

Characterization, Cultivation, and Chloroplast Genome Analysis of *Betula Fujianensis*

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Research

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

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16

17 **Abstract**

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

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

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

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

39

40 **Background**

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

49 Birch is a strong pioneer species that produces abundant seeds dispersed by wind;
50 hence it has high genetic variability and tolerance to environmental conditions(Koski
51 and Rousi 2005; Perala and Alm 1990), which enable it to adapt to a wide range of
52 sites, even including the contaminated site (Ulbrichová et al. 2005). Birch is highly
53 resistant to chemical pollutants and has great phytoremediation potential (Jonczak et
54 al. 2020; Renou-Wilson et al. 2010). Besides, birch shows rapid growth in early
55 stages, and can quickly dominate a new area, thus improving the soil functioning to
56 create favorable conditions for other tree species (Dubois et al. 2020). In recent
57 decades, there has been a growing interest in afforestation using birch (Dubois et al.
58 2020).

59 *Betula fujianensis* (*B. fujianensis*), is a new birch species reported in Luoboyan
60 Nature Reserve in Shaxian County, Sanming City, Fujian Province
61 (117°34'15"E~117°36'00"E, 26°25'45"N~26°27'30"N)(Zeng et al. 2008b) in 2008. It
62 is endemic to Fujian Province and is an endangered species in the IUCN Red List of
63 Threatened Species because only 500 are found in Luoboyan Nature Reserve
64 (Ashburner et al. 2013; Shaw et al. 2014). No special protection measures have been

65 taken. The existing ones are mainly large-diameter old trees that cannot regenerate
66 naturally, and there is little information about its seed germination and cultivation.
67 Current research only focused on the plant taxonomy (Zeng et al. 2008a; Zeng et al.
68 2014). It has been found that the *B. fujianensis* community has several other plants,
69 and the strong interspecific competition and poor regeneration puts this species at
70 greater extinction risk. Moreover, there is no information about its cultivation and
71 molecular data. It is important to systematically observe its phenology and develop a
72 seedling cultivation method to conserve it. In this study, we observed its annual life
73 and morphological characteristics. We comparatively analyzed it with its close
74 relatives, such as *B. luminifera*, to identify its detailed characteristic, especially the
75 bark and leaves. A cultivation method was also developed to ensure it is not exploited
76 to extinction. We successfully cultivated its seedlings for afforestation and scientific
77 research. This study reports the whole chloroplast genome sequences of *B. fujianensis*
78 and *B. luminifera*, conducts a comparative analysis, and constructs a phylogenetic
79 relationship using 27 other tree species. This information could facilitate its
80 well-controlled exploitation, and also enrich the molecular data of forest trees.

81 **Methods**

82 **Plant materials**

83 We cultivated the seedlings in a nursery in May 2018 using the above-described
84 methods and transplanted about 6000 seedlings to 2 hm² forest in March 2019. The
85 samples were obtained from Luoboyan Nature Reserve in Shaxian Country, Sanming
86 city, Fujian province (117°34'15"E~117°36'00"E, 26°25'45"N~26°27'30"N) in
87 November 2019. We used the 1.5-year-old (1.5 y) trees planted in nursery and forest
88 to calculate the net photosynthetic rate to exclude the habitat factor effect.

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

93 **Tree-ring data and tree age determination**

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

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

105 We obtained tree-ring data of *B. luminifera* from a previous publication about natural
106 trees in Qingyuan Forest Farm, Zhejiang province, about 240 km from Luoboyan
107 Nature Reserve (Chen Yi-liang et al. 2009). The fitted equation for *B. luminifera* was:
108 $y = 1.4251x^{1.0979}$ with coefficient determination (R^2) =0.98199. *B. luminifera* with
109 DBH of 23 and 43 cm were approximated to be 45 and 89 y, respectively.

110 **Chlorophyll content determination**

111 We used SPAD-502 Chlorophyll Meter (Minolta Camera Corporation, Ltd., Osaka,
112 Japan) with transmittance measurements at red (650 nm) and infrared (940 nm)
113 wavelengths to measure SPAD reading. Transformed CCI of *Betula papyrifera* leaves
114 from SPAD values of *Betula papyrifera* leaves was derived using the equation $y =$
115 $5.52E-04 + 4.04E-04x + 1.25E-05x^2$ with coefficient determination (R^2)=0.96
116 (Richardson et al. 2002).

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

127 chlorophyll b (C_b) concentrations were calculated using equations:

128 $C_a=13.95 \times A_{665} - 6.88 \times A_{649}$; $C_b=24.96 \times A_{649} - 7.32 \times A_{665}$. The total chlorophyll content

129 $(\text{mg/g}) = (C_a + C_b) \times \text{Volume} / \text{Weight}$.

130 **Photosynthesis light response curves**

131 We collected all data on sunny days between Nov. 9 to Nov. 10, 2019, using the

132 LI-6400 Portable Photosynthesis System (LI-COR Corporate, USA). We used red and

133 blue LEDs Light Source (6400-02B) to produce gradient light intensity of 0, 15, 30,

134 60, 120, 250, 500, 1000, 1500 and 2000 $\mu\text{mol (photon) m}^{-2} \text{ s}^{-1}$. All the tested leaves

135 were kept under natural conditions and induced using a light intensity of 1000 μmol

136 $\text{photon m}^{-2} \text{ s}^{-1}$ and the period was set to 2-3 min and a match was made after every

137 count.

138 We simulated the light response curve of photosynthesis using the non-rectangular

139 hyperbola model:

$$140 \quad P_n = \frac{\alpha I + P_{\max} - \sqrt{(\alpha I + P_{\max})^2 - 4\theta\alpha I P_{\max}}}{2\theta} - R_d$$

141 Where P_n is net photosynthetic rates [$\mu\text{mol (CO}_2\text{) m}^{-2} \text{ s}^{-1}$], I is photosynthetic photon

142 flux density [$\mu\text{mol (photon) m}^{-2} \text{ s}^{-1}$], α is intrinsic quantum yield, P_{\max} is maximum

143 net photosynthetic rate [$\mu\text{mol (CO}_2\text{) m}^{-2} \text{ s}^{-1}$], θ is convexity, R_d is the rate of dark
144 respiration [$\mu\text{mol (CO}_2\text{) m}^{-2} \text{ s}^{-1}$].

145 The initial values were: $\theta=0.5$, $P_{\text{max}}=8 \mu\text{mol (CO}_2\text{) m}^{-2} \text{ s}^{-1}$, $\alpha=0.05$, $R_d=2 \mu\text{mol (CO}_2\text{)}$
146 $\text{m}^{-2} \text{ s}^{-1}$. The limination values were: $\theta \leq 1$, $P_{\text{max}} \leq 50 \mu\text{mol (CO}_2\text{) m}^{-2} \text{ s}^{-1}$, $\alpha \leq 0.125$,
147 and R_d had no limination. We used the linear portion of the light response curve of
148 photosynthesis ($I < 250 \mu\text{mol (photon) m}^{-2} \text{ s}^{-1}$) to calculate the light compensation point
149 (LCP) and light saturation point (LSP) where:

150
$$P_n = \alpha \cdot I - R_d$$

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

152 **Microscopic observations**

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

159 **Sampling and DNA extraction**

160 We obtained fresh and healthy *B. fujianensis* and *B. luminifera* leaves from Luoboyan
161 Nature Reserve in Sha Country, Sanming city, Fujian province
162 (117°34'15"E~117°36'00"E, 26°25'45"N~26°27'30"N) and immediately soaked the
163 samples in liquid nitrogen and stored at -80°C. We used a Plant Genomic DNA kit,
164 DP305 (Tiangen, Beijing, China) for DNA extraction. Pair-end DNA libraries were
165 construct using the standard Illumina protocol and conducted sequencing using
166 Illumina HiSeq™ 2500 platform (Illumina, San Diego, CA, USA).

167 **Chloroplast genome assembly and annotation**

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

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

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

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

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

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

195 **Phylogenetic analysis and RNA editing sites predictions in the chloroplast**
196 **genome**

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

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

206 **Resules**

207 **Phenological observations and morphological characteristics of *B. fujianensis***

208 *B. fujianensis* had a height of 36 m and diameter at breast height (DBH) up to 60 cm.

209 We discovered it at Luoboyan Nature Reserve in Shaxian County, Sanming City,

210 Fujian Province. Luoboyan Nature Reserve lies 500 m above the sea level, and has a

211 mild and humid climate, with a mean annual temperature of about 12°C and annual

212 precipitation of about 2000 mm. There are only about 500 trees at Luoboyan Nature

213 Reserve presently. A general introduction of *B. fujianensis* was reported in 2008

214 (Zeng et al. 2008b). We observed trees in the forest for three years and found that *B.*

215 *fujianensis* leaves first unfolded in March and fell in September (Fig. 1). *B.*

216 *fujianensis* leaf blades are ovate with regular small tooth-like indentations, densely

217 glandular punctate, and have short and rare pubescences. Staminate inflorescences

218 axes are produced in June and wither in March, with 2-3 arranged on top of branches.

219 Pistillate inflorescences are racemes, which were produced and pollinated in March.

220 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

237 punctation and short and rare pubescences (Fig. 2B, C). Only the dorsal surface of *B.*
238 *luminifera* was punctated with irregular-shape punctation, and both sides were
239 covered with conspicuous and fine pubescences, giving it a fuzzy feel (Fig. 2B and
240 2C). We used phloroglucinol to stain the transverse sections of the 1.5-year-old stem
241 and lignified tissues, phloem, xylem, and pith(Fig. 2D). *B. fujianensis* stem is
242 relatively beardless, while *B. luminifera* stem is covered with pubescences. Therefore,
243 *B. fujianensis* is different from its close relative (*B. luminifera*).

244 **Seedling cultivation**

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

248 a) Ripe seeds were collected from healthy *B. fujianensis* at the end of April 2018 in
249 Luoboyan Nature Reserve in Shaxian County, Sanming city, Fujian province. The
250 yellow seeds with a relatively light-weight were selected and sterilized with 0.3-0.4%
251 potassium permanganate solution for 8-12 minutes (fresh seeds or seeds collected
252 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/m²) 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/m²). Soil moisture was maintained at 95%, and temperature at 28 °C 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 hm² 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 $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ 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***

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

313 We identified 295 simple sequence repeats (SSRs), including 165 mono-nucleotide,
314 17 dinucleotide, 74 trinucleotide, 15 tetranucleotide, four pentanucleotide, and two
315 hexanucleotide repeats in the cp genome of *B. luminifera* (Fig.S1, Table S1). We also
316 identified 295 SSRs, including 165 mono-nucleotide, 17 dinucleotide, 73 trinucleotide,

317 14 tetranucleotide, 2 pentanucleotide, and 1 hexanucleotide in *B. fujianensis* (Fig.S1,
318 Table S2). A and T patterns were the highest two SSRs in these two species. *B.*
319 *luminifera* had 58 types of repeat patterns, and *B. fujianensis* had 55 types with three
320 patterns (TAATA, TATTT, TTTAAT) absent. There was no significant difference on
321 each repeat pattern counts (Table S3). We further identified the repeat sequence above
322 20 bp in cp genomes, and they were mainly located in intergenic regions, except for a
323 few genes (*ycf1*, *ycf2*, *rrn4.5*, and *tRNA-GCU*). Both *ycf1* and *ycf2* had four repeat
324 sequences in *B. luminifera*, and three in *B. fujianensis*. Gene *tRNA-GCU* had one
325 repeat sequence in both *B. luminifera* and *B. fujianensis*. There were four repeat
326 sequences in the *rrn4.5* gene (*B. luminifera*) (Table S4 and S5).

327 **IR/SC boundary.**

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

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

341 **Phylogenetic analysis of *B. fujianensis***

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

349 **RNA editing**

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

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

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

375 **Discussion**

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

381 *fujianensis*. We acquired the chloroplast genomes of *B. fujianensis* and *B. luminifera*
382 to understand the phenomenon. While chloroplast genome was conserved, we found
383 out that *rpl16*, encoding a large subunit of ribosome, is absent in *B. fujianensis*.
384 1128-bp intron interrupted *rpl16* in *B. luminifera*, used to estimate phylogeny in
385 various plants, such as Tribe Cactaceae(Butterworth et al. 2002), Poaceae(Zhang 2000),
386 and Apiaceae(Downie et al. 2000). Chloroplast *rpl16* 5'UTR has a targeting binding
387 site for Pentatricopeptide repeat (RRP) proteins in maize(Hammani et al. 2016), i.e.
388 PPR proteins that can regulate organellar RNA metabolism, organelle function, and
389 organismal development via *rpl16*(Barkan and Small 2014). However, more research
390 should be conducted to clarify if *rpl16* absence is related to chlorophyll content
391 changes and the distinct features of *B. fujianensis*.

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

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

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

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

430 Birches have received a growing interest because of their high tolerance to
431 environmental conditions and helping in afforestation (Dubois et al. 2020). The
432 available data on birch is largely from silver birch (*Betula pendula*) and white birch
433 (*Betula pubescens*) studies, which were the most common species (Jonczak et al. 2020).

434 Notably, there is little information on *B. fujianensis* effects on forest ecosystems,
435 particularly soil environment, and it should be highly evaluated before using it for
436 large-scale afforestation. However, the cultivation method we developed enabled us to
437 study it in detail, such as its influence on water regime, soil physical and chemical
438 properties, and soil biota. Furthermore, more studies on its soil contamination tolerance
439 and its phytoremediation potential should be conducted. Several *B. fujianensis* studies
440 should be undertaken to add more information on its conservation, application, and
441 afforestation.

442 **Conclusions**

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

451 **Ethics approval and consent to participate**

452 Not applicable

453 **Consent for publication**

454 Not applicable

455 **Availability of data and material**

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

459 under accession numbers: GWHAOXU000000000 and GWHAOXT000000000
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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479 **Authors' contributions**

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

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689

690 **Legend:**

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

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

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

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

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

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

Figures

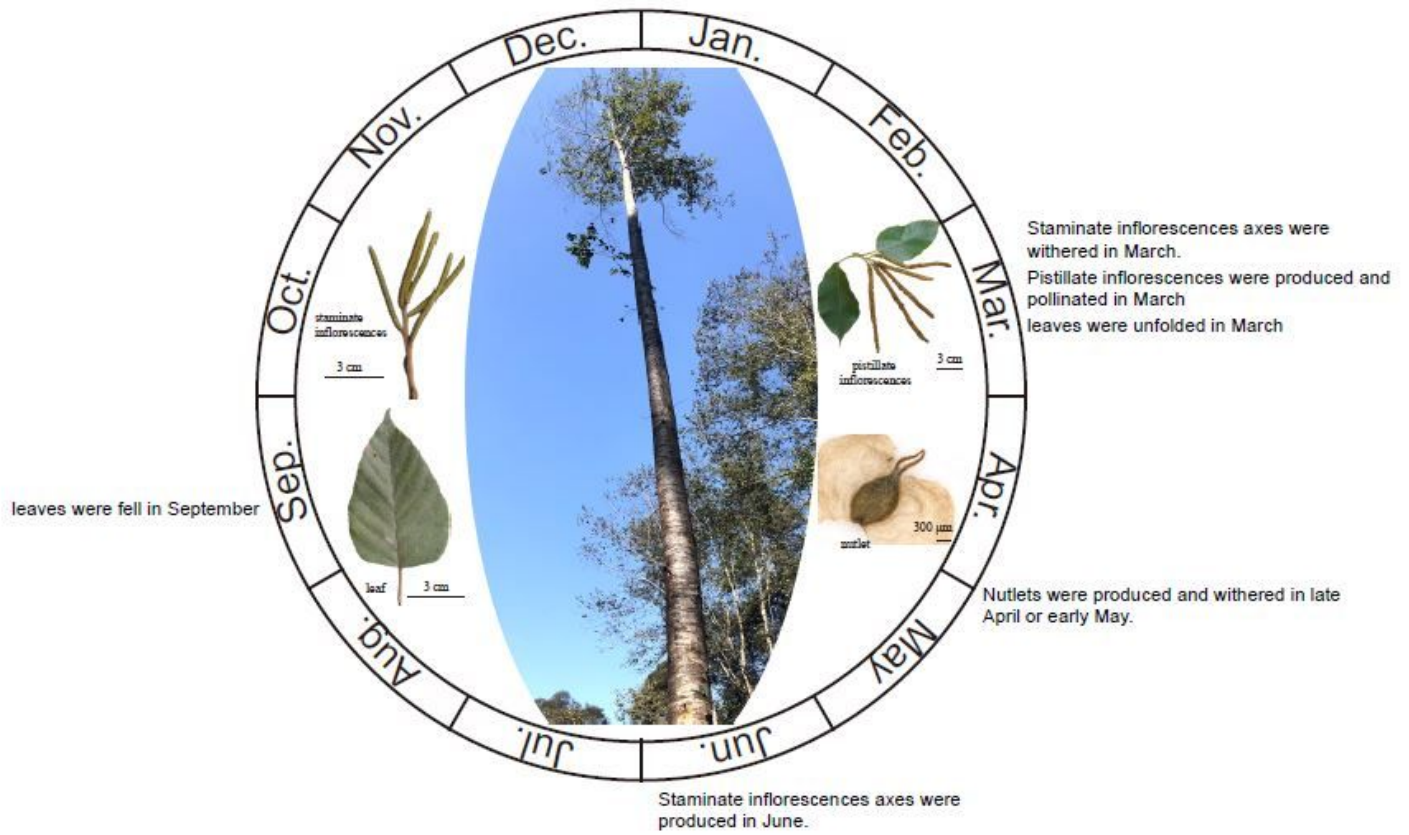


Figure 1

Annual life cycle of *B. fujianensis*.

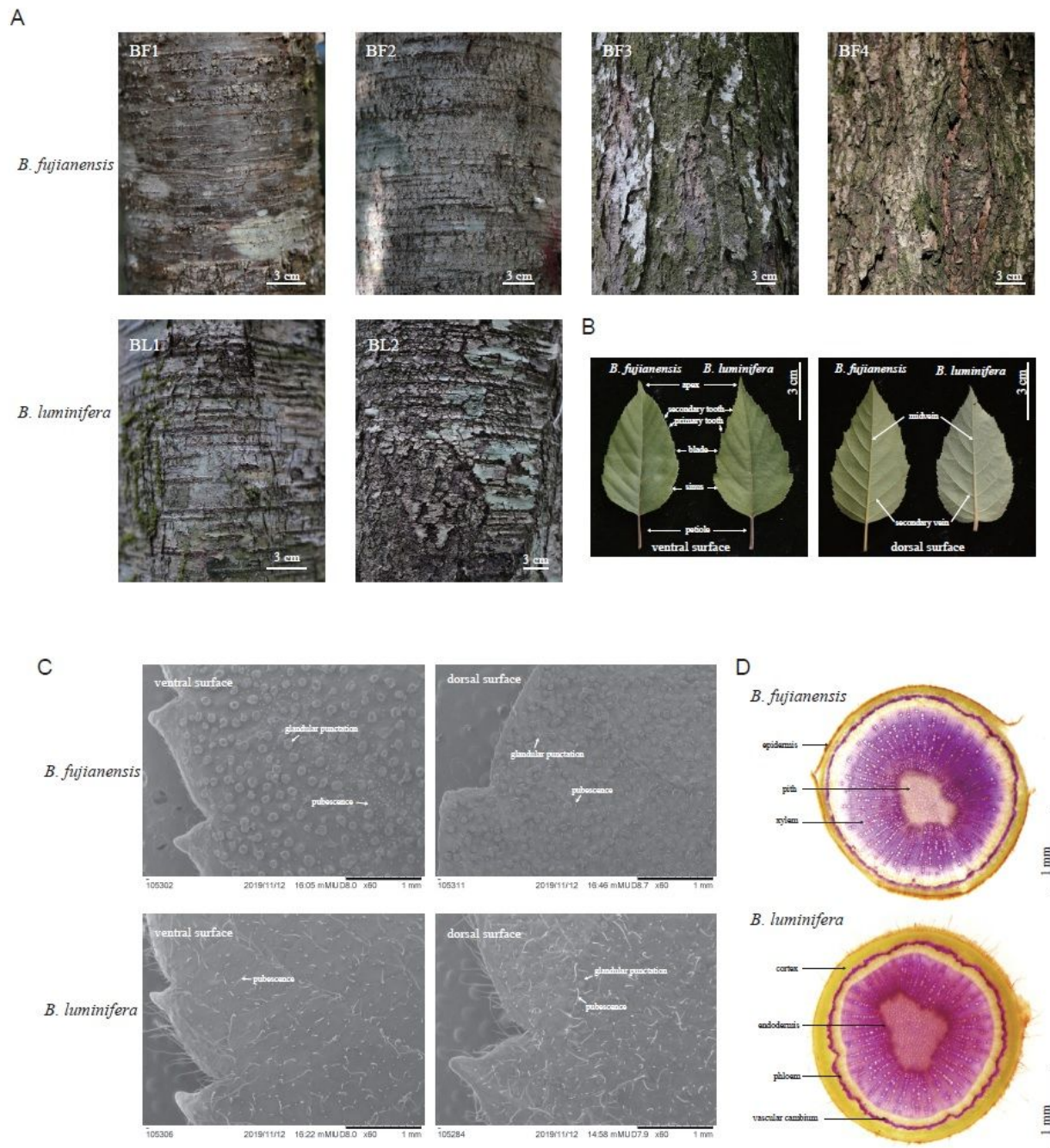


Figure 2

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.



Figure 3

The process of seedling cultivation and afforestation.

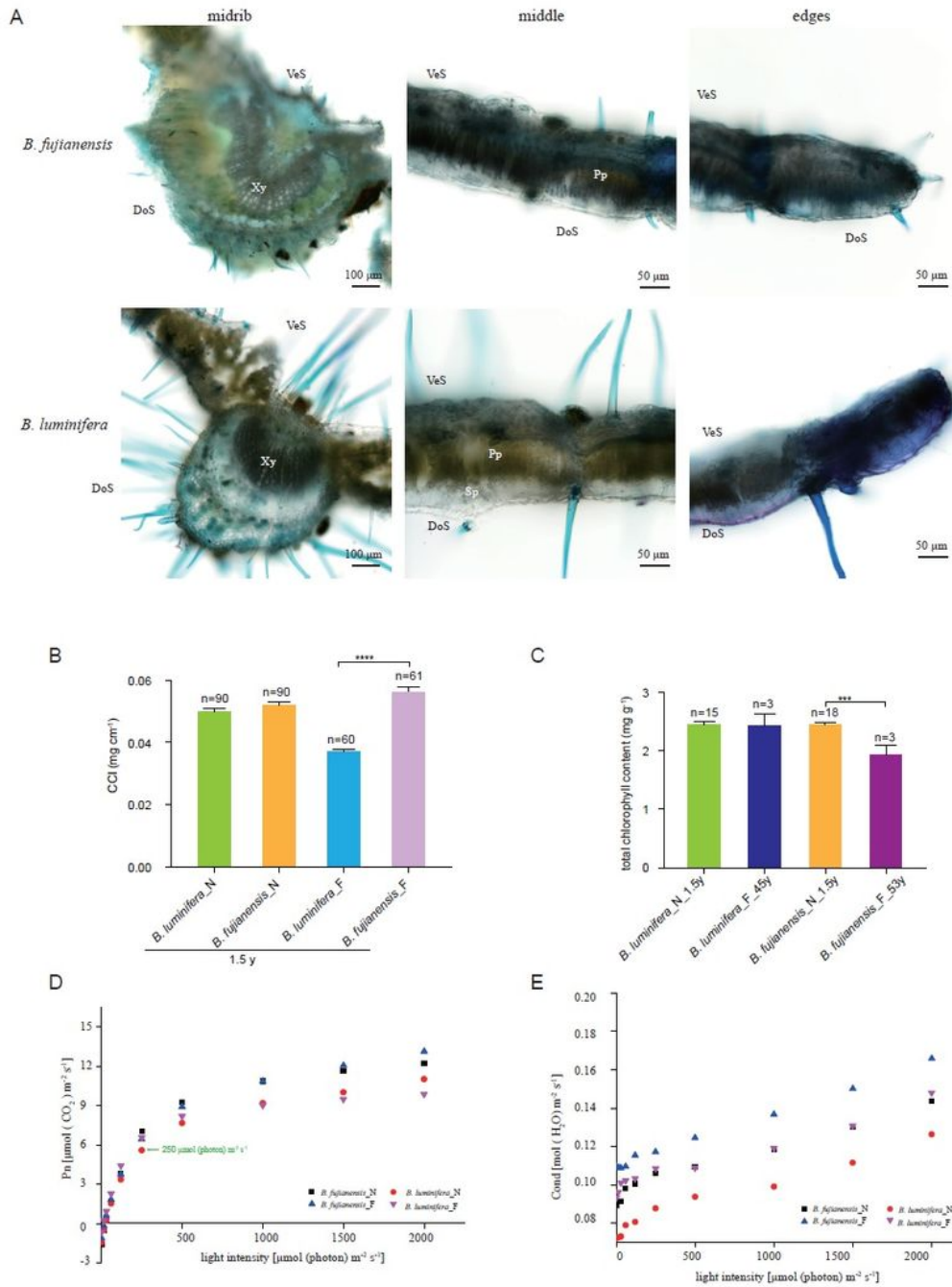


Figure 4

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 \pm SEM from replicates, and the bars represent SEM. Student t test was used to evaluate the difference at the 0.05

probability level. “***”: $P < 0.001$; “****”: $P < 0.0001$. The number of seedlings is presented in the parentheses on the bars. C) Total Chlorophyll content of 1.5a *B. fujianensis* and *B. luminifera* from nursery area, and total chlorophyll content of 45a *B. fujianensis* and 53a *B. luminifera* from forest area. D) The correlation between the net photosynthetic (P_n) rate and light intensity both in *B. fujianensis* and *B. luminifera*. E) The correlation between stomatal conductance (Cond) and light intensity both in *B. fujianensis* and *B. luminifera*.

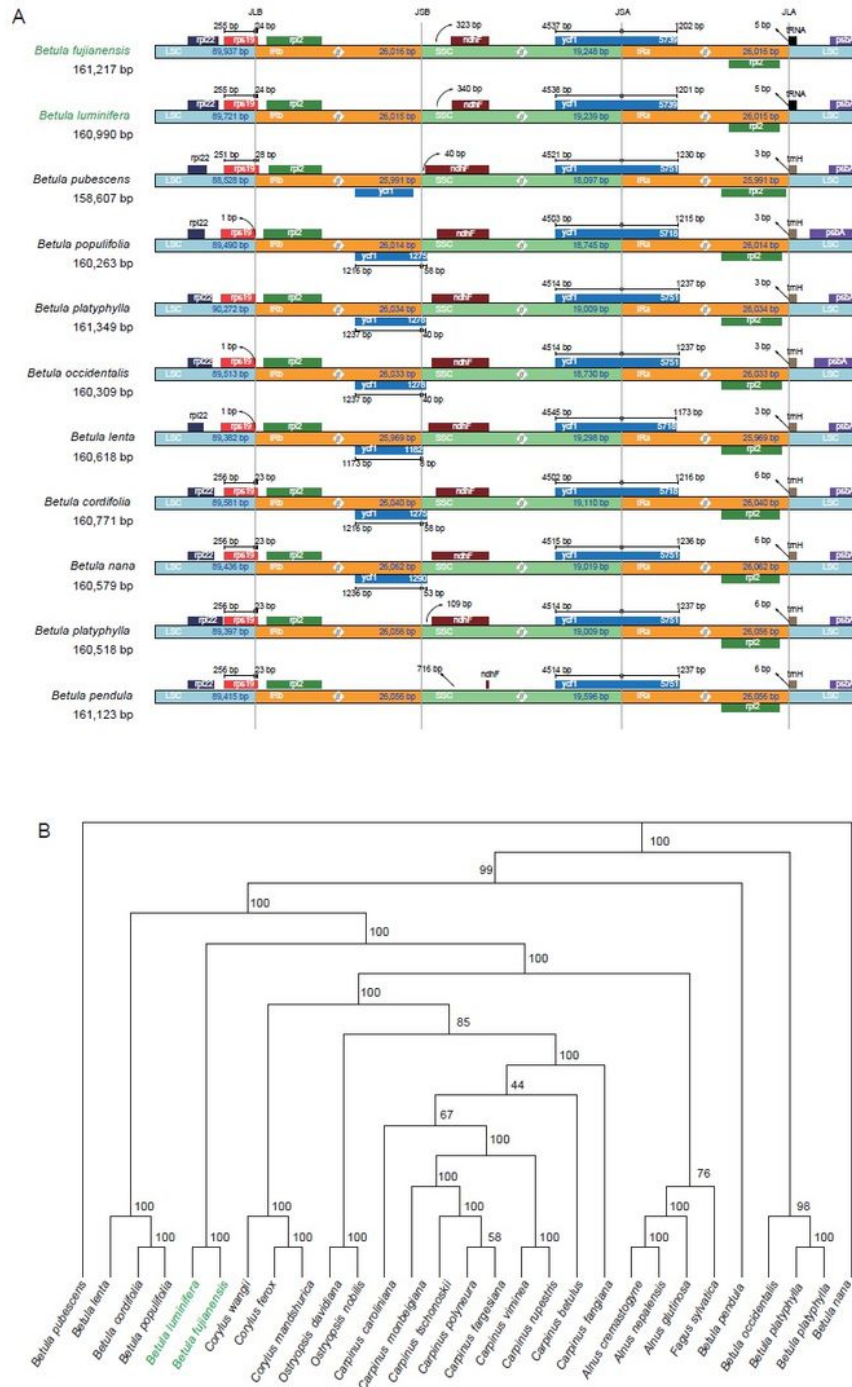


Figure 5

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

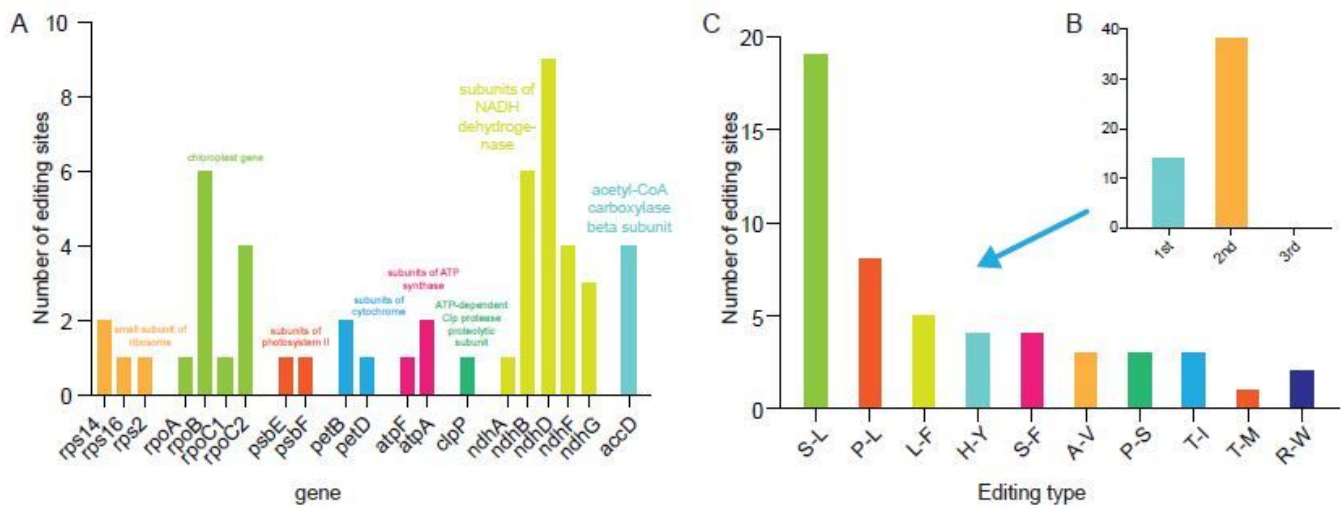


Figure 6

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.

Supplementary Files

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- [Figs1.pdf](#)
- [TableS1SSRsofchloroplastgenomeofBetulaluminifera.xlsx](#)
- [TableS2SSRsofchloroplastgenomeofBetulafujianensis..xlsx](#)
- [TableS3ComparisonofSSRs.xlsx](#)
- [TableS4RepeatedsequencesinBetulaluminifera.xlsx](#)
- [TableS5RepeatedsequencesinB.fujianensis.xlsx](#)

- [TableS7RNAeditingsites.xlsx](#)
- [TableS6SPADvalueandchlorophyllcontent.xls](#)
- [Table1CleanData.xlsx](#)
- [Table2listofgenespresentinthechloroplastgenomes.xlsx](#)