High-quality genome assembly of *Cinnamomum burmannii* (chvar. Borneol) provides insights into the natural borneol biosynthesis

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28 **Abstract:** *Cinnamomum burmannii* (chvar. Borneol) is a well-known
29 medicinal and industrial plant cultivated in the Lingnan region of China. It
30 is the key source from organism of natural borneol (D-borneol), one of the
31 precious and widely used Chinese herbal medicines with a variety of
32 medicinal effects. Here, we report a high-quality chromosome-scale
33 genome assembly of *C. burmannii* (chvar. Borneol) using Pacbio single-
34 molecule sequencing and Hi-C technology. The assembled genome size
35 was 1.14 GB with a scaffold N50 of 94.30 Mb, while 98.77% of the
36 assembled sequences were anchored on 12 pseudochromosomes including
37 41549 protein-coding genes. Genomic evolution analysis revealed *C.*
38 *burmannii* and *C. micranthum* shared two Lauraceae unique ancestral
39 whole-genome duplication (WGD) events. Likewise, comparative
40 genomic analysis showed strong collinearity between these two species.
41 Besides, the analysis for Long Terminal Repeat Retrotransposons (LTR-
42 RTs) indicated the outbreak of LTR-RTs insertion made a great contribution
43 to the size difference of genomes between *C. burmannii* and *C. micranthum.*
44 Furthermore, the candidate genes in pathway associated with natural

borneol synthesis were identified on the genome and their differential expressions were analyzed in various biological tissues. We considered that several of genes in Mevalonate (MVA) Methylerythritol Phosphate (MEP) pathways or in downstream pathway have the potential to be the key factors in the biosynthesis of D-borneol. We also constructed the genome database (CAMD; http://www.cinnamomumdatabase.com/) of *Cinnamomum* species for a better data utilization in the future. All these results will enrich the genomic data of Lauraceae plants and facilitate genetic improvement of this commercially important plant.

Key words: *C. burmannii* (chvar. Borneol), Genome, D-borneol

Introduction

Natural borneol, also known as D-borneol, borneol, plum borneol, plum flower, etc., is a highly desirable natural product, as well as a precious Chinese herbal medicine. It has been widely used as folk medicine and food additives in China and India (Chen et al., 2010). Natural borneol has the effect of opening the orifice and awakening the mind, clearing away heat and relieving pain. It is often utilized for the treatment of multiple diseases such as sore throat, convulsive phlegm and shiver. Previous researches indicated D-borneol has remarkable effects on the aspects of anti-myocardial ischemia, brain tissue protection, nerve protection and anti-infection *in vivo* or *in vitro* (Armaka et al., 1999; Lu et al., 2010). Natural borneol is also a fragrance ingredient used in decorative

cosmetics products. Its use worldwide is in the region of 1–10 metric tones per annum (Bhatia et al., 2008). As of 2016, the annual demand for natural borneol in the world was about 12,000 tons, and the total annual output of natural borneol in China was less than 200 tons, indicating a huge market gap and potential (http://www.ibaogao.com/baogao/10241a2312016.html). As an important source of natural borneol, *Cinnamomum burmannii* (chvar. Borneol) also called is a well-known medicinal and edible plant which had been widely cultivated in the Lingnan region of southern China (Wolf et al., 2011). Phytochemical studies have shown that *C. burmannii* (chvar. Borneol) contains abundant natural borneol in its leaves (Huang et al., 2019). In China, Guangdong Province is the main planting area for *C. burmannii* (chvar. Borneol), accounting for approximately 90% of its output (Chen et al., 2010).

Based on the identified metabolites and enzymes, previous studies have determined that the biosynthesis pathway of Glycerol 3-Phosphatase (GPP), the precusor of D-borneol *in vivo,* is related to the MVA pathway. Recently, MEP pathway in plants also had been reported to be associated with the GPP formation. Previous transcriptomic and synthetic biology studies also had identified that a gene (CbTPS) in TPS gene family is a key factor for downstream transformation of GPP generated by upstream pathways and natural D-borneol in *C. burmannii* (Ma et al., 2021). However, due to the lack of a complete genome, systematic researches of D-borneol

89 biosynthesis and evolutiology of *C. burmannii* at the genomic level were
90 still absent.
91 In order to fill this gap, we *de novo* assembled a chromosome-scale genome
92 of Mei Pian tree, a physiological type of *C. burmannii* (chvar. Borneol) and
93 whose leaves is rich in D-borneol. The assembly was based on single-
94 molecule real-time long reads from the Pacific Biosciences (PacBio)
95 SequeII platform, and construct pseudo-chromosomes using high-
96 throughput chromosome conformation capture (Hi-C) techniques base on
97 a chromosome number of 2n = 24 as previous report (Wu et al., 1992).
98 By evolution and comparative genomic analysis, we detected that the
99 recent insertion outbreak of LTR-RTs was an important cause of plant
100 differentiation in *Cinnamomum*. RNA-seq transcriptiomics and further
101 gene annotation analysis based on our assembled *C. burmannii* genome
102 dissected the natural D-borneol biosynthesis pathway at the level of
103 genome. The present study also demonstrated, *CbERG20* and *CbIDI1* in
104 MVA pathway, *CbDXR*, *CbCMS* and *CbHDR* in MEP pathway as well as
105 *CbTPS-1* in the downstream pathway, tend to be the key or the limiting
106 factor in the natural D-borneol biosynthesis pathway in *Cinnamomum*. To
107 encourage the data utilization, we also built a user-friendly genomic
108 database of *Cinnamomum* (CAMD;
109 http://www.cinnamomumdatabase.com/) for the data access.
110 The present study provides the first reference genome and detail genomic

information for *Cinnamomum burmannii* (chvar. Borneol), a plant rich in D-borneol which is highly desired natural product used in medicine and industry. We also dissected the D-borneol synthesis pathway and provide a novel insight into the molecular mechanisms of this pathway. Our results will be valuable resources for promoting genetic improvement and understanding the biosynthesis of active ingredients of *C. burmannii* (chvar. Borneol).

Method and materials

Plant materials and sequencing

Root, leaves, and stem samples of "Mei 3", a popular cultivated variety of *Cinnamomum burmannii* (chvar. Borneol) were collected from the Mei Zhou City (Pingyuan District, 24°28′–24°66′ N, 117.3°13′–118.1°21′ E) in Guangdong Province, China. Because of its unique-geography and environment, the Pingyuan District of Mei Zhou City is considered the authentic production area of *C. burmannii*. "Mei 3", with many excellent cultivation characteristics, including growth fast, high quality of component, and disease resistance, is the main variety of *C. burmannii* (chvar. Borneol). In Pingyuan District. High-quality genomic DNA was extracted using a QIAGEN® Genomic Kit (QIAGEN, Germany). The integrity of the DNA was checked by 0.75% agarose gel electrophoresis.

Genomic survey and assembly

The genommic size and heterozygosis were estimated by the software GCE,

133 Jellyfish, kmergenie and Genomescope (Vurture et al., 2017). The data
134 generated form Pacbio SeqII was implemented to *De novo* assemble with
135 the software MECAT2 (Xiao et al., 2017) and polished by the software
136 NextPolish (Hu et al., 2019) combining the illumina short read data
137 mentioned in survey.

138 ***Hi-C experiment and chromosome assembly construction***

139 To further anchor the contigs to chromosomes, fresh young leaves was
140 utilized to construct a Hi-C library using a NEBNext Ultra II DNA Library
141 Prep Kit with the enzyme HandIII. 78.71 GB of clean data was generated
142 for Hi-C analyses by the Illumina NovaSeq 6000 platform. For the
143 anchoring of contigs, the mounting process was conducted by the pipeline
144 Juicer (Durand et al., 2016) and 3D-DNA (Dudchenko et al., 2017).
145 Software Juicerbox (Durand et al., 2016) was implemented to visualization
146 and adjustment.

147 ***Genomic assembly quality assessment***

148 Multiple methods were implemented to assess the accuracy and
149 completeness of the assembled genome. First, the paired-end reads were
150 mapped to the genome to evaluate its completeness using BWA-MEM with
151 the default parameters (Li, 2013). RNA-seq data from different tissues
152 (leaves, stalk, and root) were also aligned to the reference genome to obtain
153 the mapping rate using HISAT2 with the default settings (Kim et al., 2015).
154 Second, GC depth scatter plots were used to evaluate any contamination in

155 the sequencing data. Finally, the accuracy and completeness of the genome
156 assembly were evaluated by using BUSCOs to identify the single-copy
157 genes in the assembled genome with the Embryophyta_odb10 database
158 (Simão et al., 2015).

159 ***Gene prediction, Repeat element identification***

160 The sequence of repeat element in *C. burmannii* genome was *de novo*
161 identified by software Repeatmodeler (Flynn et al., 2020) and masked by
162 Repeatmasker (Chen, 2004). The detection and taxonomy about the LTR-
163 RTs was accomplished by LTR_Finder (Xu and Wang, 2007), LTR_harvest
164 (Ellinghaus et al., 2008) and LTR_retriever (Ou and Jiang, 2018).

165 To generate gene annotation of *C. burmannii* genome, software Augustus
166 was utilized in *de novo* gene prediction. A total of 65.74 GB of
167 transcriptome data from roots, stems, and leaves were alignment to the *C.*
168 *burmannii* genome by Hisat2 and reassembled into transcripts by Stringties
169 (Pertea et al., 2015) and Transdecoder (Fangping et al., 2021; Kim et al.,
170 2015), which generated the site information of transcriptome. The protein
171 sequences of *C. micranthum* previously published (Chaw et al., 2019) were
172 utilized for the homologous gene prediction by GeMoMa (Keilwagen et al.,
173 2019). The information of *de novo* gene prediction, transcriptome and
174 homologous gene prediction was combining by the software EVM (Haas
175 et al., 2008). PASA and the transcripts generated by Transdecoder in
176 previous step were utilized to update the gff3 file for three rounds to add

177 alternatively spliced isoforms, adjusting the gene structure and add UTR
178 regions to gene models (Haas et al., 2008).

179 ***The Annotation of Gene function***

180 Functional annotation was achieved by using NCBI BLAST + v 2.2.2854
181 (Altschul et al., 1990) with cutoff e-values of 1e-5 and max target
182 sequences 20 to compare predicted proteins against public databases,
183 including SwissProt, Nr (Plant). The Best-hit BLAST results were then
184 considered as the gene functions. Gene Ontology (GO) identifiers for each
185 gene were obtained using InterProScan-5.25-64.0 by identify motifs and
186 domains by matching against public databases (Zdobnov and Apweiler,
187 2001). The predicted proteins were also submitted to KAAS to get KO
188 numbers for KEGG pathway annotation (M and S, 2000).

189 ***Phylogenetic analysis and Estimation of divergence time***

190 MAFFT v.7.271 (Katoh and Standley, 2013) with option --maxiterate 1000
191 was used to align 10 sets of Coding DNA Sequence (CDS) of 230 single-
192 copy orthologous groups. Each orthologous group alignment was used to
193 compute a maximum likelihood phylogeny using iqtree with the nucleic
194 acid substitution model GTR+F+R4 and 1000 bootstrap replicates
195 (Nguyen et al., 2015). The divergence time of each tree node was inferred
196 using MCMCtree of the PAML (Yang, 2007). The final species tree with
197 *Amborella trichopoda* as out-group and the concatenated translated
198 nucleotide alignments of 230 single-copy orthologues were used as input

199 of MCMCtree. The phylogeny was calibrated using various fossil records
200 or molecular divergence estimated by placing soft bounds at split node of
201 (MYA): *A. trichopoda* and *O. sativa* ('>1.73<1.99'), *D. carota* and *Salvia*
202 *splendens* ('>0.95<1.06'), *Arabidopsis thaliana* and *Populus trichocarpa*
203 ('>0.98<1.17') obtained from the TimeTree database (Kumar et al., 2017).

204 ***Synteny, Positive selection genes and WGD anaylsis***

205 The genomic alignment between *C. burmannii* and *C. micranthum* was
206 finished and visualization by MUMMER (Kurtz et al., 2004). We
207 performed multiple sequence alignment to extract the conserved paralogs
208 of the protein sequences of this data by using BLASTP (E-value ≤ 1e−5).
209 MCScanX was used to identify collinearity blocks, and Ks values were
210 calculated by software WGDI to identify and extract collinear interval and
211 corresponding gene pairs (Sun et al., 2021). The calculation of Ks with the
212 method YN00 was implemented to predict the Whole Genome Duplication
213 (WGD) events

214 ***Transcriptome profiling analysis***

215 Pipeline Hisat2 was reads alignment of the reads from RNA-seq and the
216 read counts matrix was generated by software featureCounts (Liao et al.,
217 2014) for the quantitative analysis expression. R package DESeq2 was
218 implemented to normalize the read counts data and identified the DEGs
219 between different organs with Pvalue ≤ 0.01 and an absolute log2 (fold
220 change) ≥1 as the threshold (Love et al., 2014). Software STEM was

221 utilized to the cluster analysis DEGs expression and GO enrichment
222 analysis (Ernst and Bar-Joseph, 2006).

223 ***Tps gene family anaylsis***

224 Proteome data sets including *C. burmannii* and other four sets *C.*
225 *micranthum, M. acuminate, O. sativa* and *D. carota* from NCBI were
226 utilized in TPS gene family analysis. Pfam domains: PF03936 and
227 PF01397, were used to identify against the proteomes using HMMER
228 (version 3.0; cut-off at e-10) (Finn et al., 2010; Finn et al., 2011). Putative
229 or annotated protein sequences of *TPS* were aligned using MAFFT with
230 default parameters and manually adjusted using MEGA. The TPS gene tree
231 was constructed using iqtree with 1,000 bootstrap replicates. The
232 subfamily *TPS*-a was designated as the out-group. Branching nodes with
233 bootstrap values of <80% were treated as collapsed.

234 ***Prediction of protein structure modeling and molecular docking***

235 Homology modeling was performed with Rosettafold with default *de novo*
236 prediction parameter (Baek et al., 2021). The results were inspected and
237 rendered with PyMOL (DeLano, 2002). Protein docking and binding
238 energy calculation were done with AutoDock Vina using local search
239 parameters and default docking parameters (Trott and Olson, 2010). The
240 alignment and the visualization of amino acid sequences was finished by
241 platform ClustalW and ESPript (Gouet et al., 1999).

242 ***Data and code availability***

The sequencing data, including the assembly data of *C. burmannii* was submitted to the National Genomics Data Center (NGDC) under BioProject accession number PRJCA006286. All the assembly and annotation data also can be accessed from CAMD (http://www.cinnamomumdatabase.com/)

Customized codes used in this study have been deposited in GitHub https://github.com/lifangpings/CB_genome.

Real-time Quantitative PCR (q-PCR) comfirmation

For q-PCR assays, the total RNA was extract using Aurum Total RNA Mini Kit (BIO-RAD) and cDNA was obtained by thermo scientific RevertAid First Strand cDNA Synthesis Kit according to the supplier's protocols respectively. All the primers' sequence was obtained from the National Centre for Biotechnology Information (NCBI) database. According to kit instruction 10 μl reaction system was prepared and q-PCR was operated using CFX96 Real-Time System (BIO-RAD). For data analysis, all detected genes expression were normalized against Actin-F (CBB03T203270) of *C. burmannii*.

Database construction.

All genomic sequence, annotation, were stored via MySQL on a Centos7 server. A user-friendly website was developed using HTML5, JavaScript and PHP7, which can be accessed through different browsers, such as Google Chrome and Firefox. Gene models and transcript isoforms were

265 provided via JBrowse (Skinner et al., 2009; Buels, R. et al., 2016). The
266 query searches were achieved via PHP7. Common utilities for genomic
267 studies such as BLAST, and Sequence exactor were also deployed and
268 accessible.

269 **Result**

270 ***Genomic assembly***

271 To evaluate the genome size and heterozygosity of *Cinnamomum*
272 *burmannii* (chvar. Borneol), a total of 61.4 GB short reads from the
273 MGISEQ-2000 sequencing platform were subjected to K-mer analysis.
274 The 17-mer frequency curve showed a unimodal distribution, with the
275 highest peak occurring at a depth of 54 (Supplementary Fig. S1a). Based
276 on the total number of K-mers, the genome size and heterozygosity of *C.*
277 *burmannii* (chvar. Borneol), were estimated to be 0.94-1.25 GB and
278 ~0.67%, respectively (Supplementary Fig. S1b; Supplementary Table. 1).
279 A total of 170.1 GB single molecule sequencing long read data with the
280 average length about 32 kb (N50=21.38kb) was utilized in the genomic *de*
281 *novo* assembly. After polishing combining the short-read data and
282 removing redundant/contaminated sequences, the size of the final contig
283 was 1.15 GB with a contig N50 of 1.13Mb (Table.1). A Hi-C interaction
284 heatmap indicated 12 highly obvious interacted range of the signal which
285 is corresponding with the previous research about the chromosome number
286 of *C. burmannii* (chvar. Borneol) (Supplementary Fig. S2). Based on the

287 signal, a total of 98.77% of the assembly was anchored to 12 pseudo-
288 chromosomes. The contig sequences were connected in the determined
289 order and direction by adding 100 N to obtain the final chromosome-level
290 genome sequence with a chromosome mount rate of 98.77% the size
291 ranged from 66.1Mb to 140.5Mb, and with Scaffold N50 = 94.90Mb. The
292 ordering, and orientation of the contigs was also arranged, providing the
293 first high-quality chromosome-scale genome assembly for *C. burmannii*
294 (Table.1).
295 Multiple method was utilized for the quality assessment of the novel *C.*
296 *burmannii* genome. The method of Benchmarking Universal Single-Copy
297 Orthologs (BUSCOs) was implemented to evaluate the assembly quality
298 of *C. burmannii* genomes, which identified 89.7% of the complete
299 BUSCOs in the assembly by using the reference of the conservative
300 database of dicotyledons (Supplementary Table S2). For the assessment of
301 genomic consistency, the short reads were mapped to the genome using
302 bwa software. This analysis indicated ~99.78% of the clean data were
303 mapped to the genome assembly. The mapping ratio of the RNA-seq reads
304 from different tissues was in the range of ~92.08%–94.93%. The GC
305 content of this genome is 39.02% while the sequencing depth was
306 concentrated at 110–150×. All these methods of assessment elucidated a
307 high completeness and consistency of the *C. burmannii* genomic assembly
308 (Table.1).

309 ***Repeat element identification and Genome annotation***

310 The *de novo* prediction based on RepeatModeler indicated 673.32 Mb of
311 repetitive sequences, accounting for 63.24% of the assembled genome.
312 Combining the result of *de novo*, homolog-based, and transcriptome-based
313 predictions, a total of 41549 non-redundant protein-coding genes were
314 generated across 12 chromosomes (Supplementary Fig. S3). The average
315 gene length and coding sequence size were 2962.35 and 1142.14 bp,
316 respectively, with an average of five exons per gene. The result of function
317 annotation indicated that 37799 genes (90.97 %) was annotated in at least
318 one pathway of the public database. Further functional annotation using
319 InterProScan estimated that of the genes 33582 (80.82%) contained
320 conserved protein domains recorded by Panther and Pfam database. 54.38%
321 of the genes could be classified with Gene Ontology (GO) terms. 8401
322 (20.22%) of the genes mapped to known plant biological pathways based
323 on the KEGG Pathway database.

324 ***Phylogenetic analyses***

325 To identify the phylogenetic relationship and position of *C. burmannii* and
326 *C. micranthum* we constructed a phylogenetic tree based on 230 strictly
327 single-copy orthologous sets. Consistent with expectations, phylogenetic
328 tree indicated clustering of two species of *Cinnamomum* on one branch.
329 The maximum likelihood (with a high bootstrap index) and MCMCtree
330 with fossil calibrations analysis indicated that the divergence between

331 magnoliids and eudicots tended to happen in 201 Mya (95% confidence
332 interval: 196.1-209.1). The time of divergence between *C. micranthum* and
333 *C. burmannii* to be 8 Mya (95% confidence interval: 7.5-8.5) (Figure .1).

334 ***LTR-RTs promote species differentiation among Cinnamomum***

335 In the identification of LTR-RTs for these two species, a total of 186.62Mb
336 (25.1% of the whole genome) LTR-RTs sequence was detected in *C.*
337 *micranthum* while 459.42Mb (40.59% of the whole genome) was detected
338 in *C. burmannii* (Figure. 2a and Supplementary Table S2). The insert time
339 analysis elucidated these two species experienced a significant recent
340 outbreak of LTR-RTs insertions. However, much more insertions of LTR-
341 RTs were speculated to occur in 0-8Mya in *C. burmannii* than in *C.*
342 *micranthum,* which was also corresponding with the divergent time
343 between these two species. These LTR-RTs were also evenly distributed in
344 the genome of *C. burmannii* (Figure. 2b).

345 This ~250 Mb length difference of LTR-RTs tends to be a preliminary
346 explanation for the interspecific genome size differences in *C. micranthum*
347 and *C. burmannii* genera.

348 ***Whole-genome duplication and Synteny analysis***

349 To more precisely infer the timing of the two rounds of WGD evident in
350 the *Cinnamomum,* the intragenomic and interspecies homologue *Ks*
351 (synonymous substitutions per synonymous site) distributions were
352 estimated with the genome of *C. micranthum, C. burmannii* and *A.*

353 *coerulea* (as outgroup). The curve of *Ks* distribution indicated that two
354 peaks at ~0.5 to 1.5 in both *C. micranthum* and *C. burmannii* genomes,
355 which elucidated these two species shared the same two ancient WGD
356 events (Two ancestral WGD events endemic to Lauraceae before
357 divergence of eudicots and magnoliids) before their divergence and no
358 recent independent WGD event was detected (Figure. 2c).
359 Consistent with the predictability, both the results of whole genome
360 comparison and gene collinear comparison between *C. micranthum* and *C.*
361 *burmannii* indicated an obvious synteny presence (Figure. 3a and
362 Supplementary Fig. S4). Based on the collinearity of *C. burmannii* and *C.*
363 *micranthum* genomes, we sorted and renamed *C. burmannii* genome
364 sequences. We found that the relative proportions of chromosome sizes
365 were consistent between the two species (Table. 2). Besides, large
366 fragments of chromosomal translocations were displayed in Chr2, Chr 4,
367 Chr5, and Chr12, while a large fragment inversion occurs in front of Chr6
368 in comparison between this two *Cinnamomum* species. This phenomenon
369 suggested that the translocations and inversion of chromosomes might
370 have been occurred during the process of *Cinnamomum* species
371 differentiation.
372 ***Characteristic analysis of genes showing organ-specific expression***
373 A total of 31537 genes were detected to expression in the organs of *C.*
374 *burmannii*. The gene expression levels analysis indicated that 1318, 1208,

375 672 genes expressed explicitly in roots, stalks, leaves respectively, while
376 24681 genes were expressed in all tissues (Figure. 3b).
377 To elucidate the similarities and differences of gene expression patterns in
378 different tissues, we performed a k-means cluster analysis. A total of 20444
379 differentially expressed genes (DEGs) were divided into 10 clusters. We
380 focused on clusters containing tissue-specific expressed genes, particularly
381 those that were stable in roots and stalks while increased in leaves (3348
382 genes in Cluster1) (Figure. 3c). Apart from the genes involving in
383 photosynthesis, genes in the terpene synthase (*TPS*) activity pathway (GO:
384 0010333) were shown to be expressed and enriched in leaves, which was
385 consistent with the higher content of terpenoids in leaves including D-
386 borneol in *C. burmannii* reported by previous studies. Besides, a number
387 of methylation related genes (GO: 0004222 and GO: 0008173) were also
388 significantly increased and enriched in leaves. This phenomenon suggested
389 the regulation associated with epigenetic may play a role in development
390 and compound synthesis in the leaves of *C. burmannii* (Figure. 3d).
391 ***TPS gene family analysis***
392 Similar to another plant of *Cinnamomum, C. micranthum*, a large number
393 of *TPS* genes (*CbTPS*) located in the genome of *C. burmannii.* A total of
394 73 *CbTPS* genes were predicted and annotated. Phylogenetic analysis of
395 *CbTPS* placed these genes into six *TPS* gene subfamilies that have been
396 described for seed plants. Among this *CbTPS* genes, 1 and 2 were placed

397 in the *TPS-c* and *TPS-e* subfamily respectively. These two subfamilies have
398 been reported to encode diterpene synthases like copalyl diphosphate
399 synthase and *ent*-kaurene synthase (Chaw et al., 2019). These are key
400 enzymes catalyzing the formation of the 20-carbon isoprenoids
401 (collectively termed diterpenoids; C20s). The remain *CbTPS* genes
402 attributed to *TPS*-a, *TPS*-b, *TPS*-f and *TPS*-g, which probably encode the
403 10-carbon monoterpene (C10) synthases, 15-carbon. With 29 and 23
404 homologues, respectively, *TPS*-a and *TPS*-b subfamilies are most diverse
405 in *C. burmannii*, presumably contributing to the mass and mixed
406 production of volatile C15s and C10s, including D-borneol (Figure. 4 and
407 Supplementary Table S4).
408 Besides, *CbTPS* genes are not uniformly distributed throughout the
409 chromosomes and clustering of members from individual subfamilies was
410 observed as tandem duplicates. Among these genes, forty three copies
411 (58.9%) of *CbTPS* genes belonging to different subfamilies were found in
412 the 53–59Mb region of chromosome 6, and 56–61Mb region of
413 chromosome 9. Two to nine *TPS* family genes were detected in tandem on
414 chromosomes 1, 3, 4, 7, 10 and 11 while only one gene of *TPS* family was
415 detected in chromosomes 5. chromosomes 7 contains 29 *CkTPS* genes
416 belonging to several subfamilies, including all of the eight *CkTPS*-a, 12
417 *CkTPS*-b, five *CkTPS*-e and three *CkTPS*-f Genes belonging to this cluster
418 were not grouped together in their corresponding subfamily phylogen,

419 suggesting that their arrangement might have occurred more recently than
420 the last WGD event (Supplementary Fig. 4b).

421 ***Identification of genes related to the D-borneol biosynthesis pathway***

422 Previous research indicated that MVA pathway play a key role in the
423 upstream biosynthesis pathway of D-borneol. Our gene annotation results
424 indicated that all MVA pathway genes (*CbERG10, CbERG13, CbtHMG1,*
425 *CbERG12, CbERG8, CbERG19, CbIDI1, CbERG20*) distributed in 12
426 chromosome of *C. burmannii.* The transcriptome analysis elucidated the
427 expression level of genes *CbERG20, CbIDI-1,* was significantly elevated
428 in leave comparing to stem and root. *CbERG20* is the key enzyme to
429 promote the synthesis of GPP, which is a directly related raw material of
430 D-borneol synthesis.

431 Besides, MEP pathway also have been reported to participate in the
432 precursor GPP formation in the D-borneol formation process. In this
433 pathway, the expression of multiple genes such as *CbCMK, CbMCS* and
434 *CbHDR* showed similar expression pattern with above key genes in MVA
435 pathway. (Figure. 5).

436 For the downstream pathway, *CbTPS-1* (*CBB06T352390*), encoded by a
437 gene of *TPS*-b gene subfamily, has been reported to directly mediate the
438 transformation of GPP to D-borneol (Ma et al., 2021). The expression
439 levels of these key genes for D-borneol synthesis were much higher in
440 leaves than in stem or root implied they may be the key or the limiting

441 factor in the biosynthesis of D-borneol in *C. Burmannii* leaves (Figure. 5).
442 Gene cloning and qPCR analysis to above potential key genes were further
443 conducted, and results are highly consistent with our transcriptomic and
444 gene annotation results, indicating the accuracy and reliability of our
445 results (Supplementary Fig. S6).

446 ***Sequences differences cause configuration alteration of the docking site***
447 ***of CbTPS-1 and CkTPS-1***

448 The 3D structure model of protein generated by Rosettafold was
449 implemented to model the protein structure of *CbTPS-1* and its highly
450 homologous *CkTPS-1*(*RWR98053.1*) in *C. micranthum*, and molecular
451 docking analysis with small micromolecule GPP was also performed. This
452 analysis showed an obvious GPP binding pocket in the middle of these two
453 *TPS* proteins folding structure (Figure. 6a). The GPP docking pocket
454 located in the ~300aa to ~500aa of these two TPS protein. A total of 26
455 amino acid mutations were detected in this region. Among these, 15
456 mutations changed the polarity of the amino acids at that location, which
457 have potential to affect their protein folding (Figure. 6b). Therefore, due to
458 folding differences caused by changes in amino acid sequence, GPP can
459 form more hydrogen bonds with more A-helices (4 > 1) in *CbTPS-1*
460 binding pockets (9 > 8) and its binding energy in *CbTPS-1* is also higher
461 than that of *CkTPS-1* (-7.7<-7.4). The higher binding capability of *CbTPS-*
462 *1* may cause of higher D-borneol production in *C. Burmannii* than other

463 These differences may be the main cause of *C. Burmannii* to produce
464 higher D-borneol than other *Cinnamomum.* plants (Figure. 6c).

465 ***Construction of genome database of Cinnamomum species***

466 To meet demand for pitaya genome and multiomics data resources, we
467 established an integrated pitaya genome and multiomics database (CAMD;
468 http://www.CinnamomumDatabase.com). The CAMD provides a
469 comprehensive consultation service including genomic blast, gene browser
470 and sequence fetch with the data from the latest assemblies of *C. burmannii*
471 and *C. micranthum* genomes (Chaw et al., 2019) (Figure. 7).

472 **Discussion**

473 *Cinnamomum*, a genus of about 250 species of the Lauraceae, is widely
474 distributed in tropical Asia, Australia to Pacific islands, and tropical
475 America. The plants of *Cinnamomum* are rich in volatile oil, terpenoids,
476 tannins and their analogues, aromatic compounds and polysaccharides.
477 Multiple species of this genus are important raw materials in chemical
478 industry and medicine. However, the only one assembled genome in this
479 species is *C. micranthum* (Chaw et al., 2019) . There are few reports on
480 further genome research of *Cinnamomum* genus. The lack of high-quality
481 genome and detailed genomic information has limited our understanding
482 of molecular mechanisms underlying valuable natural product and genetic
483 improvement of these plants.

484 In the present study, we assembled a chromosomal scale, high-quality

genome of *Cinnamomum burmannii* (chvar. Borneol), as well as detailed genomic and transcriptomic information. Compared with the first published genome of *Cinnamomum species, C. micranthum,* the assembly of *C. burmannii* achieved much higher quality, such as a longer sequence length of contigs (1.14 Mb > 498.92Kb) and a higher chromosome mount rate (98.31% > 90.1%) with a larger genomic size (1.13Gb > 730.7 Mb). These improvements suggested that the assembly of *C. burmannii* tends to be a valuable and better reference genome for future genomic study of *Cinnamomum* species.

The construction of the *C. burmannii* genome and the comparative genome analysis also shed light on the understanding of genome evolution of Lauraceae. Our results confirmed that Lauraceae and camphor plants shared two ancestral WGD events. As the representative of magnoliid, the phylogenetic relationship analyses of *C. burmannii* also resolve magnoliids to be closer to eudicots than to monocots. Furthermore, the outbreaks of LTR-RTs have been considered to be an important impetus of increasing genetic diversity of multiple species (Sun et al., 2020; Lin et al., 2018). The combination of LTR-RTs size and insertion time indicated that recent LTR-RTs insertion events also promoted the differentiation between species *C. micranthum* and *C. burmannii* in *Cinnamomum.*

Our assembled genome and transcriptomic analysis results also help to elucidate the molecular mechanism of borneol biosynthesis. As a medicinal

507 plant, *C. burmannii* (Chvar. borneol) is an important source of D-borneol.
508 D-borneol, which is also called natural borneol, is one of the most famous
509 Chinese traditional medicines. It has the effect of awakening, clearing heat
510 and relieving pain. It is often utilized popularly for fever, dizziness,
511 convulsion, stroke, phlegm, sore throat, purulent ear canal and other similar
512 symptoms. Since its discovery in the 1980s, *C. Burmannii* (Chvar. borneol),
513 which is rich in natural borneol, has been widely grown as a medicinal crop
514 in city Meizhou in Guangdong province of China. It is very important to
515 fully elucidate the molecular pathway of D-borneol biosynthesis for further
516 natural plant utilization and future synthetic biology application. Ma et.al
517 had reported the reconstitution of D-borneol biosynthetic pathway in
518 *Saccharomyces cerevisiae*, which is a critical step for borneol biosynthesis
519 application. However, genes responsible of borneol synthesis, and theirs
520 underlying regulation mechanisms in plants are still largely unknown.
521 With the help of high-quality reference genome of *C. burmannii* (Chvar.
522 borneol) assembled in the present study, we were able to fully dissect the
523 natural borneol synthesis pathway from a genome scope. Previous studies
524 have confirmed MVA pathway is the key pathway for the synthesis of GPP,
525 which serve as the precursor of D-borneol (Ma et al., 2021). The MEP
526 pathway has also been reported to be involved in GPP synthesis in plants
527 (Banerjee and Sharkey, 2014). The transformation of GPP and D-borneol
528 was completed with the participation of *TPS* family, while *CbTPS-1* has

529 been confirmed to participate in D-borneol biosynthesis directly (Ma et al.,
530 2021). In the present study, all genes participate in the both natural borneol
531 biosynthesis pathway were identified in *C. burmannii* (Chvar. borneol)
532 genome. Transcriptomic analysis showed key genes in both pathways tends
533 to highly expressed in leaves, rather than in stem or root. All these results
534 provided important genomic information of genes for the following
535 research about D-borneol biosynthesis in *C. burmannii*.
536 Furthermore, the analysis of *TPS* gene family elucidated multiple *TPS* gene
537 clusters were detected in *C. burmannii* genome, especially in Chr6 and
538 Chr9. Recent studies have shown that several plants biosynthetic gene
539 clusters (BGCs) of natural products exist in plant genomes. These plant
540 biosynthetic gene clusters generally participate in the process of natural
541 product synthesis synergistically (Liu et al., 2020; Mao et al., 2020). In the
542 present study, the function and mechanisms of BGCs formed by *TPS*
543 family genes are worth more profound exploration.
544 Additionally, our transcriptomic analysis further identified genes
545 participate in methylation process were significantly enriched in genes
546 specific expressed in leaves, but not in stem or root. Several researches
547 have reported the epigenetic processes, such as DNA or RNA methylation
548 played important roles in the synthesis of secondary metabolites in
549 multiples species (Zuo et al., 2020; Wang et al., 2019). Leaves is the major
550 organ for borneol synthesis in *C. burmannii*. Together with the results that

551 expressions of genes of borneol biosynthesis pathway were much higher in
552 leaves than in stem and root, and the results that methylation related genes
553 enrichment in leaves specific expression genes, implied methylation may
554 play important roles in the expression regulation of gene related to borneol
555 synthesis in *C. burmannii* leaves. Methylation and gene expression
556 regulation may be further leverage as target for borneol production
557 improvement in *C. burmannii* (Chvar. borneol). The mechanisms behind
558 these results need further investigation.
559 In the present study, we also constructed a genome database (CAMD),
560 which combined our results of *C. burmannii.* and genome information from
561 another *Cinnamomum* plant, *C. micranthum.* (Chaw et al., 2019). CAMD
562 is the first database of the *Cinnamomum* and will become an excellent
563 central gateway to better understand the biology and genetics of Lauraceae.
564 The release of CAMD will benefit for studies of genetic diversity and
565 improvement in *Cinnamomum*.

566 **Conclusion**

567 Here we presented a high-quality complete genome, as well as detailed
568 genomic information of *C. burmannii* (Chvar. borneol). The high-quality
569 genome provides novel insights about genome evolution of *C. burmannii* ,
570 as well as *Cinnamomum* genus. With the help of assembled and annotated
571 genome, we dissected the pathways of borneol synthesis, and identified
572 key genes in these pathways. Transcriptomic analysis further shed light on

573 the further investigation of regulation and molecular mechanisms of these
574 pathways. We also constructed the genome database (CAMD) of
575 *Cinnamomum* species for better data utilization in the future. The
576 availability of *C. burmannii* complete genome and its genomic information
577 from this work will provide a valuable genome resource for understanding
578 the evolution, active ingredient biosynthesis, and genetic improvement for
579 not only *C. burmannii* itself, but also *Cinnamomum* genus.

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589 **Author contributions**

590 LF, ZJ and WJ conceived and designed this research. LF, HS, XS, WB, HZ,
591 ZP, MY, ZJ, LQ, HZ and WJ performed the experiments. LF, HS, WB, ZJ
592 and WJ prepared the article.

593 **Competing interests**

594 The authors declare no conflict of interest.

Figure

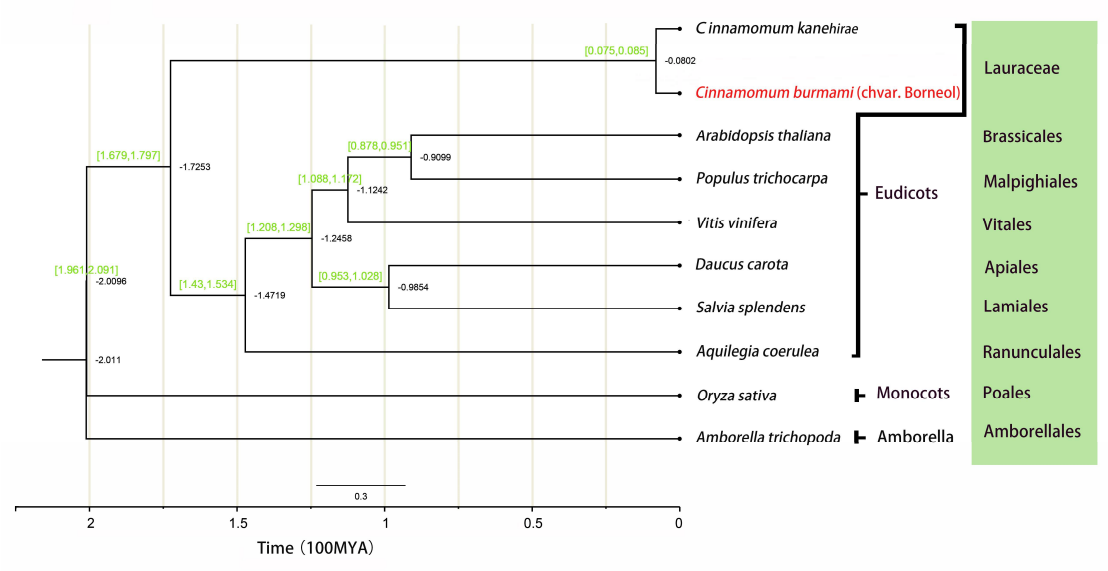


Figure.1

The phylogenetic relationship of *C. burmannii, C. micranthum* and other eight plant species of different family. The green numbers in the brackets denote divergence time estimates. All nodes' bootstrap support was 100 unless stated otherwise.

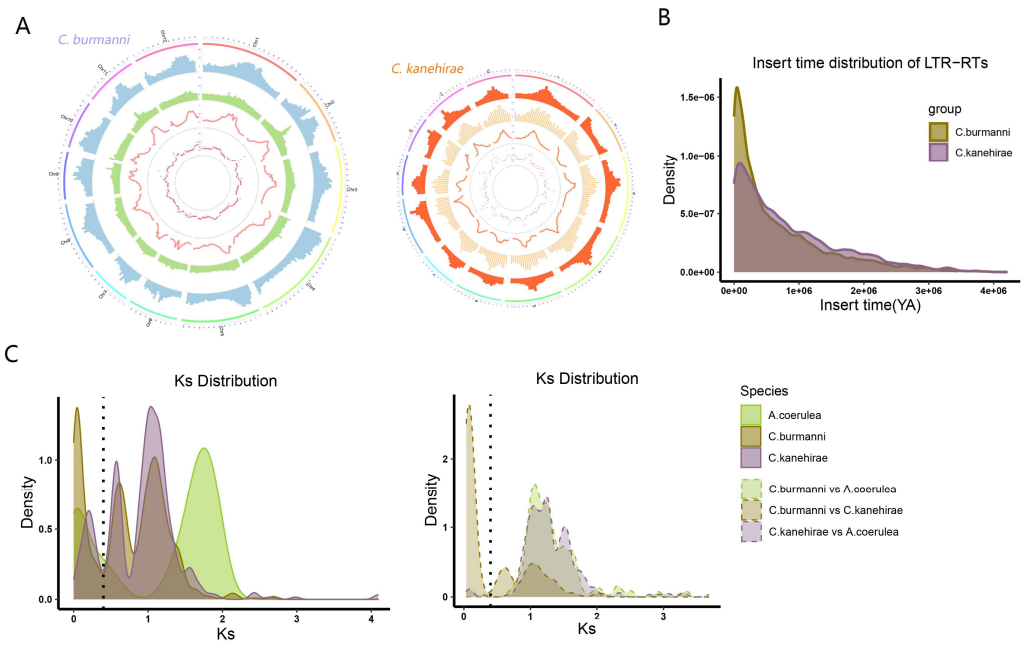


Figure. 2

The distribution and insertion times of the LTR-RTs in *C. burmannii* and *C. micranthum* genomes as well as the WGD event identification. **A:** LTR-RTs Characterization of the *C. burmannii* and *C. micranthum* genome; the circle from outside to inside represents chromosomes LTR-Copia density, LTR-Gypsy density, Total LTR density and GC content All distributions are drawn in a window size of 3 Mb. **B:** Quantity and time distribution of LTR-RTs **C:** Ks distribution among *C. burmannii*, *C. micranthum* and *A. coerulea* Lines show Ks distribution within (continuous) and between genomes (dashed).

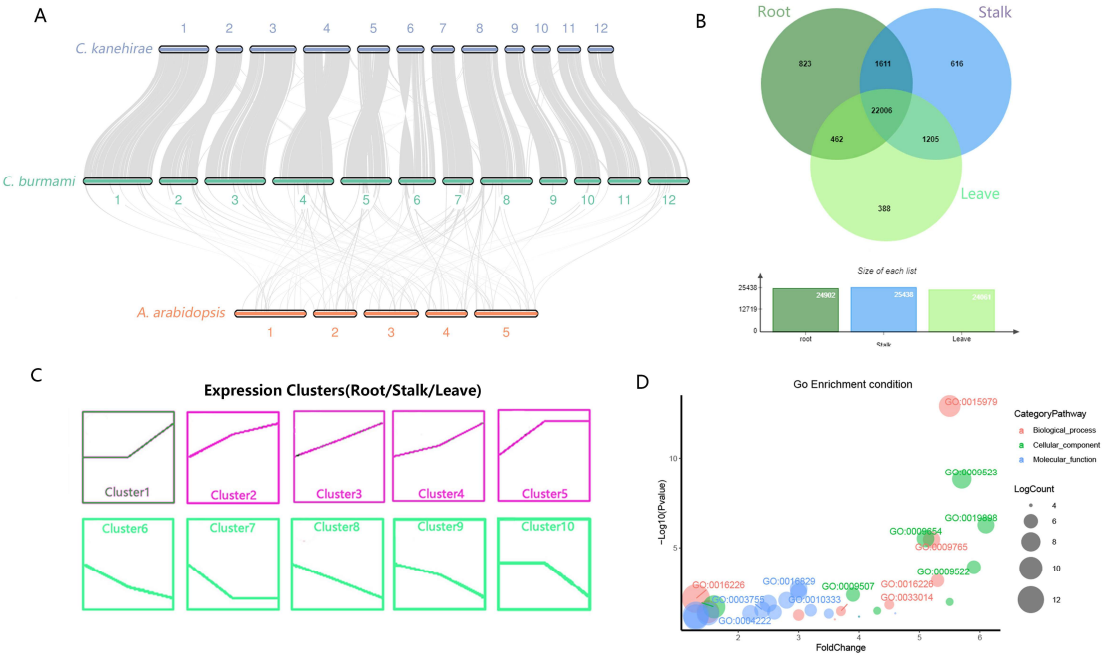
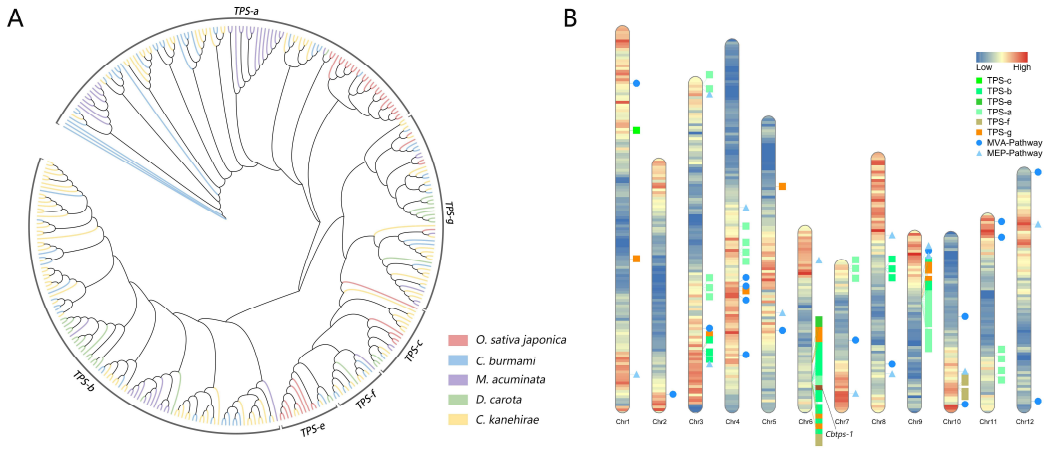


Figure. 3

Collinearity analysis between *C. burmannii, C. micranthum* and *A. Arabidopsis* as well as the gene's organ-specific expression of different organs *of C. burmannii*. **A:** Gene Collinear relationship of *C. burmannii*

619 and *C. micranthum* and *A. arabidopsis*. The gray line connects matched
620 gene pairs. **B:** The Venn diagram visualized the expressed genes in roots,
621 stalks, and leaves. The overlapping regions represent genes expressed in at
622 least two tissues, while the non-overlapping regions represent tissue-
623 specific genes. **C:** Hierarchical clustering showing the expression patterns
624 of DEGs in roots, stalks, and leaves.

625



626

627 **Figure. 4**

628 The gene density and the gene distribution involving in *TPS* gene family
629 and D-borneol formation; Phylogenetic placements of the 73 *TPS* family
630 genes of *C. burmannii*. **A:** The phylogenetic tree was constructed using
631 putative or characterized *TPS* genes from 5 plant genomes including *C.*
632 *burmannii*. **B:** Gene density distribution as well as the location of the gene
633 involving in *TPS* gene family and D-borneol formation.

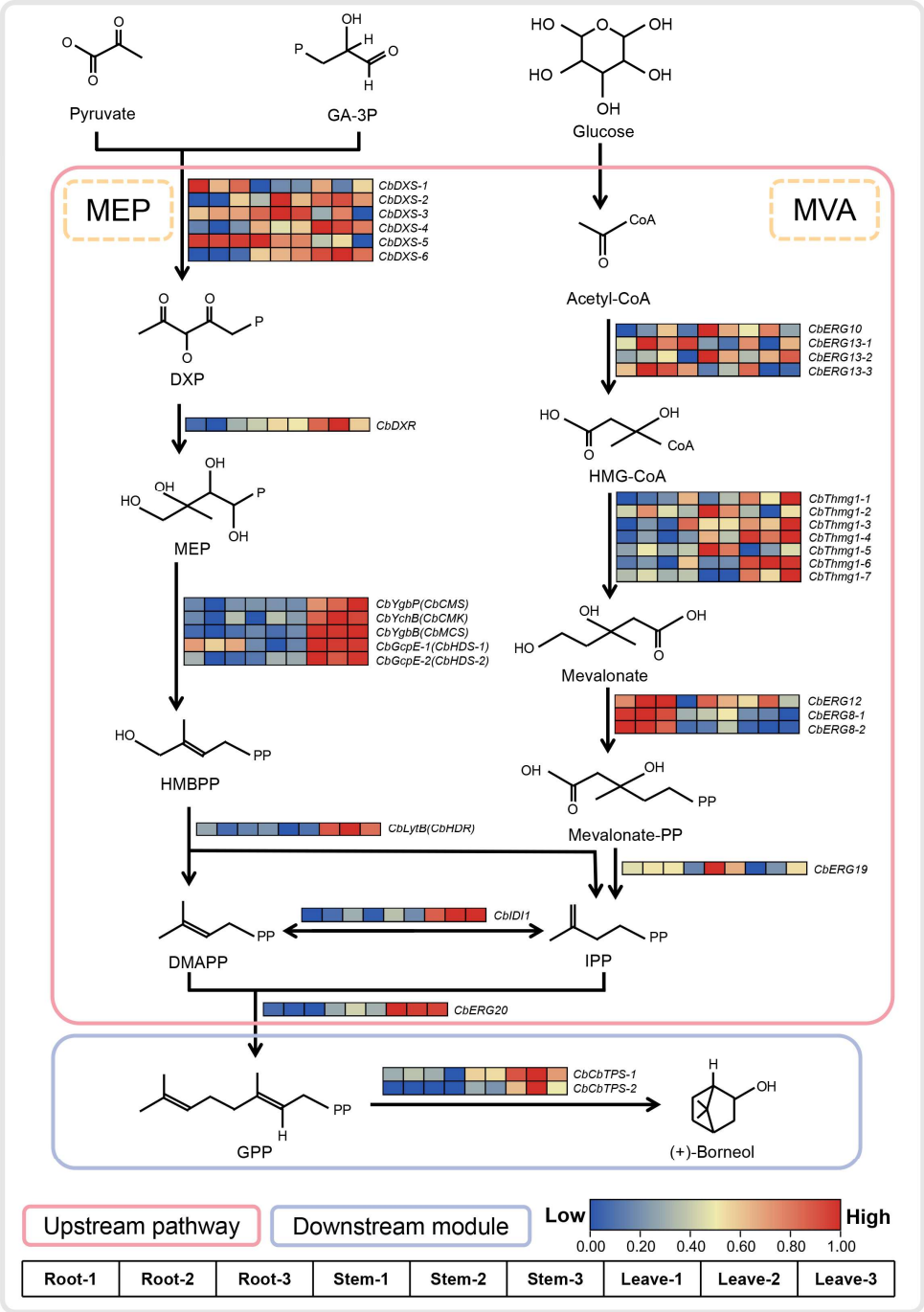


Figure. 5

Identification and expression analysis of genes involved in MVA and MEP pathway for key material GPP production (pink ellipses) nd the biosynthetic pathway of D-borneol (blue ellipses). Heat maps show the expression of genes involved in these pathways

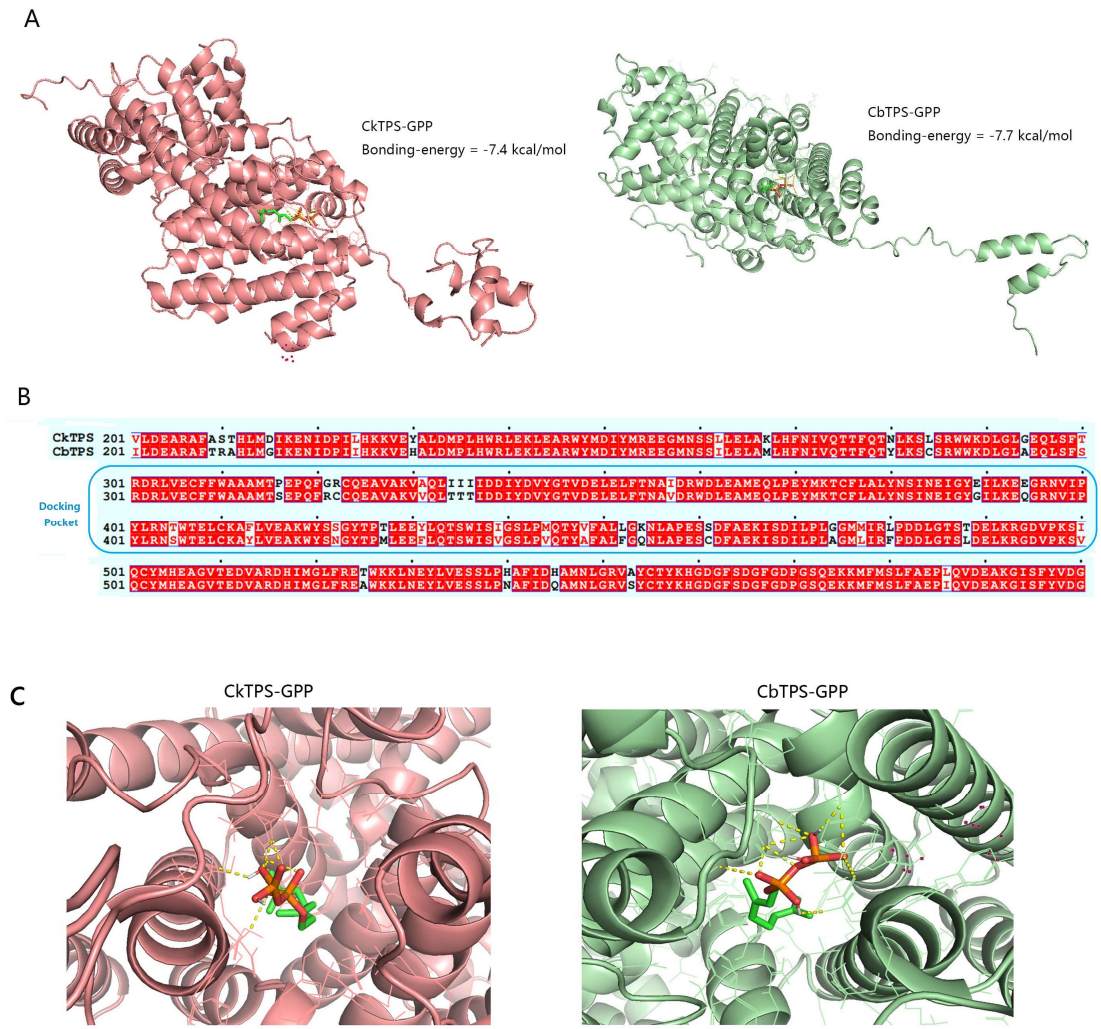


Figure. 6

Molecular docking result between *CbTPS-1* and its highly homologous gene *CkTPS-1* in *C. micranthum.* **A:** 3D structure of proteins and the overall docking situation (docking pocket position; *CkTPS-1*-GPP/*CbTPS-1*-GPP); **B:** The sequences comparison of Amino acid docking pocket position **C:** The details of docking (*CkTPS-1*-GPP/*CbTPS-1*-GPP).

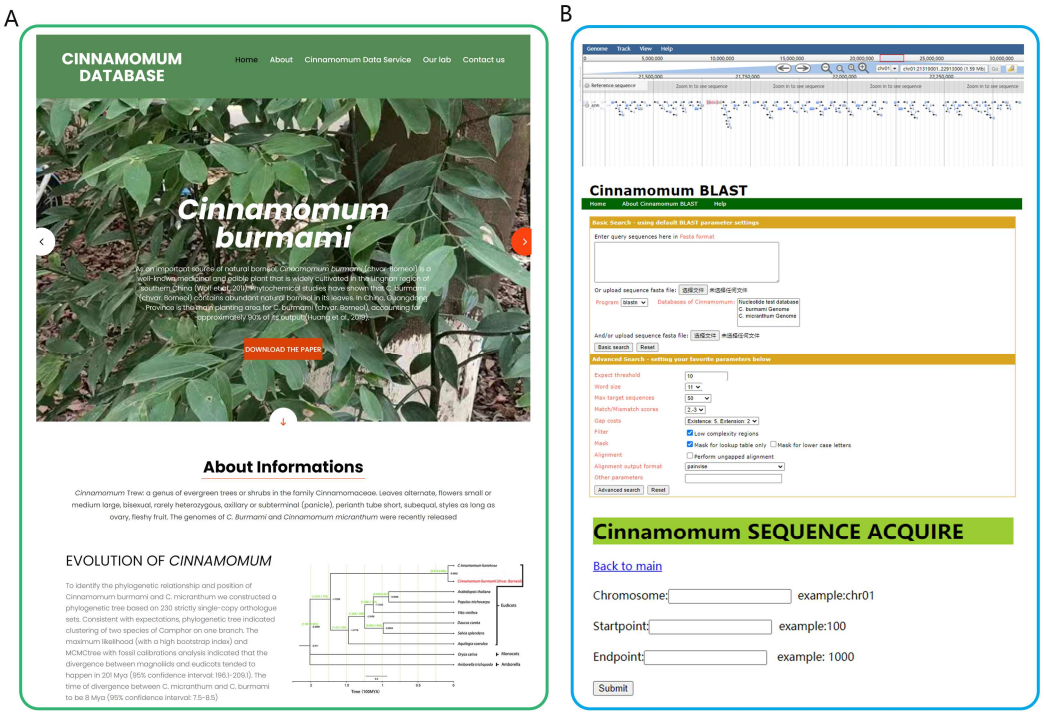


Figure. 7

The screenshots of representative resources and action page example of CAMD; **A:** Main pages of CAMD; **B:** Partial function pages displayed including Genomic blast, Gene Brower and sequence fetch.

653 **Table.1** Summary and comparison of *C. burmannii* and *C. micranthum*

654 assembly

Statistics without reference	*C. burmannii* (contig)	*C. burmannii* (Chromosome)	*C. micranthum* (Chromosome)
Contigs	4653	76	2150
Contigs (>= 50000 bp)	2109	76	268
Largest contig	5837401	140537727	87013940
Total length	1154926090	1132685329	730416403
Total length (>= 0 bp)	1154926090	1132685329	730416403
Total length (>= 50000 bp)	1115042729	1132685329	701509862
N50	1144013	94903400	50350591
N75	484956	72773248	45778809
L50	292	5	6
GC (%)	39.15	39.02	38.34

655

656 **Table.2** Statistics of the sizes of each chromosome of *C. burmannii* and

657 *C. micranthum*

Chromosome	*C. burmannii*	*C. micranthum*	Corresponding Chromosome No. (*C. micranthum*)
Chr1	140537727	87013940	QPKB01000001.1
Chr2	92646709	50350591	QPKB01000006.1
Chr3	122226000	81065106	QPKB01000002.1
Chr4	135862046	77926444	QPKB01000003.1
Chr5	108037362	58596509	QPKB01000005.1
Chr6	68311217	48047431	QPKB01000007.1
Chr7	56091752	39555212	QPKB01000011.1
Chr8	94903400	58831143	QPKB01000004.1
Chr9	66575986	41984183	QPKB01000010.1
Chr10	66169134	37069385	QPKB01000012.1
Chr11	72773248	45778809	QPKB01000009.1
Chr12	89292919	46631104	QPKB01000008.1

658

659

660 **List of Supplementary materials**

661 *Supplementary Figure*

662 Supplementary Figure.1 Evaluation of genome size and heterozygosity

663 Supplementary Figure.2 HI-C heatmap of *C. burmannii*

664

665 Supplementary Figure.4 Collinearity of genomic sequences between *C.*

666 *burmannii* and *C. micranthum*

667 Supplementary Figure.5 qPCR confirmation of partial gene involving in

668 D-borneol biosynthesis in *C. burmannii*

669 Supplementary Figure.6 Gene clone of *CbERG20* and *CbTPS-1* from *C.*

670 *burmannii*

671 *Supplementary Table*

672 Supplementary Table.1 Genomic survey result of *C. burmannii* generated

673 by software Gce

674 Supplementary Table.2 Busco assessment results of *C. micranthum*

675 Supplementary Table.3 Length and types Statistics of LTR-RTs

676 Supplementary Table.4 Numbers of *TPS* subfamilies in the 5 genomes

677 Supplementary Table.5 List about the gene in *TPS* family or involving in

678 D-borneol biosynthesis in *C. burmannii*

679 Supplementary Table.6 Sequence and List of primers in qPCR and gene

680 clone confirmation assays.

681

682

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