Characterization, cultivation, and chloroplast genome analysis of *Betula fujianensis*

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Abstract

Background: *Betula fujianensis* (*B. fujianensis*), a new *Betulaceae* species reported in 2008, is endemic and endangered in the Fujian province of China. However, little is known about it, and no special protection measures have been taken. So it is urgent to take some activities for its well conservation.

Results: In this study, we systematically described its morphological characteristics, developed a cultivation method, and were the first to report its whole chloroplast genome sequence. We conducted a comparative analysis using its synonymous species (*B. luminifera)* and identified its distinct characteristic, especially in bark and leaves. It has serrated leaves covered with glandular punctation and short and rare pubescences, and its bark gradually changes with age from smooth to exfoliate. We also observed that its chlorophyll content substantially changes with age. Furthermore, we also sequenced *B. luminifera*'s chloroplast genome sequence, compared it with that of *B. fujianensis,* and found that *gene rpl16* is absent in *B. fujianensis*. Phylogenetic analysis also showed that *B. fujianensis* is relatively close to *B. luminifera*.

Conclusions: These observations and chloroplast genome sequence results provide more information about *B. fujianensis*. The comparative analysis with *B. luminifera* will provide some clues to different environmental adaptation. The cultivation method make further scientific researches possible, contribute to its good conservation and promote its utilization in afforestation and industrial application in the future.

Keywords: Chloroplast genome, *Betula*, Cultivation, *Betula fujianensis.*

Background

Betulaceae, a family with approximately 130 species in six genera, is one of the core components of Hamamelidae, and is mainly found in the temperate regions of the Northern Hemisphere (Chen et al. 1999). Most researchers have divided the family into two subfamilies: Betulaceae (*Alnus* and *Betula*) and Corylaceae (*Corylus*, *Ostryopsis*, *Carpinus*, and *Ostrya*). While extensive research has been conducted, the phylogenetic relationship of Betulaceae genera is unknown and should be further studied(Chen et al. 1999). The birch (*Betula*) is an important ecological and economic genus in the Betulaceae family, consisting of a few dozen species (Wang et al. 2016).

Birch is a strong pioneer species that produces abundant seeds dispersed by wind; hence it has high genetic variability and tolerance to environmental conditions(Koski and Rousi 2005; Perala and Alm 1990), which enable it to adapt to a wide range of sites, even including the contaminated site (Ulbrichová et al. 2005). Birch is highly resistant to chemical pollutants and has great phytoremediation potential (Jonczak et al. 2020; Renou-Wilson et al. 2010). Besides, birch shows rapid growth in early stages, and can quickly dominate a new area, thus improving the soil functioning to create favorable conditions for other tree species (Dubois et al. 2020). In recent decades, there has been a growing interest in afforestation using birch (Dubois et al. 2020).

Betula fujianensis (B. fujianensis), is a new birch species reported in Luoboyan Nature Reserve in Shaxian County, Sanming City, Fujian Province (117°34′15″E~117°36′00″E, 26°25′45″N~26°27′30″N)(Zeng et al. 2008b) in 2008. It is endemic to Fujian Province and is an endangered species in the IUCN Red List of Threatened Species because only 500 are found in Luoboyan Nature Reserve (Ashburner et al. 2013; Shaw et al. 2014). No special protection measures have been

taken. The existing ones are mainly large-diameter old trees that cannot regenerate naturally, and there is little information about its seed germination and cultivation. Current research only focused on the plant taxonomy (Zeng et al. 2008a; Zeng et al. 2014). It has been found that the *B. fujianensis* community has several other plants, and the strong interspecific competition and poor regeneration puts this species at greater extinction risk. Moreover, there is no information about its cultivation and molecular data. It is important to systematically observe its phenology and develop a seedling cultivation method to conserve it. In this study, we observed its annual life and morphological characteristics. We comparatively analyzed it with its close relatives, such as *B. luminifera*, to identify its detailed characteristic, especially the bark and leaves. A cultivation method was also developed to ensure it is not exploited to extinction. We successfully cultivated its seedlings for afforestation and scientific research. This study reports the whole chloroplast genome sequences of *B. fujianensis* and *B. luminifera*, conducts a comparative analysis, and constructs a phylogenetic relationship using 27 other tree species. This information could facilitate its well-controlled exploitation, and also enrich the molecular data of forest trees.

Methods

Plant materials

We cultivated the seedlings in a nursery in May 2018 using the above-described methods and transplanted about 6000 seedlings to 2 hm^2 forest in March 2019. The samples were obtained from Luoboyan Nature Reserve in Shaxian Country, Sanming city, Fujian province (117°34′15″E~117°36′00″E, 26°25′45″N~26°27′30″N) in November 2019. We used the 1.5-year-old (1.5 y) trees planted in nursery and forest to calculate the net photosynthetic rate to exclude the habitat factor effect.

SPAD reading and light response curves of photosynthesis were collected from the same three nursery trees and four forest trees of each species for accuracy and reliability. At least 15 leaves from each tree was measured successively and the nursery tree measurements repeated once in the afternoon.

Tree-ring data and tree age determination

We obtained increment cores from 21 healthy and representative *B. fujianensis* trees using an increment borer (5.15 mm Core 3-Thread Increment Borer, 8″, Hagölf,

Sweden) at breast height (1.3 m above ground level) in October 2018. The DBH of the selected trees ranged from 9.5 cm to 71cm. All the trees used were from the Luoboyan Nature Reserve in Shaxian County, Sanming City, Fujian Province. We naturally dried the tree in a wooden trough and sanded them using 120 mesh and 240 mesh sandpaper until the ring boundaries were clear enough to determine tree ages. The tree age was determined by the equation $y = -0.0055x^2 + 2.0592x - 2.1202$ using coefficient determination (R^2) =0.803, where x is DBH. The tree ages of the *B. fujianensis* with DBH of 18, 29, 44, 53, 23, and 43 cm were estimated to be 33, 53, 78, and 92 y, respectively, using the equation above.

We obtained tree-ring data of *B. luminifera* from a previous publication about natural trees in Qingyuan Forest Farm, Zhejiang province, about 240 km from Luoboyan Nature Reserve (Chen Yi-liang et al. 2009). The fitted equation for *B. luminifera* was: $y = 1.4251x^{1.0979}$ with coefficient determination (R^2) =0.98199. *B. luminifera* with DBH of 23 and 43 cm were approximated to be 45 and 89 y, respectively.

Chlorophyll content determination

We used SPAD-502 Chlorophyll Meter (Minolta Camera Corporation, Ltd., Osaka, Japan) with transmittance measurements at red (650 nm) and infrared (940 nm) wavelengths to measure SPAD reading. Transformed CCI of *Betula papyrifera* leaves from SPAD values of *Betula papyrifera* leaves was derived using the equation y= 5.52E-04+4.04E-04x+1.25E-05x^2 with coefficient determination (R^2)=0.96 (Richardson et al. 2002).

We chose five 1.5 y nursery trees from both *B. fujianensis* and *B. luminifera* to determine leaf chlorophyll content using modifying methods described by Zhao (Jie et al. 2013). Six healthy and intact leaves of each tree were randomly sampled from top to bottom, and then cut into pieces and mixed. About 0.2 g fresh leaves were ground in a mixture of quartz sand, calcium carbonate powder and 10 ml 95% ethanol, until the samples turned white. The extracts were filtered into a brown volumetric flask, the mortar and the draff were washed repeatedly, and 95% ethanol was added to a final volume of 25 ml. UV-vis spectrophotometer (LAMBDA 265, Perkin Elmer, Korea) was used with 95% ethanol as the blank control to determine absorbance values of samples at 664 nm (A665) and 649 nm (A649) The (mg/L) of chlorophyll a (C_a) and

chlorophyll b (C_b) concentrations were calculated using equations: C_a=13.95×A_{665}-6.88×A_{649}; C_b=24.96*A_{649}-7.32A_{665}. The total chlorophyll content (mg/g)=(C_a+ C_b) × Volume / Weight.

Photosynthesis light response curves

We collected all data on sunny days between Nov. 9 to Nov. 10, 2019, using the LI-6400 Portable Photosynthesis System (LI-COR Corporate, USA). We used red and blue LEDs Light Source (6400-02B) to produce gradient light intensity of 0, 15, 30, 60, 120, 250, 500, 1000, 1500 and 2000 μmol (photon) m^{-2} s^{-1}. All the tested leaves were kept under natural conditions and induced using a light intensity of 1000 μmol photon m^{-2} s^{-1} and the period was set to 2-3 min and a match was made after every count.

We simulated the light response curve of photosynthesis using the non-rectangular hyperbola model:

$$P_n = \frac{\alpha I + P_{max} - \sqrt{(\alpha I + P_{max})^2 - 4\theta\alpha I P_{max}}}{2\theta} - R_d$$

Where P_n is net photosynthetic rates [μmol (CO_2) m^{-2} s^{-1}], I is photosynthetic photon flux density [μmol (photon) m^{-2} s^{-1}], α is intrinsic quantum yield, P_{max} is maximum

net photosynthetic rate [μmol (CO_2) m^{-2} s^{-1}], θ is convexity, R_d is the rate of dark respiration [μmol (CO_2) m^{-2} s^{-1}].

The initial values were: θ=0.5, P_{max}=8 μmol (CO_2) m^{-2} s^{-1}, α=0.05, R_d=2 μmol (CO_2) m^{-2} s^{-1}. The limination values were: $\theta \leq 1$, $P_{max} \leq 50$ μmol (CO_2) m^{-2} s^{-1}, $\alpha \leq 0.125$, and R_d had no limination. We used the linear portion of the light response curve of photosynthesis (I<250 μmol (photon) m^{-2} s^{-1}) to calculate the light compensation point (LCP) and light saturation point (LSP) where:

$$P_n = \alpha \cdot I - R_d$$

Where I is LCP when Pn equals 0, and I is LSP when Pn equals Pmax.

Microscopic observations

Scanning electron microscope (Model TM3030Plus Tabletop microscope, Hitachi, Japan) was used to visualize the leaf surfaces of the 1.5 y forest trees, corresponding to samples in light response photosynthesis curve. Free-hand transverse sections from random branches and were stained with phloroglucinol-HCl (1% (weight/volume) phloroglucinol in 6 mol/l HCl) for 5 min for lignin visualization (Ye et al. 2019; Zhang et al. 2018) using stereomicroscope (Leica M205FA, Germany).

Sampling and DNA extraction

We obtained fresh and healthy *B. fujianensis* and *B. luminifera* leaves from Luoboyan Nature Reserve in Sha Country, Sanming city, Fujian province (117°34′15″E~117°36′00″E, 26°25′45″N~26°27′30″N) and immediately soaked the samples in liquid nitrogen and stored at -80℃. We used a Plant Genomic DNA kit, DP305 (Tiangen, Beijing, China) for DNA extraction. Pair-end DNA libraries were construct using the standard Illumina protocol and conducted sequencing using Illumina HiSeq^{TM} 2500 platform (Illumina, San Diego, CA, USA).

Chloroplast genome assembly and annotation

We used HiSeq2500 to obtain about 24 million reads for each sample, and mapped clean data to the chloroplast genome database with bowtie2 (v2.2.4) using "very-sensitive-local"(Langmead and Salzberg 2012). The mappable reads were then used to assemble the chloroplast genome with SPAdes (v3.10.1) (kmer settings 55, 87, 121)(Bankevich et al. 2012) and scaffolds were assembled using SSPACE (v2.0)(Boetzer et al. 2011) while Gapfiller (v2.1.1) was used to fill the gap(Boetzer

and Pirovano 2012). We remapped using clean reads and compared with the reference sequence (NCBI accession No. MG386368.1) to reconfirm the assembling.

We annotated the final assembly using blast (v2.6) with coding sequences of cp genomes in NCBI using “Evalue=1e-10”(Boratyn et al. 2013); rRNA with hmmer (v3.1b2) using “Evalue=1e-10”(Mistry et al. 2013); and tRNA using Aragorn (v1.2.38) with “-gcbact -i -t -c”(Laslett and Canback 2004). We finally used Ogdraw (v1.1.1) to draw the cp genome maps(Lohse et al. 2007).

Misa (v1.0) identification tool (unit_size,min_repeats:1-8 2-5 3-3 4-3 5-3 6-3)(Beier et al. 2017) was used to identify simple sequence repeats. REPuter vmatch (v2.3.0, (hamming distance)=3) was used to identify four repeat sequences (forward, reverse, complement, palindromic) with length above 30 bp (Kurtz et al. 2001).

We used the mauve program (snapshot 2015-02-13)(Darling et al. 2004) to conduct cp genome alignments of *B. luminifera* and *B. fujianensis* with nine other Betula species (*Betula pendula* (GenBank accession NO.:MG966529.1), *Betula platyphylla* (GenBank accession NO.:MH205735.1)(Wang et al. 2018a), *Betula nana* (NCBI accession NO.:NC_033978.1), *Betula cordifolia* (NCBI accession NO.:NC_037473.1),

Betula lenta (NCBI accession NO.:NC_039992.1), *Betula occidentalis*(NCBI accession NO.:NC_039993.1), *Betula platyphylla* (NCBI accession NO.: NC_039994.1), *Betula populifolia* (NCBI accession NO.:NC_039995.1), *Betula pubescens* (NCBI accession NO.:NC_039996.1). IRscope (Amiryousefi et al. 2018) was used to compare the border positions

Phylogenetic analysis and RNA editing sites predictions in the chloroplast genome

We downloaded 27 whole cp genomes of tree species, including nine *Betula*, three *Corylus*, two *Ostryopsis*, nine *Carpinus*, three *Alnus*, *and one Fagus*, from the public database and used them for phylogenetic analysis. Multiple alignments with MAFFT (v7.427)(Rozewicki et al. 2019), and phylogenetic tree was build using RAxML (v8.2.10,bootstrap=1000) with GTRGAMMA model(Miller et al. 2015; Stamatakis et al. 2008).

The online tool PREP(http://prep.unl.edu)(Mower 2009), was used according to the manual to predict RNA editing sites, and only genes with known edit sites were predicted.

Resules

Phenological observations and morphological characteristics of *B. fujianensis*

B. fujianensis had a height of 36 m and diameter at breast height (DBH) up to 60 cm. We discovered it at Luoboyan Nature Reserve in Shaxian County, Sanming City, Fujian Province. Luoboyan Nature Reserve lies 500 m above the sea level, and has a mild and humid climate, with a mean annual temperature of about 12℃ and annual precipitation of about 2000 mm. There are only about 500 trees at Luoboyan Nature Reserve presently. A general introduction of *B. fujianensis* was reported in 2008 (Zeng et al. 2008b). We observed trees in the forest for three years and found that *B. fujianensis* leaves first unfolded in March and fell in September (Fig. 1). *B. fujianensis* leaf blades are ovate with regular small tooth-like indentations, densely glandular punctate, and have short and rare pubescences. Staminate inflorescences axes are produced in June and wither in March, with 2-3 arranged on top of branches. Pistillate inflorescences are racemes, which were produced and pollinated in March. Nutlets were produced and ripened in late April or early May. The ovate nutlet is

221 densely pubescent at the apex and has membranous wings that enable it to travel
222 hundreds of meters using wind.
223 *B. fujianensis* phenology is synonymous with that of *Betula luminifera* (Zeng et al.
224 2008b). *B. fujianensis* can reach a height of 36 m, and *B. luminifera* only 20 m. The
225 young bark of a 33-year-old *B. fujianensis* was smooth with cross grains (DBH: 18
226 cm, Fig. 2A, BF1) and that of a 53-year-old (DBH of 29 cm, Fig. 2A, BF2) gradually
227 changed from smooth to exfoliate. The older bark of a 78-year (DBH: 44 cm, Fig. 2A,
228 BF3) and a 92-year-old (DBH: 53 cm, Fig. 2A, BF4) *B. fujianensis* was completely
229 vertically furrowed and exfoliated. However, the old bark of a 45- and a 89-year-old *B.*
230 *luminifera* was smooth with cross grains(DBH: 23 cm and 43 cm, Fig. 2A, BL1, and
231 BL2).
232 The reticulate-veined leaf blades of *B. fujianensis* and *B. luminifera* are ovate with
233 small tooth-like indentations, and petioles are villous and punctate (Fig. 2B). There
234 were one to three secondary teeth between primary teeth in *B. fujianensis*, while that
235 of *B. luminifera* are one to seven. *B. fujianensis* had less leaf serration (Fig. 2B). Both
236 ventral (upper) and dorsal (lower) surface of *B. fujianensis* had densely glandular

punctation and short and rare pubescences (Fig. 2B, C). Only the dorsal surface of *B. luminifera* was punctated with irregular-shape punctation, and both sides were covered with conspicuous and fine pubescences, giving it a fuzzy feel (Fig. 2B and 2C). We used phloroglucinol to stain the transverse sections of the 1.5-year-old stem and lignified tissues, phloem, xylem, and pith(Fig. 2D). *B. fujianensis* stem is relatively beardless, while *B. luminifera* stem is covered with pubescences. Therefore, *B. fujianensis* is different from its close relative (*B. luminifera*).

Seedling cultivation

Presently, *B. fujianensis* is only found in Luoboyan Reserve, Fujian province, with a total number of 500 (Zeng et al. 2008b). We developed a cultivation method (patent application Number is 201911265286.5) described below (Fig 3):

a) Ripe seeds were collected from healthy *B. fujianensis* at the end of April 2018 in Luoboyan Nature Reserve in Shaxian County, Sanming city, Fujian province. The yellow seeds with a relatively light-weight were selected and sterilized with 0.3-0.4% potassium permanganate solution for 8-12 minutes (fresh seeds or seeds collected within 1-7 days are recommended due to their high germination rate of 48%-62%).

253 b) A reasonably level and a dry terrain field was selected as the seedbed site (A
254 slightly acidic or slightly alkaline sandy loam is the most appropriate soil for growth).
255 The field was sterilized with 30% chlorpyrifos (2 ml/m^2) at one week before sowing.
256 The nursery beds (50 cm in height, 1.1 m in wide) were arranged in parallel
257 surrounded by 35 cm x 25 cm deep ditches. Before sowing, the nursery beds were
258 covered with humus soil (0.6 cm-0.8 cm) and red loam (2.5 cm), and ditches were
259 irrigated with water to a height of two thirds to keep nursery beds wet.
260 c) Seeds were mixed with wet sand (seeds:wet sand, 1:5) (Sowing rate was about
261 20g/m^2). Soil moisture was maintained at 95%, and temperature at 28 ^oC through
262 plastic film mulching.
263 d) The ditch was drained after germination, and the plastic film uncovered after
264 seedling growth, ensuring proper field management and disease and insect pest
265 control where possible.
266 e) Upon attaining a height of 30 cm (after about one-year), the seedlings were
267 transplanted in the forest.
268 This method yielded a seedling survival rate of at least 10%, and we successfully

269 planted about 6000 seedlings to 2 hm^2 forest in March 2019.

270 **photosynthetic physiological characteristic**

271 Leaf veins transport water, nutrients, and carbon. Of note, the midrib, middle, and leaf

272 edges of *B. fujianensis* and *B. luminifera* are similar (Fig.4A). The pubescences of *B.*

273 *fujianensis* leaf veins are relatively shorter and rare than *B. luminifera*.

274 We used similar trees of about 1.5 years (y) in nursery and forest to calculate SPAD

275 (Soil and Plant Analyzer Development) reading, and converted the chlorophyll

276 content index (CCI) from SPAD (Table S6, Fig.4B). CCI was almost similar in both

277 species while nursery area, but it was significantly higher in *B. fujianensis* after grown

278 in the forest after about a half year (from 2019-03 to 2019-11). The forest

279 environment, such as soil, light, and water, influenced the two species differently. The

280 total actual chlorophyll (Chl) content of trees in *B. fujianensis* in the nursery (1.5 y)

281 ranged between 2.21 and 2.88 mg/g, with a mean of 2.50 mg/g, while for *B.*

282 *luminifera* ranged between 2.12 to 2.88 mg/g, with an average of 2.67 mg/g. The

283 actual Chl content of *B. fujianensis* grown in the forest (53 y) was about 1.93 mg/g,

284 which is lower than that of *B. luminifera* (45 y) (2.43 mg/g) (Fig. 4C, Table S6).

285 Chlorophyll content in *B. fujianensis* (53 y) decreased with age, and this is not
286 observed in *B. luminifera* probably because of chloroplast gene or structure
287 difference.

288 The net photosynthetic (Pn) rate in both *B. fujianensis* and *B. luminifera*, was
289 positively correlated with light intensity (I) (Fig.4D), and Pn rapidly increased at the
290 beginning until approximately 250 μmol m^{-2} s^{-1} reaching the maximum Pn value
291 (Pmax). *B. fujianensis* had a higher Pmax than *B. luminifera* both in nursery and
292 forest, indicating that *B. fujianensis* used light more efficiently.

293 Stomata regulate plants gas exchange and water vapor, and stomatal conductance is
294 closely associated with the photosynthetic rate for various plant species, including
295 soybeans(Soleh et al. 2016), wheat(Faralli et al. 2019), rice(Yamori et al. 2020), and
296 several crop species(McAusland et al. 2016). *B. fujianensis* had higher stomatal
297 conductance in nursery and forest than *B. luminifera* (Fig.4E), enhancing
298 photosynthesis in *B. fujianensis*, thus increasing yields. The relatively higher
299 photosynthetic rate could be causing the difference in height between the two species.

300 **Chloroplast genome features of *B. fujianensis* and *B. luminifera***

We sequenced the chloroplast genomes of *B. fujianensis* and *B. luminifera* to understand their Chloroplast genome features further and found that *rpl16*, encoding a large ribosome subunit, was absent in *B. fujianensis*. The complete cp genome of *B. fujianensis* was 161,217 bp in length, similar to *B. luminifera* (160,990 bp). GC contents were 35.92% (*B. fujianensis*) and 35.97% (*B. luminifera*) (Table 1). Both cp genomes had a typical quadripartite structure as the majority plant cp genomes consisting of two inverted repeats (IRs) regions separated into a large single-copy (LSC) and a small single-copy (SSC) regions (Fig. S1). The numbers and positions of tRNAs and rRNA genes were similar and had 37 tRNA genes, eight rRNA genes (Table 2). There were 88 mRNAs in *B. luminifera*, and 87 in *B. fujianensis*. 14 genes had a single intron, *clpP* gene had two introns in both species (Table 2), and *rpl16*, specific in *B. luminifera* cp genemes, had an 1128-bp intron.

We identified 295 simple sequence repeats (SSRs), including 165 mono-nucleotide, 17 dinucleotide, 74 trinucleotide, 15 tetranucleotide, four pentanucleotide, and two hexanucleotide repeats in the cp genome of *B. luminifera* (Fig.S1, Table S1). We also identified 295 SSRs, including 165 mono-nucleotide, 17 dinucleotide, 73 trinucleotide,

14 tetranucleotide, 2 pentanucleotide, and 1 hexanucleotide in *B. fujianensis* (Fig.S1, Table S2). A and T patterns were the highest two SSRs in these two species. *B. luminifera* had 58 types of repeat patterns, and *B. fujianensis* had 55 types with three patterns (TAATA, TATTT, TTTAAT) absent. There was no significant difference on each repeat pattern counts (Table S3). We further identified the repeat sequence above 20 bp in cp genomes, and they were mainly located in intergenic regions, except for a few genes (*ycf1, ycf2, rrn4.5,* and *tRNA-GCU*). Both *ycf1* and *ycf2* had four repeat sequences in *B. luminifera*, and three in *B. fujianensis*. Gene *tRNA-GCU* had one repeat sequence in both *B. luminifera* and *B. fujianensis*. There were four repeat sequences in the *rrn4.5 gene (B. luminifera)* (Table S4 and S5).

IR/SC boundary.

We analyzed the border structure by comparing with nine other *Betula* species, as shown in Fig. 5A. While *rps19* gene is located in the LSC region in the positive strand, it is located in JLB (junction between LSC and IRb) boundary in *B. fujianensis*, *B. luminifera*, *Betula pubescens*, *Betula cordifolia*, *Betula nana*, *Betula platyphylla*, and *Betula pendula*. In *Betula populifolia*, *Betula occidentalis*, and *Betula lenta,* it extends

into the LSC region by 1 bp. Besides, *ndhf* gene is located in the SSC region, ranging between 40 bp (*Betula pubescens*) to 716 bp (*Betula pendula*) away from the JSB (junction between SSC and IRb) border. JSA (junction between SSC and IRa) and JLA (junction between LSC and IRa) border performance is relatively stable with *ycf1 gene* located in JSA border and *trnH* in 3- 6 bp upstream of the JLA. There was only one copy of *ycf1* in *B. fujianensis* and *B. luminifera*, similar to *Betula platyphylla* and *Betula pendula*. *ycf1* gene is located in JSB border in the negative-strand gene, and in the IRb region in *Betula pubescens*.

Phylogenetic analysis of *B. fujianensis*

We acquired 27 cp genomes of tree species from the public database and built the phylogenetic relationship of *B. luminifera* and *B. fujianensis* to determine an accurate *B. fujianensis* phylogenetic relationship (Fig.5B). *B. luminifera* and *B. fujianensis*, in *Betula*, were much similar to other tree species. They formed a sister clade with 100% bootstrap, consistent with the deduced phenotype features. *B. luminifera* and *B. fujianensis* were much similar to *Corylus*, *Ostryopsis*, *Carpinus*, *Alnus*, *Fagus*, indicating that they have similar origin as the Betulaceae.

RNA editing

C-to-U RNA editing is the most prevalent type of base-changing RNA modification in the chloroplast (Ichinose and Sugita 2016) and they primarily add variations to evolutionarily conserved genes. We predicted RNA editing sites in protein-coding regions from chloroplast genome sequences of *B. fujianensis* and *B. luminifera*. We identified 52 C->U conversions from 20 genes in both species (Fig.6A, Table S7). Three genes for small ribosome subunit (*rps14, rps16,* and *rps2*) had 2,1 and 1 editing sites, respectively. The four chloroplast genes (*rpoA, rpoB, rpoC1,* and *rpoC2*), encoding DNA dependent RNA polymerase, had 1, 6, 1, and 4 editing sites, respectively. Five out of twelve NADH dehydrogenase subunits in photosynthetic genes (*ndhA, ndhB, ndhD, ndhF,* and *ndhG*) predicted had 1, 6, 9, 4, and 3 editing sites, respectively, with the highest editing frequency. Two subunit genes of photosystem II (*psbE* and *psbF*) both had one modified site. Two subunit genes of cytochrome (*petB* and *petD*) had 2 and 1 editing sites, respectively. Two subunit genes of ATP synthase (*atpF* and *atpA*) had 1 and 2 editing sites, respectively. *clpP* gene encoding ATP-dependent Clp protease proteolytic subunit also had one editing

site and *accD* gene that encodes acetyl-CoA carboxylase beta subunit had four editing sites.

A total of 14 sites were located in the first codon position, and 38 in the second codon position. These C-to-U RNA editing in these codons led to 10 amino acid alterations (Fig.6B), with Serine (S) to Leucine (L) being the most edited codons (19 counts). Five NADH dehydrogenase subunits (*ndhA*, *ndhB*, *ndhD*, and *ndhF*) accounted for about 47% (9 counts). S and L are two physical-chemical-property that differentiate amino acids (Venkatarajan and Braun 2001), eg. hydrophobicity, size and alpha-helix. Therefore, such alterations can influence the function of corresponding proteins and should be further studied.

Discussion

In this study, we identified the annual life and morphological features of *B. fujianensis*. While *B. fujianensis* was morphologically similar to *B. luminifera*, we found some distinct characteristics, especially in their bark and leaves. Chlorophyll content analysis results revealed that chlorophyll content changes with environment and age. Chlorophyll content was significantly lower in 53 y than in 1.5 y *B.*

fujianensis. We acquired the chloroplast genomes of *B. fujianensis* and *B. luminifera* to understand the phenomenon. While chloroplast genome was conserved, we found out that *rpl16*, encoding a large subunit of ribosome, is absent in *B. fujianensis.* 1128-bp intron interrupted *rpl16* in *B. luminifera*, used to estimate phylogeny in various plants, such as Tribe Cacteae(Butterworth et al. 2002), Poaceae(Zhang 2000), and Apiaceae(Downie et al. 2000). Chloroplast *rpl16* 5'UTR has a targeting binding site for Pentatricopeptide repeat (RRP) proteins in maize(Hammani et al. 2016), i.e. PPR proteins that can regulate organellar RNA metabolism, organelle function, and organismal development via *rpl16*(Barkan and Small 2014). However, more research should be conducted to clarify if *rpl16* absence is related to chlorophyll content changes and the distinct features of *B. fujianensis*.

The photosynthetic physiological analysis implied *B. fujianensis* uses light more efficiently. Therefore, it is important to sequence the genome of *B. fujianensis* to confirm if several factors influence light signal transduction and use(46), which can provide more information on molecular adaptation signatures in their genomes and open up extensive research activities. The silver birch genome sequence (*B.pendula*)

has been recently reported(Salojärvi et al. 2017), and it could serve as a good reference. Besides, “multi-omics” and co-expression analysis can also contribute to discovering key factors that influence developmental processes, secondary wall biosynthesis, and environmental stress-tolerant, thus providing comprehensive information about these processes.

In this study, we developed a cultivation method with a 10% seedling survival rate. Efficient genetic regeneration and transformation system is essential to its rapid propagation, well preservation, and genetic improvement. Presently, many woody plants such as *Populus trichocarpa (Song et al. 2006), Phellodendron amurense* (Azad et al. 2005), Ma Bamboo (Ye et al. 2017), Manchurian Ash (Liu et al. 2020), plum (Petri et al. 2008), *Tripterygium wilfordii* (Zhao et al. 2018), and Salix (Guan et al. 2018) have developed such system. This system provides a novel tool for tree biotechnology, making genetic engineering information available. A protoplast transformation system can also be developed. The protoplast transformation method used in *Populus trichocarpa*(Lin et al. 2014) has been successfully applied in Moso Bamboo(Wang et al. 2019). Transformation technology can help us tag and clone

413 genes, identify the phenotypes they can produce, and explore their regulatory
414 networks.
415 It is important to use genetic editing modification in *B. fujianensis* to improve its traits
416 and broaden its latitudinal range because of its limited distribution. Genetic
417 engineering methods, such as the *Agrobacterium*-mediated gene transformation
418 system and CRISPR-associated protein 9(CRISPR/Cas9) system, have been widely
419 used to improve various woody plants, including apple (Osakabe et al. 2018),
420 grapevine(Osakabe et al. 2018; Wang et al. 2018b), *Populus trichocarpa* (Fan et al.
421 2015; Song et al. 2006), *Leptadenia pyrotechnica* (Dutta et al. 2013), and *Eucalyptus*
422 *camaldulensis(Tournier et al. 2003).* Superior trait data, such as resistance to cold,
423 drought, and salinity, can provide valuable information for molecular breeding. While
424 some low-temperature-responsive genes have been identified in birch (*Betula*
425 *platyphylla* Sukaczev) (Yan et al. 2020), there are still limited function analyses of
426 these genes. Major factors that evolved in secondary wall biosynthesis, growth,
427 developmental processes, and stress-tolerant, could be good targets for producing

superior genetic traits. These techniques once introduced, can help us understand quantum leap, thus promoting *B. fujianensis* plantation.

Birches have received a growing interest because of their high tolerance to environmental conditions and helping in afforestation (Dubois et al. 2020). The available data on birch is largely from silver birch (*Betula pendula*) and white birch (*Betula pubescens*) studies, which were the most common species (Jonczak et al. 2020). Notably, there is little information on *B. fujianensis* effects on forest ecosystems, particularly soil environment, and it should be highly evaluated before using it for large-scale afforestation. However, the cultivation method we developed enabled us to study it in detail, such as its influence on water regime, soil physical and chemical properties, and soil biota. Furthermore, more studies on its soil contamination tolerance and its phytoremediation potential should be conducted. Several *B. fujianensis* studies should be undertaken to add more information on its conservation, application, and afforestation.

Conclusions

Our study observed the annual life and morphological features of *B. fujianensis*. Although its relative *B. luminifera* was morphologically similar, we found some distinct characteristics, especially in bark and leaves. Studies comparing within the chloroplast genome sequences firstly provide genomic information about *B. fujianensis* and *B. luminifera*, and some clues to different environmental adaptation. The cultivation method will enable further scientific researches, and also contribute to its good conservation and utilization in the future, both in afforestation and industrial application.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The whole-genome sequence data reported in this study are available at the Genome Warehouse in National Genomics Data Center (Zhang et al. 2020), Beijing Institute of Genomics (China National Center for Bioinformation), Chinese Academy of Sciences,

459 under accession numbers: GWHAOXU00000000 and GWHAOXT00000000

460 (https://bigd.big.ac.cn/gwh).

461 **Supplementary data**

462 **Fig. S1** Chloroplast genome map of *B. fujianensis* and *B. luminifera.*

463 **Table S1** SSRs of chloroplast genome of *B. luminifera.*

464 **Table S2** SSRs of chloroplast genome of *Betula fujianensis*.

465 **Table S3** Comparison of SSRs in *Betula fujianensis* and *B. luminifera*.

466 **Table S4** Analysis of repeated sequences in *B. luminifera*.

467 **Table S5** Analysis of repeated sequences in *B. fujianensis.*

468 **Table S6** SPAD value and chlorophyll content of *Betula fujianensis* and *B.*
469 *luminifera*.

470 **Table S7** RNA editing sites of *Betula fujianensis* and *B. luminifera*.

471 **Competing interests**

472 No potential conflict of interest was reported by the authors.

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Authors' contributions

WG, YRH and HXZ conceived the study. WG, YRH, HW, SDH, GFY, MGH, XSW, APC, SWX performed the observation and experiments. HXZ analyzed the sequencing data. HXZ and WG designed the study and wrote the manuscript. All authors have read and approved the final manuscript.

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689

690 **Legend:**

691 **Fig.1:** Annual life cycle of *B. fujianensis*.

Fig.2: Morphological comparison between *B. fujianensis* and *B. luminifera*. A) The comparison of barks. The young bark of *B. fujianensis* with a diameter at breast height (DBH) of 18 cm (BF1). The bark of *B. fujianensis* with a DBH of 29 cm (BF2). The old bark of *B. fujianensis* with a DBH of approximately 44 cm (BF3) and 53 cm (BF4). The bark of *B. luminifera* with a DBH of 23 cm (BL1) and 43 cm (BL2). B) The comparison of ventral and dorsal surface of leaves between *B. fujianensis* and *B. luminifera*. C) Scanning electron microscope observations of ventral and dorsal surface of leaves of *B. fujianensis* and *B. luminifera*. D) The transverse sections of one-year-old stems of *B. fujianensis* and *B. luminifera* were stained by phloroglucinol and tissues, phloem, xylem and pith, were lignified.

Fig.3: The process of seedling cultivation and afforestation.

Fig.4: A) Transverse section from midrib, middle and edge of leaves of *B. fujianensis* and *B.luminifera*. Pp:palisade parenchyma, Xy:xylem, Ph:phloem, Sp: spongy parenchyma, VeS: ventral surface, DoS: dorsal surface.B) The chlorophyll content index (CCI) of 1.5a *B. fujianensis* and *B. luminifera*. N indicates trees are planted in nursery area, and F means trees are from forest area. Values were Mean ± SEM from

708 replicates, and the bars represent SEM. Student t test was used to evaluate the
709 difference at the 0.05 probability level. "***":P<0.001; "****":P<0.0001. The
710 number of seedlings is presented in the parentheses on the bars. C) Total Chlorophyll
711 content of 1.5a *B. fujianensis* and *B. luminifera* from nursery area, and total
712 chlorophyll content of 45a *B. fujianensis* and *53a B. luminifera* from forest area. D)
713 The correlation between the net photosynthetic (Pn) rate and light intensity both in *B.*
714 *fujianensis* and *B. luminifera*. E) The correlation between stomatal conductance (Cond)
715 and light intensity both in *B. fujianensis* and *B. luminifera.*

716 **Fig 5:** A) Comparison of the border positions of large single copy (LSC), small single
717 copy(SSC), and inverted repeat (IR) regions among the 11 chloroplast genomes from
718 11 Betula species. The lengths of chloroplast genome are depicted below of each
719 species. Genes transcribed in positive are presented above of their corresponding
720 tracks, and genes in negative strands are presented below. The arrows are indicating
721 the distance of a given gene to the corresponding boundary sites, which are not to
722 scale. JLB, junction between LSC and IRb; JSB, junction between SSC and IRb; JSA,
723 junction between SSC and IRa; JLA, junction between LSC and IRa. B) Phylogenetic

relationships of the 27 tree species constructed with the complete chloroplast genome by maximum likelihood (ML) methods. Numbers above the node are bootstrap support values for ML.

Fig. 6 A) The prediction of RNA editing sites in protein-coding regions of chloroplast genome sequences. B) C-to-U RNA editing in codon position. C) The frequently edited codons due to RNA editing.