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REGULAR PAPER

Towards the conservation of the Mesozoic relict fern *Christensenia*: a fern species with extremely small populations in China

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Abstract

The Chinese occurrences of the marattioid fern genus *Christensenia* have been considered as requiring protection because of its extreme rarity and very small population size. Here, we explored different biological aspects to enable protection of these rare ferns, well known as Mesozoic living fossils. Firstly, we documented the cytology of the Chinese occurrences for the first time. This is the second tetraploid record of *Christensenia* worth for further studies to confirm its taxonomic status. Secondly, we obtained the first complete plastid genome of this genus, which confirmed the proposed conservatism of the plastid genome structure in marattioid ferns. By comparing the chloroplast genome with other marattioids, we identified several candidate regions to develop highly variable markers to investigate the intra-species diversity of marattioid ferns. Thirdly, phylogenetic analyses of *rbcL* sequences implied that there are at least two distinct species of *Christensenia*. Finally, we re-assessed the conservation status of *Christensenia* in the context of its local and global distribution by assessing specimen information extracted from publications and digitized voucher information. This assessment confirmed the need to obtain more accurate information about the distribution of this genus to assess the status incorporating the disjunct distribution from southern China and India in the North towards the Solomon Islands in the South.

Keywords Chloroplast genome · Genome size · Chromosome number · Phylogeny · Conservation assessment

Introduction

Christensenia aesculifolia (Blume) Maxon is a morphological distinct (Fig. 1) but locally rare fern, occurring in damp lowland forests throughout Southeast Asia and Malesia from the Solomon Islands in the South to Assam (India) and Yunnan (China) in the North (Amoroso et al. 2012; Camus 1990; Chen et al. 2017; He and Christenhusz 2013; Murdock 2008a; Praptosuwiryo 2013; Rolleri 1993; Takeuchi 2013; Fig. 2a). This fern was recorded only from two limestone forests located in the South Yunnan counties Jinping and Hekou in China (He and Christenhusz 2013). Despite extensive fieldwork, only one of these two recorded populations has been recovered (Cai et al. 2018). Based on its rarity, *C. aesculifolia* is recognized to be protected under Chinese national law and also categorized as a plant species with extremely small populations (PSESP, Cai et al. 2018; Ma et al. 2013; Sun 2013; Sun et al. 2019). In contrast to the proposed protection status in China, *C. aesculifolia* was considered as least concern by Lindsay and Middleton in their survey of the Thailand ferns (see https://rbg-web2.rbge.org.uk/thaiferns/factsheets/index.php?q=Christensenia_aesculifolia.xml) although this species has been only collected in the southern parts of Thailand (Tagawa and Iwatsuki 1968). Consistent with the observed rarity in China, this fern has been considered to be endangered in the Philippines (https://www.philippineplants.org/Families/Pteridophytes.html) and vulnerable in Assam (https://indiabiodiversity.org/checklist/show/248). A recent report of the fern diversity

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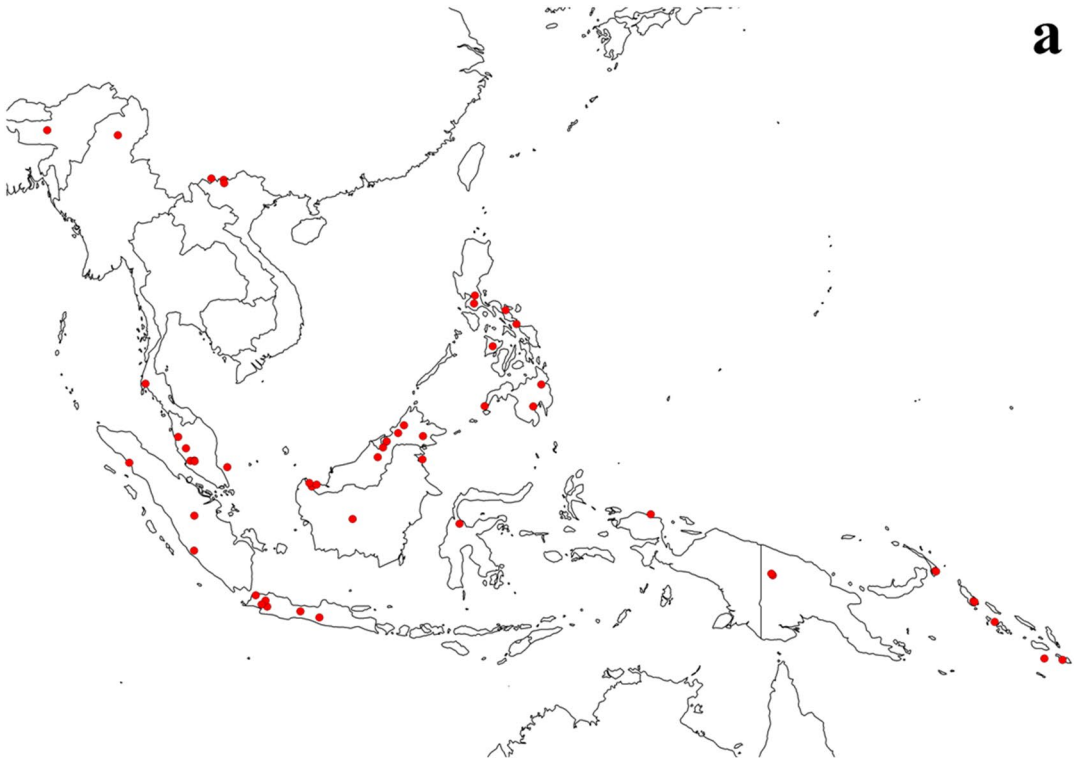
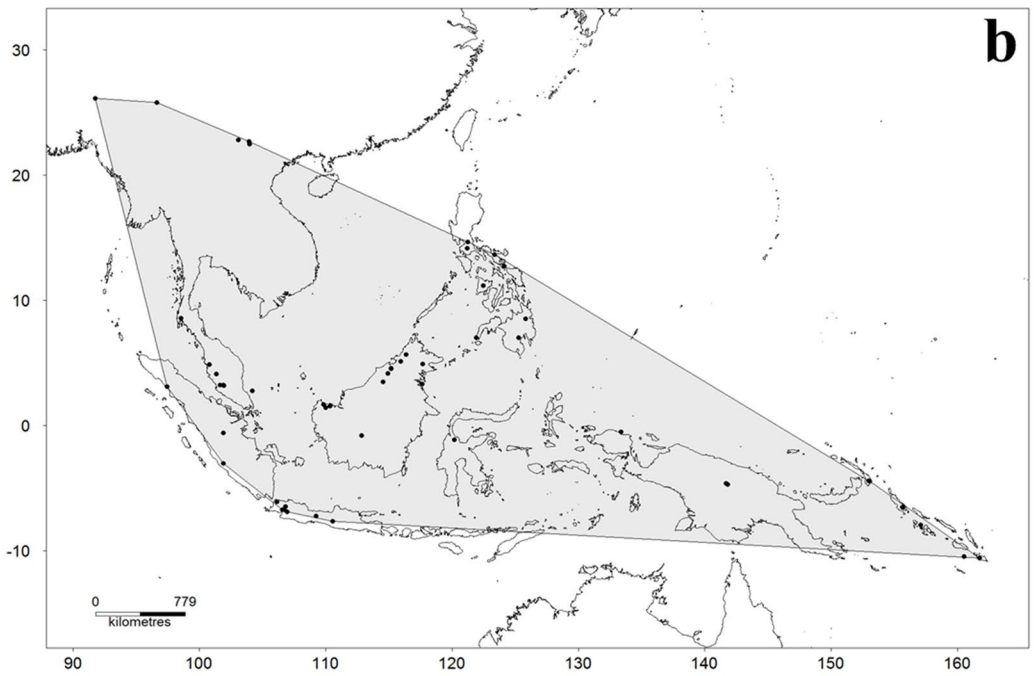
◂**Fig. 1** Sporophytes of *Christensenia*. **a**, **b** Living plants cultivated at XTBG. **c** Rhizome of *Christensenia*. **d** Pedately lobed leaf. **e** Fertile leaf with young synangia. **f** Fertile leaf with mature synangia

on Gunung Slamet (Java, Indonesia) stressed the rarity and small population size of *C. aesculifolia* in the central parts of Java (Praptosuwiryo 2013). Together, the existing records suggest a highly fragmented northern range with occurrences in northeastern India (Assam), northern Myanmar (Warzup), Southwest Yunnan of China (Jinping, Hekou), and northern Vietnam (Lao Cai) that are disjunct from the geographically closest known populations in the most southern parts of Thailand and the Malay Peninsula (Fig. 2a; see Table S1). However, the existing evidence provides limited information on the important biological parameters such as local ranges and numbers of individuals that are required to assess the local and regional extinction threats. These ferns are known to be adapted to extremely shady conditions in dense lowland rainforests (Boyce and Mohamed 2006), and some populations have been recorded to occur on limestone and in dipterocarp forests (Table S1). As a consequence, the species is expected to be highly threatened by logging of the lowland rainforests throughout its range, especially by the replacement of these forests by economic plantations, such as rubber trees, oil palm as well as banana or ginger (Hughes 2018; Vijay et al. 2016).

The potential highly enhanced extinction risk to this unique fern is especially concerning given the long phylogenetic history of this fern lineage (Rothwell et al. 2018a, b) together with its proposed status as "molecular living fossils" (Soltis et al. 2002). The Marattiales, including the extinct Psaroniaceae and extant Marattiaceae, were among the most successful fern lineages during the late Paleozoic and most of the Mesozoic (Ching 1958; Hill and Camus 1986; Rothwell et al. 2018a, b). *Christensenia* is nested in the crown group Marattiaceae (e.g., Murdock 2008a, b; PPG1 2016; Pryer et al. 2004; Qi et al. 2018; Rothwell et al. 2018a, b; Testo and Sundue 2016), and was estimated to diverge from remaining crown group genera during the Jurassic (Testo and Sundue 2016). Although this genus is easily distinguished by the palmate leaves and circular synangia from other marattioid ferns, the Mesozoic representatives of the *Christensenia* lineage have not been discovered in the fossil record (Rothwell et al. 2018a, b). Extant Marattiaceae include six genera, the three relatively species-rich genera of the lineage—namely the Neotropical *Danaea* Sm. [ca. 50 spp.], the Paleotropical *Angiopteris* Hoffm. [ca. 30 spp.], and *Ptisana* Murdock [20 spp.]—diversified after the Cretaceous-Paleogene boundary, whereas the remaining three genera show low species diversity, including the Paleotropical *Christensenia* [2 spp.], and the two Neotropical genera *Eupodium* J.Sm. [3 spp.], and *Marattia* Sw. [7 spp.] (Christenhusz 2010; Christenhusz et al. 2008; He and Christenhusz 2013; Liu 2016; Murdock 2008a, b; PPG1 2016). These genera may therefore be considered as Mesozoic relicts that were in evolutionary stasis for most of their phylogenetic history. Our current understanding of the evolutionary history of marattioid ferns based on their fossil record (see Rothwell et al. 2018a, b) suggests a scenario in which the genus *Christensenia* originated during the mid-Mesozoic. During this time, the Marattiaceae were widespread and arguably species-rich (Ching 1958; Rothwell et al. 2018a, b). The lineage survived through the transformation of the terrestrial environments caused by the rise of the angiosperms in the mid Cretaceous (Barba-Montoya et al. 2018), which in turn provided ecological opportunities for the diversification of the derived ferns (Schneider et al. 2004). The rarity of Marattiaceae in the Cretaceous floras and the absence of any unambiguous Marattiaceae fossils during the Cenozoic (Collinson 2001; Hu et al. 2006; Vera and Cesari 2016) imply a decline of the lineage that may have been caused by the transformation of the establishment of angiosperm dominated forests or be the consequence of the Cretaceous-Paleogene mass extinction. Therefore the genus *Christensenia* is considered to be a "molecular living fossil" and "mesozoic relict taxon" considering the low mutation rate (see Soltis et al. 2002) and the survival through the major ecological transitions since its Jurassic origin (Rothwell et al. 2018a, b).

Establishing conservation protocols for this taxon is challenged by the sparse knowledge about this unique lineage. The taxonomic status of *Christensenia* species is somewhat still controversial. Some authors accepted a single extant species (Camus 1990), whereas some recent taxonomic revisions proposed two distinct species, of which one includes two subspecies (Rolleri 1993; Rolleri et al. 1996). Most treatments show arguably some preferences towards a monotypic status of the genus. This is also the case for the most recent treatments of the occurrence in China and Thailand (He and Christenhusz 2013). The species in China has been recorded as *C. assamica* (Griff.) Ching (Ching 1958) that is according to Rolleri (1993) a synonym of *C. aesculifolia* subsp. *korthalsii* Rolleri (He and Christenhusz 2013). The morphological differentiation of these taxa is far from clear and further studies are required to confirm their taxonomic status (Murdock 2008a). Furthermore, published chromosome counts support the existing of both diploid ($n = 40$, Borneo; Walker 1979) and tetraploid ($n = 80$, Solomon Islands; Braithwaite 1977) cytotypes. Existing molecular systematic studies were able to reconstruct the relationships of the genus but did not provide information about the intra-generic diversity as only one accession of *Christensenia* was included (see Christenhusz et al. 2008; Murdock 2008b; Senterre et al. 2014). As a consequence, the existing sampling did not incorporate the geographic range and documented morphological variation. Establishing highly





EOO= 14618012 km2
AOO (grid res. 2 km)= 212 km2
Number of unique occurrences= 54
Number of sub-populations (radius 5 km)= 50
Number of locations (grid res.: 10 km) = 52
IUCN category according to criterion B: LC or NT

◂**Fig. 2** Distribution of *Christensenia.* **a** Distribution record (red dots) as given in Table S1. Records plotted had either latitude/longitude information provided or were geo-referenced based on the available occurrence information. Duplicated occurrences were deleted. **b** Polygonal plot used to assess the IUCN category of *Christensenia* as least concern or near threatened (LC or NT) according to the criterion B based on extent of occurrence (EOO) and area of occupancy (AOO)

variable genomic markers to overcome the limitations provided by the detected decelerated molecular evolution (see Soltis et al. 2002) are arguably one of the key issues besides the incorporation of a broader geographic sampling of widespread species.

Apart from the uncertain species determination, the current distribution records are arguably incomplete as indicated by the recent discoveries of this taxon in previously unknown locations in New Guinea (Takeuchi 2013) and Central Java (Praptosuwiryo 2013). The lack of recording quality is likely a result of the disjunct distribution across a large geographical range and the ecological preference of these ferns occurring in damp and dense lowland forests. A further challenge is the lack of biological information required to protect this taxon via ex situ cultivation. In vitro propagation via dormant buds at the stipules has not been exploited yet although this approach was successfully applied to other species of Marattiaceae (Cupitt et al. 2001; Huang et al. 2010; Matthes et al. 2011). The vegetative reproduction may be especially attractive given the challenges to reproduce marattioid ferns gametophyte cultures caused by slow growth rate and potential importance of fungal interactions in both generations (Chou et al. 2007; Ogura-Tsujita et al. 2012; Pressel et al. 2016).

In this study, we aim to improve our knowledge on the proposed "molecular living fossil" *Christensenia* by addressing these issues mentioned above. Since advances in sequencing technology enables the generation of whole plastid genomes, they are increasingly employed in conservation genetics to enhance the information available to study the genomic diversity of the protected organisms. We generated the first complete plastid genome for this genus. The newly generated plastid genome was compared to the two previously published plastid genomes from the same family, namely *Angiopteris angustifolia* C.Presl (NC026300; Zhu et al. 2016) and *A. evecta* (G.Forst.) Hoffm. (NC008829; Roper et al. 2007). This comparison enabled to test the hypothesis of structural conservatism in marattioids plastid genomes (Roper et al. 2007), as well as to identify variable regions to design primers to improve the recording of genotypic diversity among closely related or within species. The latter issue was motivated by the low mutation rates of marattioid ferns that challenge the resolution of species and/or intra-species relationships using well established molecular markers such as *rbcL* and other frequently used markers (Murdock 2008b; Soltis et al. 2002). Furthermore, we investigated the ploidy level of the Chinese occurrences using a combination of chromosome counts and DNA C-value measures. We also revalued the taxonomic status of the Chinese occurrences by comparing the sequence variations with those recorded in Malay Peninsula. Finally, the conservation status of *Christensenia* was assessed in the context of its local and global distribution.

Materials and methods

Plant materials

Several individuals of *Christensenia* are cultivated at Xishuangbanna Tropical Botanical Garden, CAS (Yunnan, China). All individuals originated from the same population in Hekou County, Yunnan. Fresh leaves were removed from the cultivated plants for the complete plastid genome sequencing and DNA C-value measure, and root tips were collected for chromosome counting.

Chromosome counting and cytological measurement

Cytological data was generated using the living plants cultivated at XTBG and the method described by Takamiya (1993). Root tips were fixated to obtain chromosome squashes allowing for visually counting the number of chromosomes in a diploid cell. The root tips were pretreated with mM 8-hyrdoxyquinoline for 10 h at approximately 15 °C and fixated using Carnoy's solution (3:1 ethanol:acetic acid) for 30 min. They were macerated in a 3:1 mixture of 1 N HCL and 45% acetic acid at 60 °C for about 30 s and then squashed in 2% aceto-orcein. The obtained squashes were examined and imaged using a Zeiss Axiophot microscope (Carl Zeiss AG, Oberkochen, Germany) with a 1000× objective and equipped with a Zeiss Axiocam (Fig. 3). Leaf fragments were used to generate DNA C-values using cytofluometric approach (Clark et al. 2016; Fujiwara et al. 2018). The leaf fragments were co-chopped with *Pisum sativum* L. 'Citrat' (2C = 9.09 pg; Dolezel et al. 2018) as the internal standard, using a razor blade in 1.2 ml chopping buffer. The obtained mixture was expanded by adding 0.6 ml chopping buffer, then was filtered through a 0.3 mm nylon mesh, and centrifuged for 2 min at 4,000×*g*. The supernatant was mixed with 0.5 ml chopping buffer and propidium iodide DNA stain added. The samples were analyzed using BD FACSVerse™ (BD Biosciences, San Jose, CA, USA). These measurements were carried out for three leaf fragments obtained from three individuals. To interpret the obtained cytological evidence, we assembled all DNA C-values available for Marattiaceae (Table 1) by extracting the information

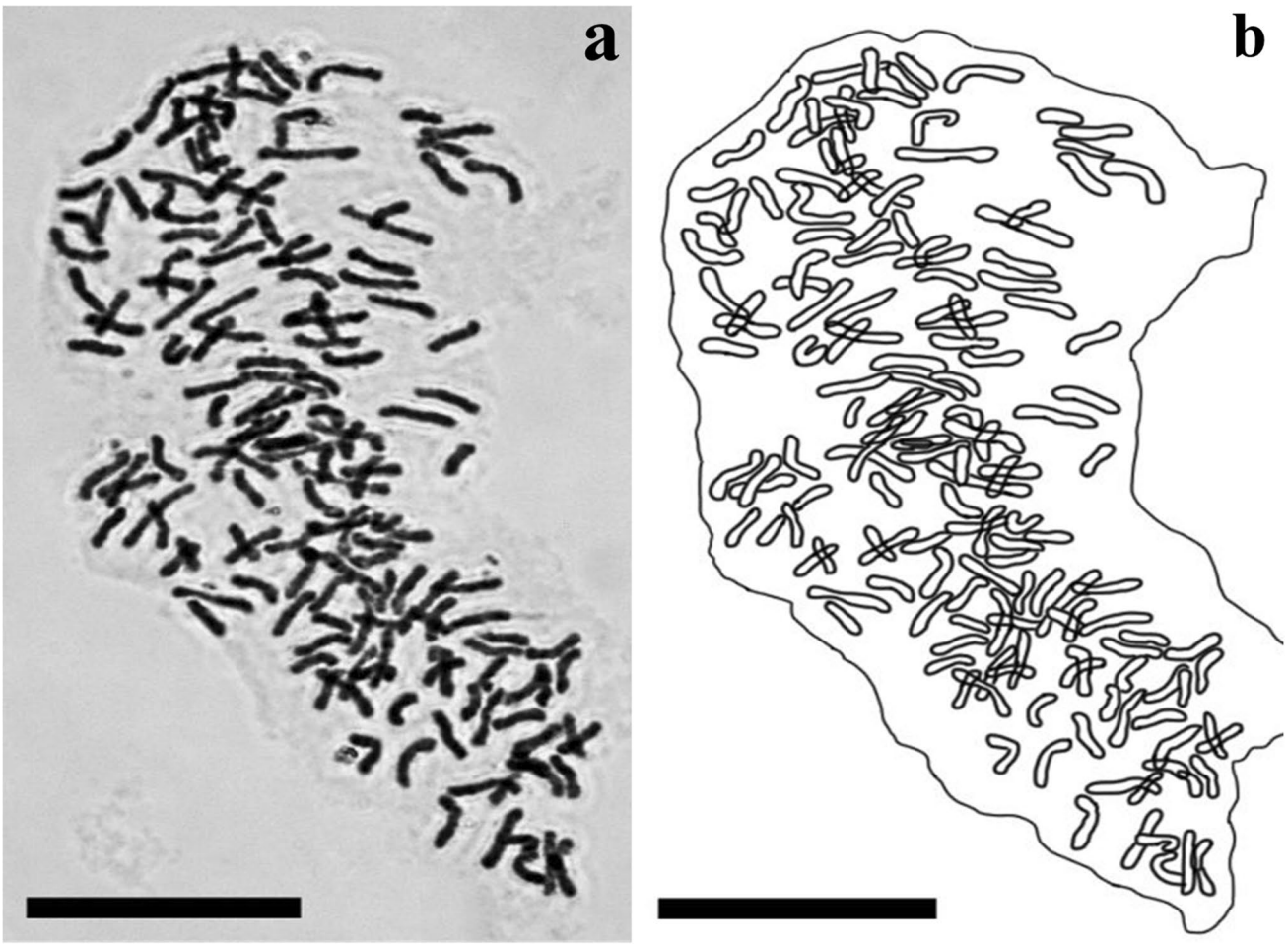


Fig. 3 Chromosome count of *Christensenia* occurrences in China based on mitotic cell division in the root tip collected from material cultivated at XTBG. Same individual as *rbcL* sequence and DNA C-value measurements. **a** Image of the mitotic chromosomes. **b** Interpretative drawing of the chromosome count. Scales bar = 20 µm

from the literature (e.g., Clark et al. 2016; Hanson and Leitch 2002; Kuo et al. 2017). Information on existing chromosome counts were obtained from the "Chromosome Count Database" (http://ccdb.tau.ac.il/browse/; Rice et al. 2014) and by checking recent publications (Hsieh et al. 2008).

Chloroplast genome sequencing, assembly, and annotation

The complete chloroplast genome was generated by extracting whole genomic DNA from silica dried leaf fragments obtained from the same individual growing at XTBG. Total genomic DNA was extracted using a modified CTAB approach (Yang et al. 2014) and used to generate a genome library employing Illumina Nextera XT DNA library preparation (Illumina, Inc. San Diego, CA, USA) based on c. 500 bp-long DNA fragments that were obtained by shearing the genomic DNA. About 200 Mb of sequences were generated by obtaining 90 bp long pair-end sequences via the Illumina HiSeq 2000 (Illumina, Inc. San Diego, CA, USA) at BGI-Shenzhen. The raw sequences were assembled into relatively long scaffolds employing the CLC Genomic workbench v10.1.1 (https://www.qiagenbioinformatics.com) involving quality control of the raw sequences with the NGS QC Tool Kit (http://nipgr.res.in/ngsqctoolkit.html) with cut-off values for read length and PHRED quality scores set as recommended in Yang et al. (2014). The assembly of whole plastid genome and annotations were made with the support of DOGMA (Wyman et al. 2004) and Geneious (Kearse et al. 2012). The two previously published plastid genomes from the same family were used to guide the sequence annotation. The newly sequenced plastid genome was submitted to the GenBank (accession MN056350) and drawn in circular shape (Fig. 4) using Organellar Genome DRAW (Lohse et al. 2013).

Table 1 Summary of genome size (DNA C-values) of Marattiaceae

Genus	Species	Authorities	2C	CV	1C	1Cx	2n	Voucher	References	Method	Buffer	Calibration standard
Angiopteris	*latipinna*	(Ching) Z.R.He, W.M.Chu & Christenh.	21.98	0.31	10.99	11	80	HM Liu s.n. SZBG (Cul.)	[a]	Cytofluometry	GPB + 3% PVP-40	*Pisum sativum* 'Citrad' [9.09 pg]
Angiopteris	*lygodiifolia*	Rosenst.	14.21	1.03	7.105	7.11	80[A]	Not stated	[b]	Feulgen	NA	*Pisum sativum* 'Citrad' [9.09 pg]
Angiopteris	*palmiformis*	(Cav.) C.Chr.	14.51	0.05	7.255	7.26	80	Not stated	[c]	Cytofluometry	Backmen buffer	*Nicotiana tabacum* 'Xanthi' [10.04 pg]
Angiopteris	*pruinosa*	Kunze	27.12	0.4	13.56	/	/	RBGK 1973-13071	[a]	Cytofluometry	GPB + 3% PVP-40	*Pisum sativum* 'Citrad' [9.09 pg]
Christensenia	*aesculifolia*	(Blume) Maxon	42.02	0.37	21.01	10.5	160[A]	XTBG, Yunnan	[d]	Cytofluometry	Ebihara	*Pisum sativum* 'Citrad' [9.09 pg]
Danaea	*antillensis*	Christenh.	24.81	0.13	12.41	/	/	Guadeloupe, M. Christenhusz 7192	[a]	Cytofluometry	Ebihara	*Allium cepa* 'Alisa Craig' [34.89 pg]
Danaea	*kalevala*	Christenh.	22.89	0.07	11.45	5.72	160	Guadeloupe, M. Christenhusz 7198	[a]	Cytofluometry	Ebihara	*Allium cepa* 'Alisa Craig' [34.89 pg]
Danaea	*mazeana*	Underw.	27.98	0.01	13.95	/	/	Guadeloupe, M. Christenhusz 7197	[a]	Cytofluometry	Ebihara	*Allium cepa* 'Alisa Craig' [34.89 pg]
Ptisana	*purpurascens*	(de Vriese) Murdock	15.3	0.49	7.65	7.65	78	RBGK 1973-13071	[a]	Cytofluometry	Citric acid	*Pisum sativum* 'Citrad' [9.09 pg]
Ptisana	*salicifolia*	(Schrad.) Senttere & Rouhan	12	0.24	6	6	78	HM Liu LHM2215	[d]	Cytofluometry	Ebihara	*Pisum sativum* 'Citrad' [9.09 pg]
Ptisana	*salicina*	(Sm.) Murdock	13.94	0.08	6.97	6.97	78	RBGK 1981-6450	[a]	Cytofluometry	GPB + 3% PVP-40	*Pisum sativum* 'Citrad' [9.09 pg]

Reported 2C, 1C, 1Cx, 2n, voucher and publication, buffers, and calibration standard. Genome size values are recoded in pg

[a]Clark et al. (2016)

[b]Hanson and Leitch (2002)

[c]Kuo et al. (2017)

[d]This study

[A]Chromosome counts and DNA C-value measure from the same specimen

/Data not available

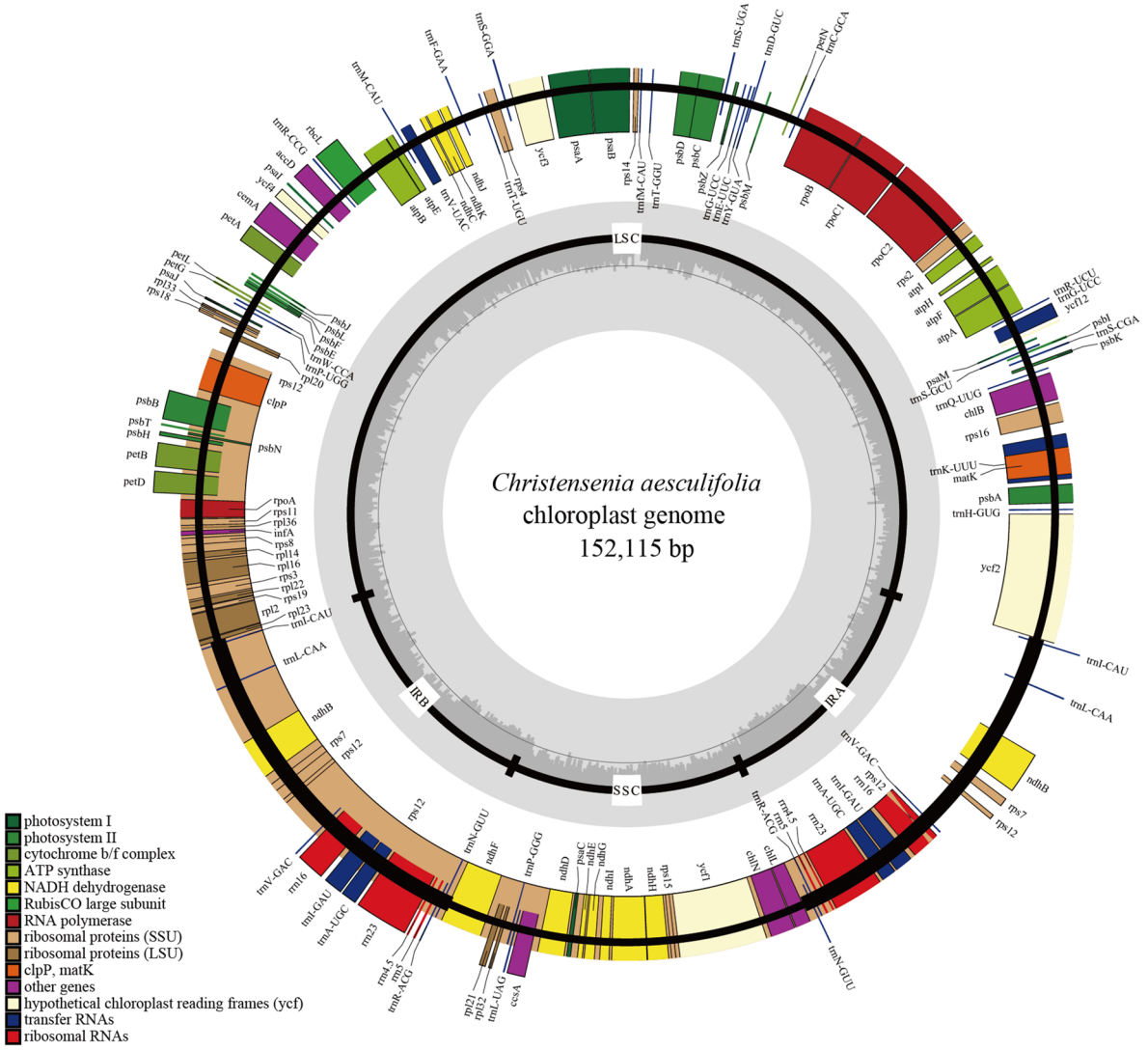


Fig. 4 Circular plot of the complete chloroplast genome of *Christensenia aesculifolia*

Phylogenetic reconstruction

To explore the phylogenetic relationship and genetic differentiation among the studied occurrences of *Christensenia*, we downloaded all *rbcL* sequences of Marattiaceae available at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) in March 2019. This region was selected because it is the most commonly used molecular marker used in ferns. The assembled data matrix contained 123 samples from 60 species representing all six genera of extant Marattiaceae. In total, the matrix included four *rbcL* sequences of *Christensenia.* The two sequences available in GenBank were likely obtained from the same accession collected at Ulu Gombak, Malay Peninsula, whereas we included two sequences obtained from individuals cultivated at XTBG. One was generated as part of the whole plastid genome sequencing whereas the other one was obtained independently from the specimen used for the cytological studies. The newly generated *rbcL* sequence was submitted to GenBank (accession number LC487993). Given the challenges observed in recent taxonomic treatments (e.g., Christenhusz 2010; He and Christenhusz 2013), the number of extant species included may be diverging from the given species diversity estimate. We aimed to update the species names using the most recent treatments (Fig. 5 for accession numbers and species names). The sequence alignment was compiled using Mesquite 3.6 (Maddison and Maddison 2019). The inclusion of the complete reading frame of *rbcL* gene extracted from three complete plastid genomes enabled us to determine the start and stop-codons as well as to inspect the amino-acid sequence changes. No stop codon was recovered besides the one at the end of the sequence. This is consistent with the proposed absence or rarity of RNA editing in the plastid genomes of Marattiaceae (Roper et al. 2007). We performed maximum likelihood analyses using PhyML (Guindon and

Gascule 2003; Guindon et al. 2010) with the mutation model GTR plus invariable sites and gamma parameter selected using jModelTest (Darriba et al. 2012). The robustness of the obtained phylogenetic hypothesis was explored by estimated non-parametric bootstrap values and SH-like test (Guindon et al. 2010). The neotropical genus *Danaea* was selected as the outgroup following the hypothesis proposed in previous phylogenetic studies (Christenhusz et al. 2008; Murdock 2008a, b). Because of the phylogenetic isolation of extant Marattiaceae, the selection of other ferns as outgroup may result in a very long branch leading to the extant crown group. Thus, recent studies have incorporated fossils as an alternative rooting of the crown group (Rothwell et al. 2018a, b), and evidently this issue requires further investigation.

Distribution range and IUCN assessment

Distribution and IUCN status were revalued using the available specimen records throughout the range of *Christensenia* considering all plants in this genus as one species (Fig. 2; Table S1). The distribution range of the genus was explored by assembling a list of vouchers extracted from recent publications (e.g., Rolleri 1993) and digitized herbarium collections using GBIF (http://www.bgif.org), JSTOR Global Plants (https://plants.jstor.org), botany collection of the Naturalis Biodiversity Center (science.naturalis.nl), the Singapore Herbarium Online (http://herbaria.plants.ox.ac.uk), The Australian Virtual Herbarium (avh.chah.org.au), the Chinese Virtual Herbarium (http://www.cvh.org.cn), and the Kingdonia (http://kun.kingdonia.org). The first two authors also checked the specimens from Herbarium of Yunnan University (PYU). The obtained list contains information about the collector, herbaria deposited, location, habitat, and related information (Table S1). The geo-referenced specimens were plotted using dot map representation and the IUCN status estimated using range wide occurrence of two-degree grid cell size and the convex hull polygon approach available in the ConR-package (Dauby et al. 2017). Duplicated occurrence data were ignored and geo-referenced data were employed as given in the digitized resource data (see above) or were newly generated based on the location information given at the voucher labels. Some older occurrences lack precise information such as those recorded in Assam. In this case, the geo-referenced points referred to the geographic center of Assam province. The same approach was used for some specimens from Java and Sumatra.

Results

Cytology of *Christensenia* in China

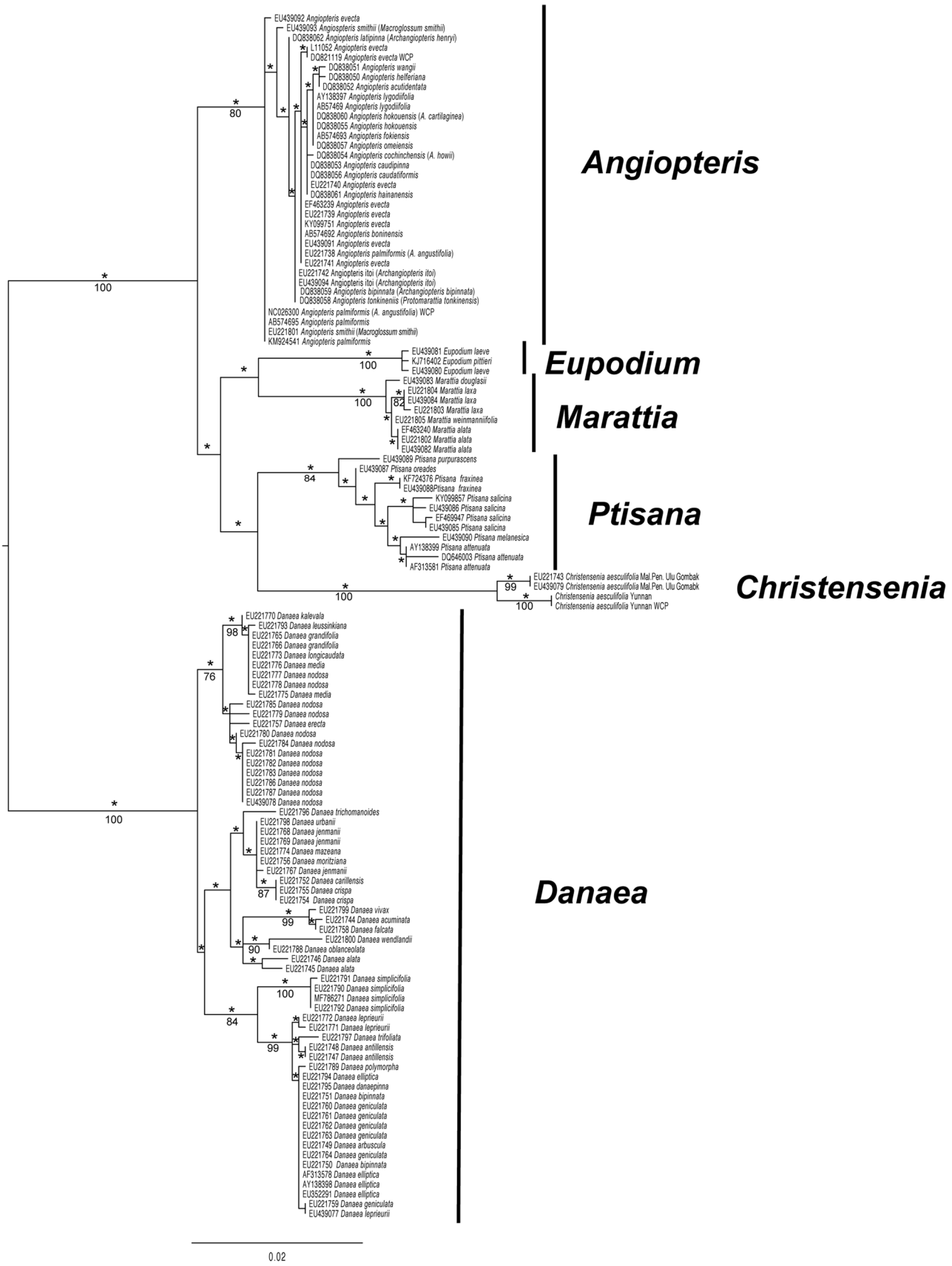
The chromosome count (Fig. 3) recovered 2n = 160, a DNA C-value of 2C = 42.02 pg and 1Cx = 10.50 pg. This was the first tetraploid record for the genus in the northern hemisphere. In turn, the Chinese occurrence of *Christensenia* had the largest holoploid genome size (2C) recorded for Marattiaceae but not the largest monoploid (1Cx) recorded (Table 1).

Characteristics of the plastid genome of *Christensenia*

The plastid genome of *Christensenia* has a length of 152,115 bp and a GC content of 34.8% (Fig. 4). Both values were similar to the two published plastomes of the closely related genus *Angiopteris* (Table 2). The whole genome displayed the typical quadripartite structure of land plants, including the large single-copy (LSC) with a length of 90,505 bp, the small single copy (SSC) with 20,603 bp, and a pair of inverted repeats (IRa and IRb) with 20,428 bp and 20,579 bp, respectively. In total, 137 genes were identified, including 90 protein-coding genes, 39 tRNA and eight rRNA loci (Table 2). No evidence for RNA editing and presence of pseudogenes was recovered. The gene content and gene order were highly similar to those of the two *Angiopteris* species (Table 2). The length of the 23 identified introns, the protein CDS, rRNAs, and tRNAs were highly similar to those found in *Angiopteris* (Tables S2–S7). Screening for SSRs recovered 3,927 sites that may be used to establish markers to identify intra-species variations. Of the SSRs, three sites had a SSR motif length of 10 bp, 10 sites with a motif length of 9 bp, nine sites with a motif length of 8 bp. The longest motif with a more than 2 repeats included one combining a motif with 9 bp and 3 repeats at the position 12, 971 bp (Table S7).

RbcL-based phylogenetic hypothesis

The two newly generated *rbcL* sequences of *Christensenia*—one was extracted from the complete plastome and one sequenced independently from the same individual cultivated at XTBG—were identical. The phylogenetic analyses supported the Chinese accessions as sister to the clade comprising two *Christensenia* accessions from Malay Peninsula (Ulu Gombak), and both lineages differ in five nucleotide sites. The *Christensenia* clade was resolved as sister to *Ptisana* (Fig. 5). The employed *rbcL* sequences failed to separate all morphologically recognized species in the genera *Angiopteris*, *Danaea*, *Eupodium*, *Marattia*, and *Ptisana*, but showed variation within some species, such as *D. nodosa* (L.) Sm., *M. alata* Sw., and *P. salicina* (Sm.) Murdock. The low level of sequence variations among the *rbcL* sequences of marattioid ferns resulted in rather low bootstrap values for the majority of clades and caused polytomies in the intra-generic topology of *Angiopteris* (Fig. 5). In turn, the observed limited sequence differentiation among


EU439092 Angiopteris evecta
EU439093 Angiospteris smithii (Macroglossum smithii)
DQ838062 Angiopteris latipinna (Archangiopteris henryi)
L11052 Angiopteris evecta
DQ821119 Angiopteris evecta WCP
DQ838051 Angiopteris wangii
DQ838050 Angiopteris helferiana
DQ838052 Angiopteris acutidentata
AY138397 Angiopteris lygodiifolia
AB574691 Angiopteris lygodiifolia
DQ838060 Angiopteris hokouensis (A. cartilaginea)
DQ838055 Angiopteris hokouensis
AB574693 Angiopteris fokiensis
DQ838057 Angiopteris omeiensis
DQ838054 Angiopteris cochinchensis (A. howii)
DQ838053 Angiopteris caudipinna
DQ838056 Angiopteris caudatiformis
EU221740 Angiopteris evecta
DQ838061 Angiopteris hainanensis
EF463239 Angiopteris evecta
EU221739 Angiopteris evecta
KY099751 Angiopteris evecta
AB574692 Angiopteris boninensis
EU439091 Angiopteris evecta
EU221738 Angiopteris palmiformis (A. angustifolia)
EU221741 Angiopteris evecta
EU221742 Angiopteris itoi (Archangiopteris itoi)
EU439094 Angiopteris itoi (Archangiopteris itoi)
DQ838059 Angiopteris bipinnata (Archangiopteris bipinnata)
DQ838058 Angiopteris tonkinensis (Protomarattia tonkinensis)
NC026300 Angiopteris palmiformis (A. angustifolia) WCP
AB574695 Angiopteris palmiformis
EU221801 Angiopteris smithii (Macroglossum smithii)
KM924541 Angiopteris palmiformis
Angiopteris
EU439081 Eupodium laeve
KJ716402 Eupodium pittieri
EU439080 Eupodium laeve
Eupodium
EU439083 Marattia douglasii
EU221804 Marattia laxa
EU439084 Marattia laxa
EU221803 Marattia laxa
EU221805 Marattia weinmanniifolia
EF463240 Marattia alata
EU221802 Marattia alata
EU439082 Marattia alata
Marattia
EU439089 Ptisana purpurascens
EU439087 Ptisana oreades
KF724376 Ptisana fraxinea
EU439088Ptisana fraxinea
KY099857 Ptisana salicina
EU439086 Ptisana salicina
EF469947 Ptisana salicina
EU439085 Ptisana salicina
EU439090 Ptisana melanesica
AY138399 Ptisana attenuata
DQ646003 Ptisana attenuata
AF313581 Ptisana attenuata
Ptisana
EU221743 Christensenia aesculifolia Mal.Pen. Ulu Gombak
EU439079 Christensenia aesculifolia Mal.Pen. Ulu Gombak
Christensenia aesculifolia Yunnan
Christensenia aesculifolia Yunnan WCP
Christensenia
EU221770 Danaea kalevala
EU221793 Danaea leussinkiana
EU221765 Danaea grandifolia
EU221766 Danaea grandifolia
EU221773 Danaea longicaudata
EU221776 Danaea media
EU221777 Danaea nodosa
EU221778 Danaea nodosa
EU221775 Danaea media
EU221785 Danaea nodosa
EU221779 Danaea nodosa
EU221757 Danaea erecta
EU221780 Danaea nodosa
EU221784 Danaea nodosa
EU221781 Danaea nodosa
EU221782 Danaea nodosa
EU221783 Danaea nodosa
EU221786 Danaea nodosa
EU221787 Danaea nodosa
EU439078 Danaea nodosa
EU221796 Danaea trichomanoides
EU221798 Danaea urbanii
EU221768 Danaea jenmanii
EU221769 Danaea jenmanii
EU221774 Danaea mazeana
EU221756 Danaea moritziana
EU221767 Danaea jenmanii
EU221752 Danaea carillensis
EU221755 Danaea crispa
EU221754 Danaea crispa
EU221799 Danaea vivax
EU221744 Danaea acuminata
EU221758 Danaea falcata
EU221800 Danaea wendlandii
EU221788 Danaea oblanceolata
EU221746 Danaea alata
EU221745 Danaea alata
EU221791 Danaea simplicifolia
EU221790 Danaea simplicifolia
MF786271 Danaea simplicifolia
EU221792 Danaea simplicifolia
EU221772 Danaea leprieurii
EU221771 Danaea leprieurii
EU221797 Danaea trifoliata
EU221748 Danaea antillensis
EU221747 Danaea antillensis
EU221789 Danaea polymorpha
EU221794 Danaea elliptica
EU221795 Danaea danaepinna
EU221751 Danaea bipinnata
EU221760 Danaea geniculata
EU221761 Danaea geniculata
EU221762 Danaea geniculata
EU221763 Danaea geniculata
EU221749 Danaea arbuscula
EU221764 Danaea geniculata
EU221750 Danaea bipinnata
AF313578 Danaea elliptica
AY138398 Danaea elliptica
EU352291 Danaea elliptica
EU221759 Danaea geniculata
EU439077 Danaea leprieurii
Danaea
0.02

◂**Fig. 5** Phylogram of the reconstructed phylogenetic relationships based on *rbcL* data of 123 accessions including all genera of marattioid ferns and rooted with the neotropical genus *Danaea.* The maximum likelihood hypothesis had a log-likelihood of −4213.199 with a gamma shape parameter of 0.468 and nucleotide frequencies f(A) = 0.28128, f(C) = 0.18360, F(G) = 0.23417, and f(T) = 0.300095. Bootstrap values > 75% are shown and stars mark clades with a p = 1.00 in a SH-like test

closely related species in the species-rich genera such as *Angiopteris* and *Danaea*, supported the hypothesis that the observed sequence differentiation between the *Christensenia* accessions from China and Malay Peninsula likely reflected two distinct species of *Christensenia*.

Discussion

We reported critical data on the cytology and plastome from the highly threatened *Christensenia* occurrences in China for the first time. We also reconstructed the phylogenetic relationships of the Chinese occurrences using *rbcL* sequences and reevaluated its distribution range. The obtained results provided new insights in the taxonomy and evolution of this Mesozoic relict genus.

Taxonomic status of the Chinese occurrences of *Christensenia*

According to the Flora of China (He and Christenhusz 2013), the Chinese occurrences belong to *Christensenia aesculifolia* (Blume) Maxon, which is a widespread species with an occurrence range including the Solomon Islands in the south and Assam in the north (Fig. 2a; Table S1). Based on morphological evidence, Rolleri (1993) recognized two subspecies of *C. aesculifolia*, of which *C. aesculifolia* subsp. *korthalsii* (de Vriese) Rolleri was recorded in Assam, Borneo, Malay Peninsula, Philippines, and Sumatra, and the other *C. aesculifolia* subsp. *aesculifolia* was recorded in Java, Bismarck Archipelago, and Solomon Islands (Rolleri 1993). The second species, *C. lobbiana* (de Vriese) Rolleri was recorded in Malay Peninsula, Myanmar, Philippines, and Sumatra. However, the morphological differentiation used by Rolleri (1993) was questioned by Murdock (2008a) who proposed that the differences might be due to phenotypic plasticity.

The two *rbcL* sequences obtained from an accession collected in Ulu Gombak, Malay Peninsula (Christenhusz et al. 2008; Murdock 2008b) differed in the sequence variation from the samples in China. These two sequences from GenBank were generated independently but arguably from the same silica sample (confirmed by Murdock Andrew G, pers. comm.). Given the general extremely slow molecular evolution in the marattioid ferns (Soltis et al. 2002), the two occurrences (China versus Malay Peninsula) seemly diverged from each other for quite some time considering the sequence variations of the *rbcL* gene. In turn, the observed sequence divergence supports the hypothesis that there are at least two independent species of *Christensenia* (Fig. 5). The two published chromosome number reports referring to two geographic occurrences of *C. aesculifolia* are also different from each other. The chromosome counts from Solomon Islands (Braithwaite 1977) and China (see above) are tetraploid, whereas the Borneo counts are diploid (Walker 1979). Together, cytological and chloroplast sequence data suggest the existence of more than one distinct species of *Christensenia*. However, the type was collected in Java and we currently lack DNA sequence data obtained from occurrences in or close to the type location. At the current state, the concepts of one and/or two species have to be taken into consideration pending for further evidence from both molecular and physical/chemical data. Morphological comparison of the Chinese specimens with descriptions provided by Rolleri (1993) was inconclusive.

Cytology and nuclear genome of *Christensenia*

Few efforts have so far been made to analyze the nuclear genomes of Marattiaceae taxa, despite some transcriptomic data have been made available for a few species including a *Christensenia* accession (Qi et al. 2018). Apart from above, the DNA C-values are known only for 10 species covering four out of the six genera of Marattiaceae (about 9% of species diversity). In present study, we found the highest holoploid genome size (2C) for *Christensenia* among all marattioid ferns (see Clark et al. 2016). However, as for the monoploid genome size (C*x*), *Christensenia* is somewhat similar to *Angiopteris latipinna* (Ching) Z.R.He, W.M.Chu & Christenh., although the chromosome number of the latter specimen needs to be confirmed.

Our data suggests a 1C-value range of 2.95 x-fault in the marattioid ferns instead the 2.03 x-fault reported previously (Clark et al. 2016). This increase is the consequence of including the tetraploid *Christensenia* accession described here, which suggests a potential further increase by expanding the exploration of the genome sizes of marattioid fern genomes. Considering the much better explored genome size variation of osmundaceous ferns (Schneider et al. 2015), ancient lineages of ferns may be characterized by limited genome size variation (Clark et al. 2016). The marattioid ferns show relative higher frequency of polyploidy than osmundaceous lineage, which evidently deserves further investigation considering both lineages not only represent the Mesozoic relict clades, but also share low mutation rates (Schneider et al. 2015; Soltis et al. 2002).

Our results confirmed tetraploid counts of *Christensenia* but did not add to the number of species in the Marattiaceae

Table 2 Comparative statistics of the chloroplast genome of *Christensenia* compared to the two available *Angiopteris* plastid genomes

Species	Accession no.	Genome size (bp)	GC (%)	LSC (bp)	SSC (bp)	IR (F)	IR (R)	Genes no.	CDs no.	Pseudogene no.	tRNA no.	rRNA no.	Introns no.	RNA editing
Angiopteris angustifolia	NC026300	153,596	35.5	89,713	20,537	21,673	21,673	135	89	0	39	8	23	Undetectable
Angiopteris evecta	NC008829	153,901	35.5	89,717	22,088	21,050	21,046	141	88	ycf1	45	8	23	Undetectable
Christensenia aesculifolia	MN056350	152,115	34.8	90,505	20,603	20,428	20,579	137	89	0	39	8	23	Undetectable

with chromosome counts of 26, representing five out of the six genera in this family. With coverage of about 23.4% of the species diversity, the Marattiaceae are among the most poorly covered fern families in respect of species with known chromosome number (Schneider Harald, Liu Hongmei unpublished). Among these 26 species, two have both diploid and tetraploid records, namely the widespread *Angiopteris evecta* and *C. aesculifolia*. The first case may result from taxonomic confusion between *A. evecta* and its close relatives such as *A. palmiformis* (Cav.) C.Chr.—a known diploid species. The occurrence of both diploid and tetraploid cytotypes of *Christensenia* has to be considered in the context of the relatively low frequency of polyploidy in Marattiaceae. About 71.4% of the taxa with chromosome counts are diploids, whereas 21.4% are tetraploids and only 7.1% are triploid. In Asia, tetraploid marattioids include beside the two tetraploid occurrences of *Christensenia*, *A. somae* (Hayata) Makino & Nemoto—endemic to Taiwan—and the widespread *A. evecta*. The interpretation of the ploidy level of the latter species is challenged by taxonomic uncertainty of this species (see above). The only known triploid, *A. itoi* (W.C.Shieh) J.M.Camus, is endemic to Taiwan (Hsieh et al. 2008). In the Neotropics, tetraploids have been recorded in the genera *Danaea* and *Marattia*. The chromosome counts of two *Marattia* species were reported with one diploid (*M. alata*.) and one tetraploid (*M. weinmanniifolia* Liebm.), whereas the four *Danaea* species with counts included one diploid, one triploid, and two tetraploids. In summary, 70.3% of extant Marattiaceae species are reported as diploids, a number exceeding the average proportion of diploid species among all extant ferns that is estimated as 55.5% (Schneider et al. unpublished). Previous analyses have revealed the frequency of polyploidy-based speciation of 34.0% and infra-generic polyploid incident of 32.9% in leptosporangiate ferns as well as 19.4% and 24.0% in eusporangiate ferns, respectively (Wood et al. 2008). At the family level, the frequency of polyploidy incidence is with 28.6% slightly higher than the average rate estimated for all non-leptosporangiate ferns (Wood et al. 2008). The genera *Christensenia* and *Danaea* have even polyploidy incidence frequency of 50% and 75%, respectively. In opposite to these genera, available data show with 17.6% a lower proportion of polyploidy enforced speciation in *Angiopteris* although this genus is with ca. 30 extant species the second most species rich genus of marattioid ferns (PPG1 2016). However, the estimated number of species has to be treated as ambiguous at least for the genus *Angiopteris* because more than 200 species names have been introduced in the past. These results suggest that polyploidy may play an important role in the diversification of these ferns but also distinct contributions among the extant genera of marattioid ferns.

The origin of the tetraploid *Christensenia* cytotype is currently unknown. Both tetraploid counts were recovered

from populations at the northern (China) and southern (Solomon Islands) border of the distribution range, respectively, whereas the diploid account originated close to the center (Sarawak, Borneo) of the distribution range (Fig. 2). The two tetraploids may have originated independently but this hypothesis needs further investigation using a sufficient geographic sampling across the whole region. The alternative hypothesis predicts the tetraploid cytotype displaying higher potential to establish new populations via long distance dispersal.

The chloroplast genome of *Christensenia*

The newly obtained complete chloroplast genome of *Christensenia* was compared with the two previously published plastid genomes of *Angiopteris* (Roper et al. 2007; Zhu et al. 2016). These genomes show great similarity in the gene content and organization (Table 2; Tables S2–S7), supporting the hypothesis of structural conservatism of the plastid genomes in the order Marattiales (Roper et al. 2007). The phylogenetic conservatism of the plastid genome structure together with low substitution rate supports these ferns as "molecular living fossils" (Soltis et al. 2002). This conclusion has to be further tested by obtaining whole plastid genomes of the other genera *Danaea*, *Eupodium*, *Marattia*, and *Ptisana* beside studies focusing on nuclear and mitochondrial genomes. However, no evidence has been reported for decoupling of mutation rates among the three genomes in ferns. The observed length variations and frequency of SSRs in studied ferns enable to develop markers with great potential to distinguish related species or study the genetic variations of geographic-isolated populations.

From local to global threats to *Christensenia*

Our understanding of the current threats to *Christensenia* has been challenged by insufficient records of occurrences and the number of individuals per population in a species that has a wide but highly fragmented distributed range (Fig. 2). Since many collections, especially those from Java and Sumatra, were obtained and deposited in the first half of the twentieth century, the survival of these occurrences requires to be confirmed. More recent collections provide evidence for continuous occurrences (Mitsuta 1984) but further fieldwork is required. Given the rapid deforestation in these areas, the recorded distribution using historical collections may exhibit a misleading positive scenario because some of these populations may have been destroyed as a consequence of habitat loss during the progress of the Anthropocene. In turn, several occurrences may still wait to be discovered especially in areas that have not been densely explored such as parts of Seram, New Guinea, Borneo, Laos, and Myanmar (e.g., Iwatsuki and Kato 1980; Kato 1989; Takeuchi 2013) but also in assumed better collected areas such as Java (Praptosuwiryo 2013). In the context of the occurrences in China, it is important to note that the two recorded occurrences are located close to the borders to Vietnam (Fig. 2) where the occurrences need further investigation. The record of *Christensenia* in both Hekou County in China and Lao Cai Province in Vietnam suggests a rather continuous distribution range along the China/Vietnam border from Hekou westwards to Jinping and perhaps even further westwards to the northern border between Laos and Myanmar. In this context it is interesting to note that the single specimen obtained from Myanmar is somewhat located among the known occurrences in Assam (India) and the occurrences in southwestern China as well as northern Vietnam.

Despite the possibility that these collections reflect correctly a highly disjunct distribution along the northern border of the taxon range (Fig. 2a), we could not reject the alternative that many of the occurrences in this region have not been recorded until now. In this context, it is worth realizing that the only known occurrence in Thailand was obtained from Phangnga, Nakhon Si Thammarat in southern Thailand (https://rbg-web2.rbge.org.uk/thaiferns/factsheets/index.php?q=Christensenia_aesculifolia.xml). This location is closer to recorded occurrences in the Malay Peninsula but far from the occurrences in China and Myanmar (Fig. 2). To establish effective conservation programs, more information has to be assembled to confirm that the observed geographic disjunction has been caused by historical disruption events in the geological past or it is the consequence of a more recent long-distance dispersal event. Addressing these questions will not only improve the assessment of the protection needs in China but also to establish effective ex situ and in situ conservation plans across the belt-and-road region of SE Asia.

A further aspect is the lack of information of the local population sizes for the majority of occurrences. Estimation of the threat to species may be further improved by not only taking the number of occurrences but also the number of individuals per site into account. Unfortunately, very little information is available at moment. Some floristic treatments (Holttum 1954) reported this fern as locally abundant in the past. This information is crucial to assess the dynamic of local population size changes under the current transformation of habitats. The Chinese occurrence of *Christensenia* has been recognized as a plant species with extremely small populations (PSESP) that requires special and urgent protection (Cai et al. 2018; Wade et al. 2016). The concept of a species with locally small population size may also apply to other newly discovered occurrences such as the one at Gunung Slamat of central Java (Praptosuwiryo 2013). The proposal of *Christensenia* as PSESP is of theoretical interest because ferns are known for their high dispersal capacity.

Until now, only three fern species out of 62 high plant species in China has been proposed as PSESP. In contrast, this fern was recorded to be locally abundant in some locations at the Malay Peninsula such as Kinta and Batang Padang (Holttum 1954).

The assessment of the IUCN Red List status of *Christensenia* is challenged by the ambiguous species delimitation and the widespread but highly disjunct distribution range (Fig. 2). The threat status has been assessed as least concern (LC) or nearly threatened (NT) under the assumption that all known occurrences belong to a single species and continuous occurrences of the populations since their first documentation. This assessment is consistent with previous propositions (Lindsay and Middleton, https://rbg-web2.rbge.org.uk/thaiferns/factsheets/index.php?q=Christensenia_aesculifolia.xml). However, this assessment has to be considered as too positive by considering several issues. Firstly, *Christensenia* seemingly includes more than one species considering the different ploidy levels and genetic variation, and each one requires independent assessment. Secondly, the survival of several occurrences recorded more than 50 years ago needs to be confirmed. This applies especially to occurrence records in Sumatra. Thirdly, the current distribution shows highly disjunct ranges. The known occurrences in New Guinea suggest a continuous range in Western and Central Malesian (Fig. 2). Currently, the single Chinese population has to be considered as highly vulnerable because the other recorded location was not rediscovered in the extensive field survey carried out by Cai et al. (2018) and our own team. Nevertheless, we also know very little about the occurrences of this genus in Vietnam. Currently, we lack information about the status of the populations found in Lao Cai (North Vietnam) by Chinese researchers in 2002 (Table S1). Thus, cross-border assessments are important to confirm the distribution and status of the populations in SW China and N Vietnam. Similarly, the status of *Christensenia* in Myanmar is ambiguous with the only known record was collected at the border region between Kachin State and Sagaing Region of northern Myanmar. In summary, the reassessed distribution range supports the consideration of conservation needs not only globally but also regionally and locally. For example, occurrences along the northern range of *Christensenia* are geographically disjunct from the distribution range in Malesia. National assessments such as China are crucial but require the incorporation of cross-border assessments.

Conclusions

Our study provided new evidence in cytology, plastid genome, and geographical distribution of the relict fern genus *Christensenia*. These data are crucial to elucidate the species delimitation and develop protection protocols for sustainable preservation the occurrences in China and adjacent regions by either in situ or ex situ procedures. The detected sequence variation in the non-coding plastid DNA regions will enable to design markers to record the variation at the population level. In summary, the current study integrated cytological and genomic evidence besides revaluation of the distribution and IUCN status, it is the first integrative study concerning the taxonomic status and diversity of this threatened Mesozoic relict and molecular living fossil. Further studies, precursor studies are already in progress will focus on nuclear genomic regions to enable the tracking of both generations, namely the maternal line via the plastid genome and the parental line via the nuclear genome. Similar studies are arguably required to improve our understanding of the protection needs of ancient fern lineages occurring in SE Asia.

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Author contributions HM designed the study, HM and HS wrote the manuscript. TF performed the cytological experiments, HS, YY and PK analyzed the data, and all authors approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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