogenome that shared the gene content and order with those previously published mitogenomes of mosses. It is important to note that *Andreaea* represents one of the most isolated lineages in mosses.

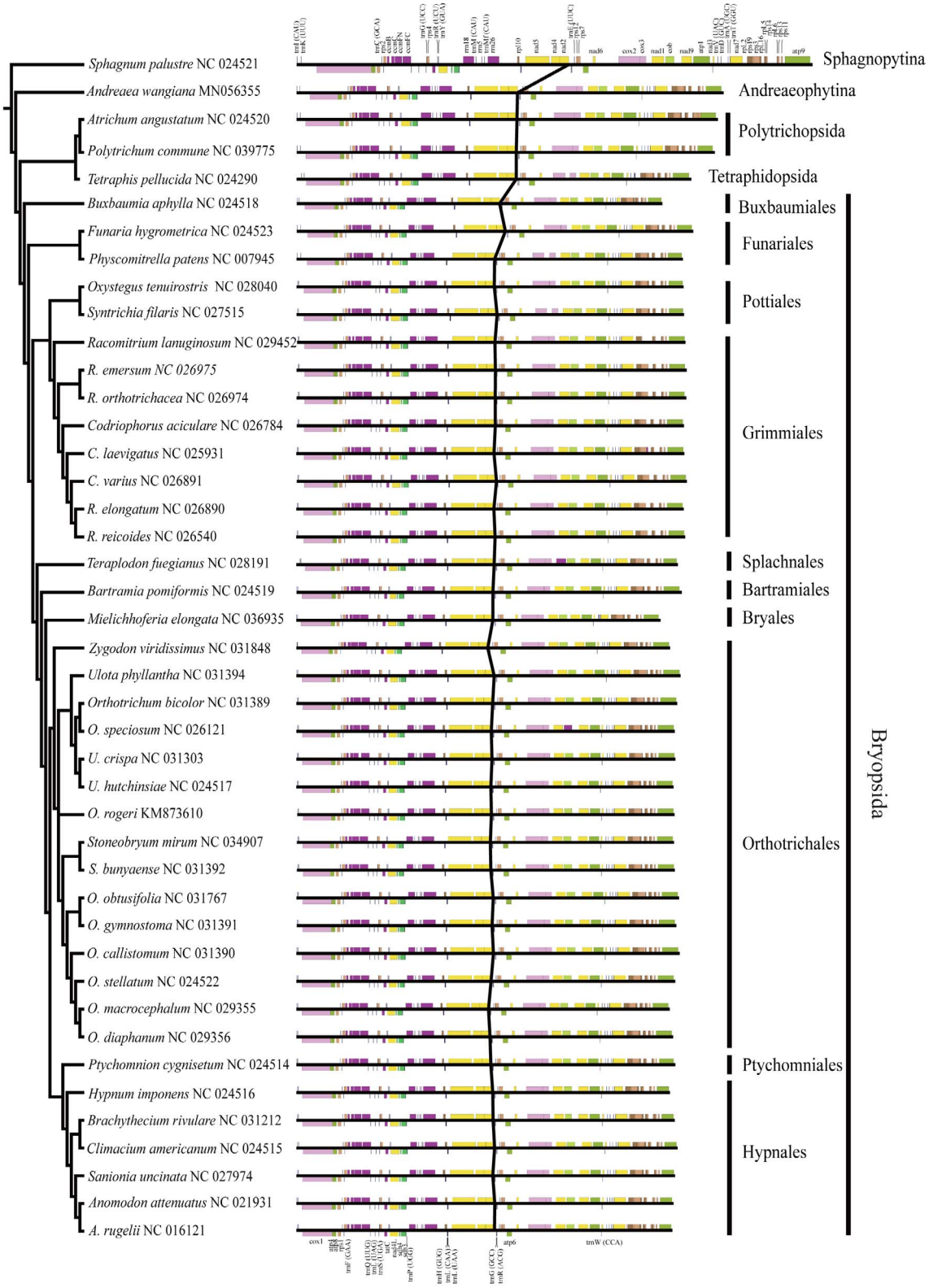

Figure 2. Synteny of mitochondrial genomes of 43 mosses organized following the phylogenetic hypotheses proposed in Goryunov et al. (2018) and Liu et al. (2019). The mitogenomes are shown in linearized form illustrating the relative gene synteny. The solid line connects the end of the *nad*2 gene, whereas the colored bars represent proteins, rRNAs, and tRNA regions. Names of the regions are displayed above and below the graphics. The sequence of the regions is highly conserved across the phylogeny of mosses.

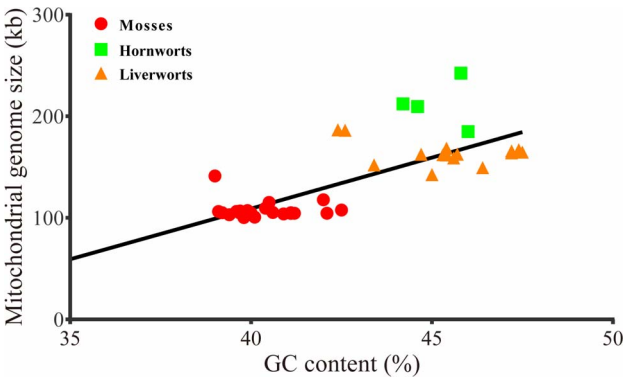

Figure 3. The correlation between the GC content and the mitogenome size among three bryophyte lineages using linear regression. Colored symbols indicate different lineages: red circles = mosses, orange triangles = liverworts, green squares = hornworts. A significantly positive correlation was supported among three lineages, as indicated by $R^2 = 0.657$ and $p < 0.001$, but not within lineages, as indicated by $R^2 = 0.001$ and $p = 0.968$ in hornworts; $R^2 = 0.107$ and $p = 0.185$ in liverworts, and $R^2 = 0.002$ and $p = 0.759$ in mosses.

Although the available data clearly revealed that early land plants almost share the similar gene assembly of plastid and mitochondrial genomes, the maintaining processes remain to be discovered (Liu et al. 2014b). Several hypotheses have been proposed to explain the structural stability of plastid and mitochondrial genomes in bryophytes (André et al. 1992; Liu et al. 2011, 2012b; Maréchal et al. 2009; Shedge et al. 2007). First, it was assumed that nonadjacent sequence repeats longer than 50 bp and 85% or more similar mediated the rearrangements that often take place during recombination (André et al. 1992; Maréchal & Brisson 2010). However, bryophyte mitogenomes usually lack such repeats, except those of *Sphagnum* and *Atrichum* (Liu et al. 2011, 2014b). Thus, this absence likely leads to rarity of rearrangement events occurring in this group. The assumption is consistent with the observation that only one nonadjacent repeat (>50 bp) was found in the mitogenome of *Andreaea wangiana*. Second, some mechanisms controlled by nuclear genes may involve the processes that maintain the stability of mitochondrial genomes in land plants (Abdelnoor et al. 2003; Davila et al. 2011; Maréchal et al. 2009; Shedge et al. 2007; Zaegel et al. 2006). Third, a large number of polycistronic operons have been found in the mitochondrial genomes of bryophytes (Liu et al. 2011, 2012b; Xue et al. 2010), parallel to those occurring in green algae and early land plants (Lang et al. 1997; Ohta et al. 1998; Turmei et al. 2002, 2003). Polycistronic operons are more likely gene clusters, in which the linkage between the genes is tight and the arrangement order is strictly controlled under particular functional selections (Liu et al. 2011). Presence of this trait may not only represent an ancestral state of gene assembly across the plant phylogeny, but also facilitate maintenance of stability of plastid and mitochondrial genomes.

Parallel to the evolutionary pattern found in size, moss mitogenomes also exhibit limited variations in the GC content—ranging from 39.0% to 42.5% (**Supplementary Table S1**). The observed range is much narrower than those documented in other plant lineages, such as algae from 22.2% to 57.2% (Smith & Lee 2008; Turmel et al. 1999) and vascular plants from 42.3 to 68.1% (Hecht et al. 2011; Park et al. 2015). Genome size has been assumed to be one of important causes or drivers indirectly or directly shaping the evolution of GC content (Rocha & Danchin 2002; Šmarda & Bureš 2012; Veleba et al. 2014), but the correlation between these two variants seems causal as it varied across different phyla and species (e.g., positive in bacteria, fungi and some land plants, negative in animals, or not significant in protists, Li & Du 2014). In this study, the regression analysis supported a positive correlation between the mitogenome size and GC content among, but not within, the three major lineages. This correlation is also found in some other plant groups (Li & Du 2014). Nevertheless, further studies are required to test the robustness of this link in bryophytes and explore the underlying processes.

Previous studies have suggested a trend towards reduction of mitogenome size in the phylogenetic history of mosses (Liu et al. 2014b). The mediate size of *Andreaea wangiana* that is smaller than those from the earlier diverging peat mosses but large than those from derived mosses, supported this hypothesis. This trend is consistent with its phylogenetic positions in the tree of mosses—diverging from the ancestors of mosses following Takakiales and Sphagnales (**Fig. 2**, **Supplementary Fig. S1**; Liu et al. 2019). Most of such size variations could be explained by the processes of hierarchical loss of introns and/or shortening of intergenic spacers. For example, two *cox*1 introns *cox1i323g2* and *cox1-i1200g2* are only found in the mitogenomes of *Sphagnum*, and the ribosomal intron *rrn18i839g1* is absent in all Bryopsida taxa. Furthermore, some introns in derived bryophytes, such as *atp9i87g2*,

atp9i95g2, *cox1i1064g2* and *cox2i373g2*, tend to be shorter than those in early diverging lineages. Apart from this evidence, the size of intergenic spacers tends to decrease during the phylogenetic history of mosses. For example, the intergenic spacers accumulated a total length of 54kb in the mitogenome of *Sphagnum* whereas it is 42kb in that of *A. wangiana*, and between 31–38kb in those of derived mosses. The evolutionary significance of the size reduction is still not understood. Thus, currently we cannot rule out that this is a consequence of a neutral or an adaptive evolutionary process.

RNA editing is a post-transcriptional mechanism that alters the identity of nucleotides in an RNA sequence, and allows for the difference between RNA and its corresponding DNA sequence (Nishikura 2010). RNA editing plays an essential role in restoration of the hydrophobicity of the conserved protein domain through correcting DNA mutations at RNA level (He et al. 2016; Takenaka et al. 2013), which may influence the genetic diversity, adaptation and environmental acclimation (Rosenthal 2015). In bryophytes, RNA editing shows high variation among sites—ranging from zero (e.g., *Marchantia*, Salone et al. 2007; Steinhauser et al. 1999) to several hundred bases (e.g., 732 in *Haplomitrium hookeri*, Myszczynski et al. 2019). In the present mitogenome, we estimated 233 putative RNA editing sites using *Physcomitrella patens* as reference. However, we propose that the exact number of RNA editing sites for the mitogenome of *Andreaea wangiana* is likely between three—occurring in start and stop codons and unambiguously defined—to 233. Despite some uncertainty associated with these analyses, it is reasonable to say that the frequency of RNA editing in moss mitogenomes is relatively low compared to those reported for some pteridophytes (e.g., 1072 and 984 editing sites in *Ophioglossum californicum* and *Psilotum nudum*, respectively, Guo et al. 2016b).

Simple sequence repeats (SSRs) are thought to be common in plant mitochondrial genomes, and may contribute to their physiological, biochemical and phenotypic characteristics (Bartom 2006; Kashi & King 2006; Li et al. 2002). Considering their rapid mutation rates, SSRs have been widely used in studies of population genetics and biogeography (Karlin et al., 2008a,b; Shaw et al. 2008). The SSRs search in the mitogenome of *Andreaea wangiana* supported the findings by Zhao et al. (2014). First, diversity of SSRs varies among regions, and non-coding regions are much richer than coding regions. Second, most of SSRs for moss mitogenomes are mononucleotides and/or dinucleotides. In general, SSRs with excellent characteristics for population genetic markers are rare in the mitogenome of these mosses.

In summary, this study reports for the first time the mitochondrial genome sequence from one of important moss lineages, Andreaeales. The mitogenome of this early diverging lineage of mosses provide support to (1) the hypothesis of structural conservatism and (2) the trend of size reduction of mitogenomes during the phylogenetic history of mosses. Furthermore, several highly variable regions were identified which could be used as markers to study the genetic diversity of populations in this group. Nevertheless, some long-standing issues of evolutionary dynamics of moss mitogenomes remain unresolved here, such as the processes maintaining the stability of genomes while still accumulating unknown key mutations to adapt to changing environments, and whether a complex trade-off exists between GC content and genome size.

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Supplementary documents online:

Supplementary Table S1. Sixty-five bryophyte mitogenomes from 60 species, 30 families and 25 orders. The newly generated mitogenome of *Andreaea wangiana* is highlighted.

Supplementary Table S2. Estimation RNA editing sites estimated for 40 protein-coding genes in the mitogenome of *Andreaea wangiana* using *Physcomitrella patens* as reference.

Supplementary Table S3. Sixty SSR-loci through the whole mitogenome of *Andreaea wangiana*. The SSR concept follows Gandhi et al. (2010). Minimal number of repeating units $\geq$10 for mononucleotides, $\geq$5 for dinucleotides, $\geq$4 for trinucleotides, and $\geq$3 for tetra-, penta-, hexauncleotides.

Supplementary Fig. S1. Phylogeny of moss orders following the hypotheses proposed in Goffinet & Buck (2019) and Liu et al. (2019).