The chromosome-scale reference genome of black pepper provides insight into piperine biosynthesis

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Black pepper (Piper nigrum), dubbed the ‘King of Spices’ and ‘Black Gold’, is one of the most widely used spices. Here, we present its reference genome assembly by integrating PacBio, 10x Chromium, BioNano DLS optical mapping, and Hi-C mapping technologies. The 761.2 Mb sequences (45 scaffolds with an N50 of 29.8 Mb) are assembled into 26 pseudochromosomes. A phylogenomic analysis of representative plant genomes places magnoliids as sister to the monocots-eudicots clade and indicates that black pepper has diverged from the shared Laurales-Magnoliales lineage approximately 180 million years ago. Comparative genomic analyses reveal specific gene expansions in the glycosyltransferase, cytochrome P450, shikimate hydroxycinnamoyl transferase, lysine decarboxylase, and acyltransferase gene families. Comparative transcriptomic analyses disclose berry-specific upregulated expression in representative genes in each of these gene families. These data provide an evolutionary perspective and shed light on the metabolic processes relevant to the molecular basis of species-specific piperine biosynthesis.
Black pepper (Piper nigrum, 2n = 52), known as the ‘King of Spices’, is one of the oldest and most widely used seasonings in the world. It had been highly valued and considered as ‘Black Gold’, occupying a preeminent status in the spice trade. It was the primary spice in early trading between Europe and Asia. The production, transportation and consumption of black pepper has influenced the destinies of nations and their people, both economically and culturally. Originating in the humid, tropical evergreen forests of the Western Ghats of South India, black pepper is now cultivated in most tropical and subtropical regions, with production primarily in Vietnam, Indonesia, Brazil, India, Sri Lanka, China, Malaysia and Cambodia. In 2017, the world’s total cultivation area was 458,731 hectares, which gives 510,045 metric tons of production and 2 billion US dollar trade value. It is also an important cash crop for small farmers in many developing countries.

Black pepper is a perennial, woody climbing vine. It belongs to the family Piperaceae, which is the largest family in the order Piperales. In current phylogenetic classification,5,6 Piperales is considered as a sister order of the Canellales, Laurales and Magnoliiales orders within the Magnoliid clade. However, the phylogenetic position of magnoliids relative to eudicots and monocots is still unsettled, even based on the two newly published magnoliid genomes, which indicates a controversial phylogenetic understanding of these long-isolated lineages6–8.

The Piperales are well known for their special phytochemistry, particularly their unique piperidine alkaloids. Piperine is the major alkaloid responsible for the pungency and flavour of black pepper. In addition to being a common culinary spice and a preservative for meat products, black pepper has been widely used in traditional medicinal systems, such as the Indian Ayurvedic system, traditional Chinese medicine and folkloric medicines of Latin America and Southeast Asia. Piperine possesses a range of pharmacological activities, including the attenuation of fat cell differentiation through the downregulation of peroxisome proliferator-activated receptor (PPAR) gamma expression, leading to its use for treatment of diabetes as a PPAR agonist.10 It has also been used as an antioxidant, antitumour, antimicrobial, anti-depressive and anti-inflammatory agent. The phytochemical and pharmacological characteristics of black pepper have received renewed attention in recent decades. However, relatively little is understood about the genetic mechanisms controlling of its biosynthetic pathway and accumulation, and few genetic resources are available for black pepper.

Here, we report a reference genome of black pepper using a combination of four technologies. Evolutionary analysis of three available magnoliid genomes from different orders provides evidence for the phylogenetic position of the magnoliids. In addition, our comparative genome and transcriptome analyses identify changes in gene expression, evolution and family size associated with piperine biosynthesis. Genomic resources provided here will be valuable for biological and agronomic research in Piper species.

Results

Genome assembly and main features. Cv. Reyin1 derived from the cultivar ‘Lampang Daun Kecl’ was used for genome sequencing. Based on the k-mer genome survey analysis (Supplementary Fig. 1), black pepper (Supplementary Note 1) was estimated to have a genome size of 761.74 Mb. K-mer analysis with a length of 17 indicates the genome had high heterozygosity (1.33%) and a repetitive sequence content of 59.54% (Supplementary Table 1).

To overcome the impact of heterozygosity and repetitive sequence content on the construction of a chromosome-scale reference genome, a comprehensive de novo assembly strategy (Supplementary Fig. 2) combining Illumina paired-end reads (137× coverage), PacBio single-molecule long reads (N50 length of 13 Kb, ~138× coverage), 10X Genomics, BioNano (Supplementary Table 2), and Hi-C sequencing (Supplementary Table 3) was adopted. The workflow is summarised as follows: (1) FALCON12 was selected for the PacBio-only assembly, using the overlap-layout-consensus algorithm and FALCON-Unzip12 for true diploid assembly; (2) fragScaff13, which leverages information derived from different barcoded pools, was used to order and orient linked contigs into longer scaffolds, which resulted in an assembly (Piper_nigrum_v1) consisting of 1277 scaffolds with an N50 of 2.3 Mb and a total length of 791.0 Mb; (3) a non-haplo-type-aware hybrid assembly with ‘no extend split’ and ‘no cut segdup’ parameters (according to BioNano’s suggestions) was performed using BioNano Solve tools, yielding an assembly with a total molecule length of 316,350.85 Mb and 128× effective coverage (Supplementary Table 2). This improved version (Piper_nigrum_v2) contains 201 hybrid scaffolds with an N50 of 7.8 Mb (3.4 fold improvement compared with the Piper_nigrum_v1) and a longest scaffold of 25.8 Mb (Supplementary Figs. 3–5); (4) additional scaffold refinement was performed in a Hi-C experiment with ~125 million unique Di-Tags read pairs (Supplementary Table 3 and Supplementary Fig. 6) and postprocessing by gap filling and polishing to generate the final version of the assembly, Piper_nigrum_v3. This final assembly contains only 45 scaffolds, with a scaffold N50 of 29.8 Mb and 99.9% of the assembled genome contained in 26 scaffolds (Table 1). Inasmuch as the chromosome number of Piper nigrum is 2n = 5214, we infer that these large scaffolds reflect a chromosome-scale assembly (Supplementary Figs. 7–14). The assembly size of 761.22 Mb (99.93% coverage of the genome) was very similar to the estimated genome size of 761.74 Mb obtained from the k-mer analysis. The higher heterozygosity estimated from the k-mer analysis was also consistent with single-nucleotide polymorphism (SNP) calling in the final reference genome (Supplementary Note 4 and Supplementary Fig. 15).

Detailed assembly data are summarised in Table 1. The extent of comprehensive gene coverage was assessed by screening for 248 core eukaryotic genes (CEGs)15, which revealed a complete and partial matches for 234 (94.35%) and 244 (98.39%) genes, respectively (Supplementary Table 4). BUSCO16 analysis against the plant-specific database containing a total of 430 genes revealed 413 (96.1%) complete BUSCOS, 80 (19.1%) of which were duplicated genes (Supplementary Table 5). These data support the interpretation that the P. nigrum genome assembly is reasonably complete.

A total of 54.85% repetitive sequences were identified in the black pepper genome. Among these repeats, 54.01% are classified as interspersed repeats (Supplementary Table 7). Similar to most

| Table 1 Major indicators of the Piper nigrum genome |
| Assembly feature | Statistic |
|------------------|----------|
| Estimated genome size (by k-mer analysis) (Mb) | 761.74 |
| Number of scaffolds | 45 |
| Scaffold N50 (Mb) | 29.8 |
| Longest scaffold (Mb) | 48.45 |
| Assembled genome size (Mb) | 761.22 |
| Assembly % of genome | 99.93 |
| Repeat region % of assembly | 54.85% |
| Predicted gene models | 63,466 |
| Average coding sequence length (bp) | 1347 |
| Average exons per gene | 5.84 |
plant genomes\textsuperscript{17}, the predominant type of transposable elements (TEs) was long terminal repeat (LTR) retrotransposons, accounting for 40.55\% of the genome, including 27.63\% LTR/Gypsy and 9.95\% LTR/Copia retro-elements (Supplementary Note 2, Supplementary Tables 8–9 and Supplementary Fig. 16). These TEs exhibit an apparently random distribution on the chromosomes, and an inverse correlation with gene density (Fig. 1; Supplementary Figs. 17–25).

We conducted Illumina strand-specific RNA sequencing (RNA-seq) using eight different tissues and organs, and performed PacBio isoform sequencing (Iso-Seq) (Supplementary Fig. 26) to provide transcriptional evidence supporting the annotation and to obtain reliable gene structure annotation. We also employed a strategy that combined ab initio and evidence-based gene prediction using the BRAKER\textsuperscript{18} pipeline. The black pepper genome encodes 63,466 inferred protein-coding genes, with an average length of 900 bp and an average GC content of 51.21\%. Annotation Edit Distance (AED)\textsuperscript{19} quantification showed a high AED of 0.04 at the nucleotide-level and 0.13 at the exon level, indicating a highly credible gene model.

Five thousand eighty-two transcription factors (TFs) from 75 gene families accounting for 8.0\% of the protein-coding genes are categorised in this report. In addition, 646 chromatin regulators (CRs), 157 transcriptional regulators (TRs), 6509 long non-coding RNAs (lncRNAs), 1514 tRNAs, 1206 rRNAs, 1533 small nuclear RNAs (snRNAs) and 256 microRNAs (miRNAs) were also

Fig. 1 Black pepper genomic landscape of diversity and expression data. a Circular representation of the pseudomolecules. b–d The distribution of the GC density, repeat density and gene density, respectively, with densities calculated in 500 Kb windows. e–l Expression of berry-specific genes (from outside to inside tracks: 2 MAP, 4 MAP, 6 MAP, 8 MAP, root, stem, leaf and flower). m Locations of genes mapped to secondary metabolism (green square) and alkaloid metabolism (red square) pathways. n Syntenic blocks. The band width is proportional to syntenic block size. Source data are provided as a Source Data file.
Comparative genomic and phylogenomic analyses. Ancient whole-genome duplication (WGD) (also known as polyploidization) events are important driving forces of the evolution of animals, fungi and other organisms, particularly plant species. Comparative genomic and phylogenomic analyses (Supplementary Fig. 28) recovered duplications within the black pepper genome that are either inter-chromosomal or intra-chromosomal duplications (Supplementary Fig. 30).

An analysis of the genomic syntenies of black pepper using MScanX indicated that most genes were classified as WGDs or segmental duplications (32,547 genes, accounting for 51.3%), followed by three other types: dispersed (19.1%), proximal (7.4%) and tandem (3.6%) duplications. We also performed a comparative genomic analysis of black pepper with *Amborella* and *Cinnamomum* and identified 3652 protein families containing 21,184 proteins and 2071 Gene Ontology (GO) terms, of which 41.63%, 13.19% and 45.18% of the genes were annotated to biological process, cellular component and molecular function categories, respectively (Supplementary Fig. 28).

Identified (Supplementary Note 3 and Supplementary Fig. 27). An InterProScan Pfam analysis identified 3652 protein families containing 21,184 proteins and 2071 Gene Ontology (GO) terms, of which 41.63%, 13.19% and 45.18% of the genes were annotated to biological process, cellular component and molecular function categories, respectively (Supplementary Fig. 28).

The RHB paralog K_γ distribution showed a single peak at ~0.1 (Fig. 2b; Supplementary Fig. 29a). Second, the synteny dot plot analysis revealed duplications within the black pepper genome that are either inter-chromosomal or intra-chromosomal duplications (Supplementary Fig. 30).

An analysis of the genomic syntenies of black pepper using MScanX indicated that most genes were classified as WGDs or segmental duplications (32,547 genes, accounting for 51.3%), followed by three other types: dispersed (19.1%), proximal (7.4%) and tandem (3.6%) duplications. We also performed a comparative genomic analysis of black pepper with *Amborella* and *Cinnamomum* and identified 1:1 and 1:2 syntetic depth ratios in the *Amborella-Cinnamomum* and *Amborella-Piper nigrum* comparisons (Supplementary Fig. 31a), respectively. Furthermore, an analysis of syntenies in black pepper with *Amborella* showed 316 syntenic blocks that covered 40% and 34% of the assembled genomes, respectively (Supplementary Fig. 29a and b).
WGD event during black pepper genome evolution. Based on the slow substitution rate of basal angiosperms, we speculate that the black pepper WGD event ($K_e = 0.106 \pm 0.002$) occurred ~17.2–17.9 MYA with a synonymous substitution rate of 3.02E−9 synonymous substitutions per year by29 (Fig. 2c).

The high-quality reference genome for black pepper enabled us to perform comparative genomics among relatively early diverging angiosperms. We compared the black pepper genome with the genomes of nine eudicots, three monocots, three magnoliids, Amborella, two gymnosperms and two species Selaginella moellendorffii and Physcomitrella patens as the outgroups.

Eighty-two single-copy orthologous gene families among the 21 species were identified using OrthoMCL to accomplish this goal, and a phylogenetic analysis of these families using BEAST placed magnoliids as a sister clade to the monocots–eudicots clade, consistent with Liriodendron genome research30, the Plastid Phylogenomic Angiosperm (PPA) tree36 and Angiosperm Phylogeny Group (APG) IV3. Furthermore, Piper nigrum was placed as a sister to Magnolidae–Laurales among the magnoliids (Fig. 2c), consistent with its phylogenetic position inferred from chloroplast genomes31. Based on our results, Piperales, represented by Piper nigrum, first diverged from the Magnoliales (Liriodendron chinense) plus Laurales (Cinnamomum kanehirae and Persea americana) approximately 175–187 MYA (95% highest posterior density (HPD) interval).

Evolution piperine biosynthesis-associated genes. Piperine is synthesised from two direct precursor substrates, piperyl-coenzyme A and piperidine, in a reaction catalysed by an acyltransferase32. Thus, piperine production is associated with three major gene groups (see below): group I: genes in the phenylpropanoid pathway (KEGG pathway: map00940), which produce cinnamoyl-CoA for piperyl-coenzyme A biosynthesis via a few complex reactions, such as amino transfer and elimination of ammonia-lyase and cinnamate 4-hydroxylation; group II: genes involved in L-lysine metabolism (KEGG pathway: map01064), which catalyse the transformation of lysine to piperidine via a series of reactions including decarboxylation, amine oxidation, cyclization and reduction; and group III: acyltransferase genes, that catalyse the synthesis of piperine in the presence of piperyl-coenzyme A and piperidine.

We acquired insights into the genomic basis of piperine biosynthesis, using OrthoMCL to identify orthologous genes and CAFE to identify gene family clusters, with a specific focus on the expansion (gain) and contraction (loss) events related to the above three gene groups in black pepper. This process identified 39,400 gene families consisting of 471,854 genes among the 21 species (Fig. 2a; for clarity, only species with a close evolutionary relationship to black pepper are shown). Four hundred twenty-three gene families containing 4228 genes were unique to black pepper.

According to the CAFE analysis, 91 gene clusters (including 21,368 genes) and 189 gene clusters (including 14,201 genes) expanded and contracted, respectively, in the black pepper genome. Among these clusters, 35 and 4 families exhibited significant (family-wide $p$-value ≤ 0.01) expansions and contractions, respectively (Fig. 3). Furthermore, the species-specific expanded genes were significantly enriched in two main types, one of which was genes with secondary metabolite-associated functions, such as glycosyltransferases (GTs; 19 genes), shikimate hydroxycinnamoyl transferases (HCTs; 69 genes), cytochrome P450s (CYP; 31 genes), lysine decarboxylases (LDCs; 75 genes), BAHD acyltransferases (BAHD-ATS; 6 genes) and serine carboxypeptidase-like acyltransferases (SCPL-ATS; 7 genes) (Fig. 4a; Supplementary Fig. 33). These expanded genes occurred in gene clusters on some chromosomes (Supplementary Fig. 34a–f). A KEGG pathway analysis of these specific gene families revealed marked enrichment in genes involved in piperine synthesis, including the biosynthesis of alkaloids from the shikimate pathway (map01063); alkaloids from ornithine, lysine and nicotinic acid (map01064); phenylpropanoid biosynthesis (map00940); and phenylalanine, tyrosine and
tryptophan biosynthesis (map00400). The other category was disease resistance-related genes, such as disease-resistance proteins (RPS5, RPP13, RGA1, RGA2, RGA3, RGA4 and RGA5), NBS-LRR disease-resistance proteins, LRR receptor-like serine/threonine protein kinases (EFR, BAM1, FLS2, GSO2 and EMS1) (Supplementary Fig. 34 g, h) and salicylic acid-binding protein 2. These genes have been widely reported to play important roles in pathogen-resistance mechanisms. The expansion of these genes might indicate that biotic stresses from pathogen infection and animal ingestion are a source of major selection pressure on black pepper in the tropical rainforest.

Upregulation of piperine biosynthesis-related genes. The piperine content determined using high-performance liquid chromatography (HPLC) (Supplementary Note 5) revealed that the organ with the highest content was berry at 8 months after pollination (MAP), followed by 6 MAP, 4 MAP, 2 MAP, flower,
root, stem and leaf (Fig. 4b). A transcriptomic analysis was performed with RNA-seq using samples from different organs of black pepper, including berries (which synthesise piperine) at different developmental stages (2 MAP, 4 MAP, 6 MAP and 8 MAP; Supplementary Fig. 35), and organs not containing piperine, i.e., the stem, root, leaf and flower (Supplementary Note 6 and Supplementary Figs. 36–38), to investigate genes involved in piperine production. A gene set enrichment analysis (GSEA) and weighted gene co-expression network analysis (WGCNA) revealed that upregulated genes in berries relative to nonberry organs were associated with the phenylpropanoid pathway (Supplementary Fig. 39) and lysine metabolism. The transcriptional module (Supplementary Figs. 40–42) of piperine synthesis in berries exhibited significant enrichment of the amino transferase (PPA-AT), arogenate dehydratase (ADT), cinnameate 4-hydroxylase (C4H/CYP73), HCT, p-coumarate 3-hydroxylase (C3H), caffeic acid-3-O-methyltransferase (CAOMT), p-coumarate-CoA-ligase (4CL), GTF, CYP72 and CYP71 clans (Fig. 4b; Supplementary Fig. 43). These genes are involved in the metabolism of phenylpropanoids to produce piperoyl-CoA. In addition, the differential expression of LDC, primary-amine oxidase (PAO) and diamine oxidase (DAO) in black pepper berries (Fig. 4b) is consistent with the hypothesis that lysine, rather than quinolizidine or indolizidine, is the intermediate precursor of piperidine synthesis in black pepper. Importantly, the expansion of the BAHD-AT and SCPL-AT gene families (11 BAHD-ATs and 3 SCPL-ATs) (Fig. 4a), accompanied by the high transcriptional activity of these genes in berries (Fig. 4b), is associated with the metabolism of phenylpropanoids and lysine into piperine (Fig. 4i).

Purifying selection of piperine biosynthesis gene families. Based on our analysis of gene expansion and expression, we synthetically investigated the gene-sequence-level features of the aforementioned three gene groups exhibiting expansion and berry-specific upregulated expression that might contribute to the unique piperine production in black pepper.

Within group I, the GTF, CYP and HCT gene families were significantly expanded in black pepper (Fig. 4a). HCT has been reported to play a crucial role in piperoyl-CoA synthesis (a precursor for piperine biosynthesis) by catalysing the transformation of p-coumaroyl-CoA into p-coumaroyl shikimic acid and caffeoyl shikimic acid into caffeoyl-CoA. The HCT gene family was expanded in the black pepper genome (69 genes in black pepper compared with one or two genes in the other species). The particularly high expression in berries (2 MAP, 6 MAP and 8 MAP; Fig. 4b) detected by analysing transcriptomes from different organs is consistent with a role for this family in the biosynthesis of piperine. In the sequence-level analysis of HCT expansion, most conserved regions (107 sites) were under strong purifying selection, with one site showing diversifying selection and eight sites showing episodic selection (Fig. 4c, d). The adaptive expansion of HCT and the maintenance of duplicates appear to have operated in concert, resulting in higher enzyme levels for the accumulation of the necessary donor precursor used for piperine biosynthesis (Fig. 4i). Despite the significant expansion of the GTF and CYP gene families in black pepper, no positively selected sites were detected (Supplementary Fig. 44a, b). The significant expansion of GFT, CYP and HCT family genes suggests the importance of the phenylpropanoid pathway, which provides precursors for the biosynthesis of alkaloids and flavonoids typical of modern pepper cultivars (Fig. 4i).

Within group II, LDC catalyses the first step in lysine metabolism (Fig. 4i) and serves as the rate-limiting enzyme in the synthesis of lysine-derived alkaloids, including piperidine alkaloids (piperine), quinolizidine alkaloids and indolizidine alkaloids. Seventy-five LDC genes were detected in black pepper, compared with just six in Ananas comosus, five in Vitis vinifera and at most one in the other species studied (Fig. 4a). An analysis of sequence evolution in the LDC family indicated that 19 sites have been under strong purifying selection, two sites have been under diversifying selection and five sites under episodic selection (Fig. 4c, f). The expansion of LDC genes suggests the unique activation of lysine-derived alkaloid synthesis in black pepper.

Within group III, BAHD-AT and SCPL-AT encode the two main acyltransferase families that use phenolic compounds as donor molecules. The BAHD-AT and SCPL-AT genes were expanded to include six and seven members, respectively, in black pepper. An assessment of sequence evolution revealed that 261 sites have been under strong purifying selection, one site has been under diversifying selection and nine sites under episodic selection in BAHD-ATs (Fig. 4g, h), and no sites showed positive selection in SCPL-ATs (Supplementary Fig. 45). This adaptive expansion may have resulted from purifying and diversifying selection and led to high levels of BAHD-AT and SCPL-AT transcripts in the berries (Fig. 4b), further explaining why piperine is uniquely accumulated in black pepper.

Overall, the main pathway leading to piperine synthesis and key genes involved in its regulation (Fig. 4i) are consequences of the expansion of multiple gene families, followed by evolutionary selection leading to transcription specifically in the berries. However, we fully recognise that a direct causal link between each identified gene and metabolite abundance remains to be

Fig. 4 Analysis of gene families involved in black pepper piperine metabolism. **a** Expansion of genes involved in the phenylpropanoid pathway, lysine metabolism and acyltransferase family in black pepper. **b** Tissue-specific upregulated expression of genes involved in the phenylpropanoid pathway, lysine metabolism and acyltransferases in different tissues. The heatmap on the right shows the piperine content in the freeze-dried fresh tissues. MAP: months after pollination. **c–h** Selection analysis of HCT, LDC and BAHD-AT gene expansion events. Red points indicate positive selection. Green points indicate episodic selection. Purple points indicate episodic positive selection. Blue bars show sequencing depth. A significance threshold of $\alpha = 0.1$ was used for both SLAC and MEME. **c** Normalised dN-dS (SLAC) values across a multispecies alignment of 94 HCT sequences with 195 sites. Points indicate statistically significant evidence for codons under selection. One site shows positive selection across the entire tree (SLAC); eight sites show episodic selection (MEME). **d** Comparison of episodic selection on particular codons across black pepper HCT genes ($n = 69$). **e** Normalised dN-dS (SLAC) values across a multispecies alignment of 72 LDC sequences with 80 sites. Two sites show positive selection across the entire tree (SLAC); five sites show episodic selection (MEME). **f** Comparison of episodic selection on particular codons across black pepper LDC genes ($n = 57$). **g** Normalised dN-dS (SLAC) values across a multispecies alignment of 87 BAHD-AT sequences with 375 sites. One site shows positive selection across the entire tree (SLAC); nine sites show episodic selection (MEME). **h** Comparison of episodic selection on particular codons across black pepper BAHD-AT genes ($n = 6$). **i** Schematic representation of the phenylpropanoid pathway and lysine metabolism branch with the reactions associated with piperine biosynthesis genes. The solid lines indicate genes catalysing major reactions that were characterised in our study. The dotted lines indicate genes catalysing reduction reactions in lysine metabolism and approximate derivative reactions in the phenylpropanoid pathway that were not characterised in our study. The source data underlying Fig. 4a–h are provided as a Source Data file.
established. Nevertheless, the genetic framework underpinning the evolution of piperidine alkaloid biosynthesis is being clarified.

Discussion

The Piperales represents a useful taxonomic group for the study of the link between evolution, ecology and phytochemistry41 because of its geographic distribution pattern, the diversity of its lineages, and its characteristic secondary metabolism. The black pepper genome will provide resources for phylogenomic analyses and studies of piperine synthesis. Piperales is one of the most diverse lineages of basal angiosperms. In terms of stem anatomy, *Piper nigrum* has a pattern of vascular bundle arrangement that is similar to monocots, whereas the vascular bundle arrangement of *Piper colubrinum* is similar to eudicots42. In addition, the cotyledonary forms of *Peponema pellucida*, *Peponema peruviana* and *Peperomia parvifolia* show an evolution typical of eudicots to monocots. Interestingly, *Liriodendron* (Magnoliidae) also shows features of both monocots and eudicots4. Because of their anti-quity and morphological diversity, the phylogenetic positions of various lineages within the magnoliid clade have remained unclear, with three primary proposed tree topologies reported9. Two newly issued genome sequences have been used to address the phylogenetic position of magnoliids, but have produced conflicting results6,8–8. In our comparative genome analysis, a combination of three representative orders (Piperales, Magnoliidae and Laurales) of magnoliids indicates that magnoliids are sisters to the monocots–eudicots clade, consistent with research into the *Liriodendron* genome9, the PPA tree20 and APG IV21. In addition, *Piper nigrum* is placed as a sister to Magnoliidae–Laurales among the magnoliids (Fig. 2c). According to Soltis et al.8, the inclusion of complete angiosperm lineages that were missing in the present study (Nymphaeales, Austrobaileyales, Chloranthales, Ceratophyllales and Canellales in magnoliids) will facilitate a better understanding of the phylogenetic relationships of these diverse and long-isolated lineages of flowering plants.

The current assembly of the black pepper genome provides insights into the underpinning genetic changes following WGD events that are responsible for the unique biosynthesis of piperine. Our current study has focused on analysing the biological events that are responsible for the unique biosynthesis of piperine, insights into the underpinning genetic changes following WGD in trobaileyales, Chloranthales, Ceratophyllales and Canellales in addition, the precursors of nicotine alkaloids in tobacco (*Nicotiana*) species, in which capsaicinoids are derived from the metabolic pathways described above. One example is the synthesis of nicotine alkaloids through the convergence of the two metabolic pathways described above. *Nelumbo nucifera* convergence of phenylpropanoid and lysine metabolism, specifically the decarboxylation and amine oxidation of lysine, and acyl transformaton represent the characteristic features of piperine synthesis (Fig. 4i), and we describe the genetic and evolutionary basis of these features in this study.

Sequencing the black pepper genome has advanced our understanding of the unique piperine biochemistry of black pepper. Our study therefore provides valuable insights that may serve as a foundation for future research on Piperales taxonomy and piperine biosynthesis, leading to a better understanding of the evolution, phytochemistry and ecology of the *Piper* genus.

Methods

**Sample collection and DNA library construction.** Fresh leaf tissues from single living black pepper plants were collected to extract genomic DNA and RNA (Supplementary Note 1). For genome survey analysis, a short paired-end Illumina DNA library with a 350 bp insert size (137 × coverage) was sequenced on the Illumina HiSeq 2500 sequencer. For PacBio Sequel sequencing, 50 µg of high-molecular-weight (HMW) genomic DNA were prepared to generate five standard SMRTbell libraries with 20 Kb insertions. PacBio long reads were sequenced using 15 SMRTcells on the PacBio Sequel System (Pacific Biosciences) with SMRTbell Template Prep Kit 1.0-SPv3 (Pacific Biosciences). HMW genomic DNA was also prepared for 10 × Genomics libraries according to the manufacturer’s protocol (Chromium Genome v1, PN-120229). Sequencing-read libraries were sequenced using HiSeq 2500 with 2 × 150 paired-end reads to generate ~96 Gb (120 × coverage) raw data.

**Transcriptome library preparation and sequencing.** RNA-seq experiments (three biological replicates) used RNA extracted from different organs of the black pepper *Cv. Reyin1* (root, stem, leaf, flower and berries at four different stages: 2 months after pollination (MAP), 4 MAP, 6 MAP and 8 MAP) (Supplementary Fig. 3). Prepared libraries were sequenced on the Illumina HiSeq 2500 platform according to the manufacturer’s recommended protocol. We generated an average of 28.0 million paired-end reads for each sample.

**Preprocessing of PacBio Iso-Seq reads.** The PacBio Iso-Seq3 pipeline (https://github.com/PacificBiosciences/IsoSeq3) was applied to obtain high-confidence transcriptome reads through the CCS (circular consensus sequence) output. The polyA tailing, cluster and polishing process. The high-quality, full-length and isoform transcript sequences were prepared for subsequent analysis.

**De novo genome assembly.** The errors in the PacBio single-molecule real-time (SMRT) sequences were initially corrected using Canu46 and the default parameters. Because of heterozygosity and repeated sequences, FALCON12 was subsequently employed for de novo assembly using the corrected reads to produce primary contigs (p-Contigs) and haplotigs (h-Contigs). Finally, the postprocessing step was used to polish using Arrow47 based on corrected PacBio long reads. The 10X Genomics LinkReads were mapped to the consensus assembly described above using BWA-MEM48 with a default parameter. Then, fragScaff49 was used to extend contigs into initiatory scaffolds (Piper_nigrum_v1) according to the recommended scripts and processes of the fragScaff software.

**BioNano optical maps and hybrid assembly.** Total of 750 ng of fresh young leaf tissues were collected from living plant material following BioNano Genomics guidelines. HMW genomic DNA with a fragment distribution ranging from 150 Kb to 2 Mb was fluorescently labelled using single-sequence-specific DLE-1 endonuclease (BioNano Genomics) based on the BioNano Direct Label and Stain (DLS) technology. The fluorescently labelled DNA was stained for at least two hours at room temperature and then loaded onto a Saphyr chips to scan on the BioNano Genomics Saphyr System by the sequencing provider Berry Genomics Corporation. BioNano data were first filtered based on molecule length, mapping rate and label density using BioNano Solve (https://bibionanogenomics.com/wp-content/uploads/2017/10/30182-Bionano-Tools-Installation-Guide.pdf). Non-haplotype de novo assembly was performed in BioNano Solve using filtered high-quality BNX files based on the Overlap–Layout–Consensus paradigm. The 350,823 filtered
DLE-1 molecules (N50 length 0.288 Mb) produced 547 genome maps with an N50 of 3.8 Mb for a total map length of 1304 Mb. The de novo assembly containing BioNano molecules was combined with Piper_nigrum_v1 input into the BioNano Solve hybrid scaffolding pipeline to produce a hybrid scaffold assembly with -T p-value set to 1e-10 in both initial alignment, final alignment and hybrid scaffold steps, and more stringent value of 1e-11 for both the merge_Tvalue and T_cutoff p-value thresholds. Finally, the hybrid assembly had an N50 of 7.8 Mb, and the longest scaffold was 25.8 Mb. This stage of assembly was termed Piper_nigrum_v2 (Supplementary Note 1).

Scaffolding with Lachesis. About 5 g of fresh young leaf tissue from living plants were macerated and crosslinked using paraformaldehyde to capture the interacting DNA segments. Chromatin was subsequently digested with HindIII (NEB), and biotinylated nucleotides were used to fill in the resulting sticky ends. Following ligation, a protease was used to remove the crosslinks. Finally, genomic DNA was extracted, sheared into 350 bp fragments using a focused ultrasonicator (Covaris, Woburn, USA), and fragments into which biotin had been incorporated were pulled down with streptavidin-coated magnetic beads purified. Purified DNA was then prepared and sequenced on an Illumina HiSeq instrument according to the trimmomatic), and then checked with HiCUP49. The scaffolding began by aligning Woburn, USA), and fragments into which biotin had been incorporated were

Post-processing. We also performed an additional round of gap filling to eliminate the gaps generated in the final scaffolding steps using PBjelly (https://github.com/alvaralmstd/Tutorials/wiki/Gap-closing-with-PBjelly) with PacBio subreads. The assembled scaffolds were further polished with Pilon (https://github.com/broadinstitute/pilon) using Illumina paired-end reads to correct base errors. The ultimate assembly consisted of 45 scaffolds with a total size of 761.2 Mb and a scaffold N50 of 29.8 Mb. This assembly is designated as the final version of black pepper genome: Piper_nigrum_v3.

For an assessment of completeness, the Piper_nigrum_v3 genome was subjected to a BUSCO analysis59 and compared with the Vitisvinifera_odb10 database (Update date: 2017–12–01) with the –long parameter. In addition, Core Eukaryotic Genes (CEGs) were also aligned to Piper_nigrum_v3 using CEGMA13 with the default parameters.

Genomic annotation. We used a combination of the de novo repeat library and homology-based strategies to identify repeat structures. TransposonPSI (http://transposonpsi.sourceforge.net/) was used to identify transposable elements; GenomeTools suite60 (LTRharvest and LTRdigest) was used to annotate LTR-RTs with protein-coding genes from the P. hybridus (Supplementary Note 2). Then, a de novo repeat library of black pepper was built using RepeatModeler software (http://www.repeatmasker.org/RepeatModeler/), and each of the three repeat libraries was classified with RepeatClassifier, followed by Merge and de-redundancy using RepeatMasker (https://www.repeatmasker.org/RepeatMasker/index.php). The de-novo repeat library was used to discover and mask the assembled genome with RepeatMasker (https://www.repeatmasker.org/RMDownload.html) with the -xsmall parameter.

For gene structure annotations, the Illumina RNA-seq data from black pepper Cv. Rejini1 were aligned to repeat-softmasked genome using GSNAp54, which generated intron hints with other aligned hints (ESTs, proteins, and nuclear genes from NCBI) by a fingerprinting approach. For PacBio Iso-Seq reads, GMAP26, a splice-aware aligner, was used to align the high-quality isoform transcripts to the repeat-softmasked genome for the detection of new isoforms.

The structural annotation of protein-coding genes was performed using BRAKER238, which integrated GeneMark-ET and AUGUSTUS by combining the aligned results from ab initio predictions, homologous protein mapping, RNA-seq mapping and GMAP PacBio mapping to produce the final gene set. Gene models from these different approaches were combined using the EVM software (version 1.1.1115). Moreover, trNA, rRNA, tRNA, lncRNAs, snRNA and miRNAs and non-protein-coding genes were annotated by performing homologous searching and deep-learning analyses across the assembled genome sequence (Supplementary Note 3).

Functional annotation of protein-coding genes. Predicted genes were subjected to functional annotation by performing a BLASTP homologue search against the UniProtKB Viridiplantae database, and the NCBI non-redundant protein database with an e-value threshold of 1e-10. In addition, a comprehensive annotation was also achieved using InterProScan (v5.31-70.0)29, which includes motifs/domains prediction, functional classifications, protein family identification, transmembrane topology, predicted signal peptides and GO annotations. KAAS (https://www.genome.jp/kaas) and KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/) were used to search the KEGG GENES database for KO (KEGG Orthology) assignments and generating a KEGG pathway membership. PlantTFnet22 was also used to systematically analyse InterProScan domain and categorise possible chromatin regulators (CRs), transcription factors (TFs) and other transcriptional regulators (TRs) in the current assembly. The plantSimVASH version 3.0.5-a04b4c5d62 software was used to cluster the plant-specific secondary metabolism genes.

Transcriptome assembly and gene expression analysis. RNA-seq reads from three replicates of the eight tissue types were preprocessed using Trimmomatic by removing adapter sequences and filtering low-quality reads. HISAT2 was used to align the samples with the genome-guided transcript assembly using StringTie57.

Read counts extracted from StringTie were filtered using the sva R package to decrease batch effects and hidden variables. Differentially expressed genes (DEGs) were detected using DESeq258, and calculated based on absolute log2 transformed fold-change values greater than 2 and p-value of 0.05 using the Benjamini–Hochberg correction59. A gene set enrichment analysis (GSEA)53 was performed to determine significant gene sets, and the WGCNA package68 in R was applied to perform a multivariate analysis of gene co-expression modules. See Supplementary Note 6 for additional details.

Synteny analyses. For an estimation of the whole genome duplication events, we selected an orthologous best hit (BR) pairwise sequences of paralogs (within the species genome) and orthologous relationships (between black pepper and other species) were identified and the synonymous divergence levels (Ks) were calculated using the YN model in KaKs_Calculator v2.090. The raw Ks distributions were used to fit a mixture model of Gaussian distributions and thus derive the mean Ks values between paralogs and orthologues in R package Mclust5. The Ks distribution of orthologues between black pepper and other species were adopted to compare the relative substitution rates in different species by plotting with the ggplot2 package. The divergence time of black pepper was calculated by combining the Ks value with synonymous substitutions at each site per year (r) for magnolids through the following equation (T): T = 1 / (2rKs), and the divergence time between species was estimated by the divergence date using BEAST v2.1.2 (Bayesian Evolutionary Analysis Sampling Trees)246. A Calibrated Yule model with a Strict Clock rate and gamma hyperparameter of prior distribution was used to estimate the divergence time. Speciation event dates for Ananas comosus-Oriza sativa (Normal model, Mean: 105 MYA, Sigma: 0.5) and Liriodendron chinense-Cinnamomum kanehirae (Normal model, Mean: 118 MYA, Sigma: 0.5), which were obtained using Timetree web service (http://www.timetree.org/), Papaver somniferum-Nelumbo nucifera (Normal model, Mean: 125 MYA, Sigma: 0.5)22, monocots–eudicots (Log Normal model, Mean: 150 MYA, SD: 4 MYMA), were used to calibrate the divergence time. The Markov chain Monte–Carlo analysis was repeated 10,000,000 times with 1000 steps.

The orthologous genes and phylogenetic tree topology inferred from the OrthoV CAPE analysis were taken as input for RAXML v8.2.13, which employed a random birth and death model, to estimate the size of each family at each ancestral node and obtain a family-wise p-value (based on a Monte-Carlo re-sampling procedure) to indicate whether has a significant expansion or contraction occurred in each gene family across species

Identification of alkaloid and pipeline biosynthesis genes. Predicted protein-coding genes from the black pepper genome were aligned against annotated alkaloid and pipeline biosynthesis pathway genes in the KEGG database by BLASTP with e-value cut-off of 1e-5, identity ≥ 50% and alignment length ≥ 50%. In addition, all orthologous gene sequences involved in alkaloid and pipeline biosynthesis were also captured from comparative genomics analyses, and
corresponding gene functions were verified by searching the published literature and the NCBI and UniProt databases.

Sequence evolution of gene families. Gene families derived from OrthoMCL identification were aligned using ClustalW (http://www.clustal.org/.clustal2) and trimmed with trimAl (v1.2) (http://trimal.cgenomics.org/introduction). Evidence of selection across genes within gene families was tested using a multispecies alignment in HyPhy with the datamonkey webserver. The genetic algorithm recombination detection method was performed to detect breakpoints at nucleotide sites. Evidence of positive selection and episodic selection sites in genes was inferred by estimating the rates of synonymous and non-synonymous changes at each site in the aligned sequence through single likelihood ancestor counting (SLAC) and a mixed effects model of episodic diversifying selection (MEME) (Supplementary Note 8).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The data sets generated and analysed during this study are available from the corresponding author upon request. All the raw sequencing data generated during this study have been deposited at NCBI as a BioProject under accession PRINAS29758. Transcriptome sequence reads have been deposited in the SRA database under BioProject number PRINAS29760. The genome assemblies and annotation files are available at the website http://cotton.ibnu.edu.cn/EN/download.php. The source data underlying Figs. 1, 2a, b, 3 and 4a–h are provided as a Source Data file.

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