Construction of Pseudomolecules for the Chinese Chestnut (Castanea mollissima) Genome

Jinping Wang,*1 Shoule Tian,* Xiaoli Sun,* Xinchao Cheng,* Naibin Duan,‡ Jihan Tao,*1 and Guangning Shen,*1

*Shandong Institute of Pomology, 66 Longtan Road, Tai’an Shandong, 271000, China, 1Biomarker Technologies Corporation, Floor 8, Shunjie Building, 12 Fuqian Road, Nanfaxin Town, Shunyi District, Beijing, 101300, China, and 2Shandong Centre of Crop Germplasm Resources, Shandong Academy of Agricultural Sciences, Jinan, Shandong, 250100, China

ORCID IDs: 0000-0002-3159-8063 (J.W.); 0000-0003-0443-0406 (N.D.)

ABSTRACT The Chinese chestnut (Castanea mollissima Bl.) is a woody nut crop with a high ecological value. Although many cultivars have been selected from natural seedlings, elite lines with comprehensive agronomic traits and characters remain rare. To explore genetic resources with aid of whole genome sequence will play important roles in modern breeding programs for chestnut. In this study, we generated a high-quality C. mollissima genome assembly by combining 90x PacBiosciences long read and 170x high-throughput chromosome conformation capture data. The assembly was 688.93 Mb in total, with a contig N50 of 2.83 Mb. Most of the assembled sequences (99.75%) were anchored onto 12 chromosomes, and 97.07% of the assemblies were accurately anchored and oriented. A total of 33,638 protein-coding genes were predicted in the C. mollissima genome. Comparative genomic and transcriptomic analyses provided insights into the genes expressed in specific tissues, as well as those associated with burr development in the Chinese chestnut. This highly contiguous assembly of the C. mollissima genome provides a valuable resource for studies aiming at identifying and characterizing agronomical-important traits, and will aid the design of breeding strategies to develop more focused, faster, and predictable improvement programs.

KEYWORDS Chinese chestnut Castanea mollissima Blume genome assembly single molecular sequencing high-throughput chromosome conformation capture

The Chinese chestnut (Castanea mollissima Blume), of the Fagaceae family (Figure 1), is one of the most anciently (>3500 years) domesticated and a multipurpose tree species (Zhang et al. 2005). It is cultivated in East Asia, primarily in China, Korea, and Vietnam (Huang et al. 1999). C. mollissima is a dominant ecological and economic tree (Zhang et al. 2005) which typically grows and flourishes in mountainous regions with poor soil. This species exhibits resistance to two major pathogens, Cryphonectria parasitica and Phytophthora cinnamomi, both responsible for the complete demise of the susceptible American chestnut (Castanea dentata), a dominant US forest species (Anagnostakis 2012; Fang et al. 2013). Thus, C. mollissima could be a useful genetic donor for the improvement of the resistance of American chestnuts to these pathogens (Fang et al. 2013). In addition to its ecological value, the nuts produced by C. mollissima are very delicious and contain many nutrients, including starch, protein, fat, fiber, calcium, phosphorus, and iron. Due to the high starch content and high nutritional value of the nuts, C. mollissima is categorized as a “woody grain and oil” plant (Li et al. 2016; Zhu 2016; Hong and Feng 2017). Over 300 C. mollissima cultivars are currently used for nut production. These cultivars are subdivided into five major regional groups: Northern, Yangtze River Valley, Sichuan and Guizhou, Southern, and Southwestern (Zhang et al. 2005). More than two million tons of C. mollissima nuts are produced in China each year (China-statistical-yearbook, http://www.shujuku.org/). However, as most of the cultivars have been artificially selected directly from natural chestnut seedlings, elite
lines with comprehensively selected character sets (e.g., nut quality, production, and processing adaptabilities) are still needed.

Because of the above important values, high-quality genomes are needed. The previous genome, especially the one supported by Staton et al. (2015) has played a huge role in the early stage of Chinese chestnut researches and utilization, such as in disease resistance QTL (Quantitative Trait Loci) location (Kubisiak et al. 2013). However, the existing genomic data of C. mollissima (Staton et al. 2015, 2019; Xing et al. 2019) are still fragmented and with no complete chromosomal information, due to the high heterozygosity of the genome and technical limitations. In order to identify important structural and functional elements of genomes and use valuable genetic variation for breeding, accurate, complete and contiguous genome assemblies are essential (Love et al. 2014). Previous studies have shown that combining Pacific Biosciences (PacBio) and illumina data significantly improve N50 length and generate more accurate assemblies (Koren et al. 2012; Jiao et al. 2017; Westbrook et al. 2019). High-throughput chromosome conformation capture (Hi-C) is a cost-effective technique for assigning, ordering, and orienting genomic sequences onto chromosomes (Lin et al. 2018). This technique has been successfully used to improve the assemblies of several complex genomes, including animals (Bickhart et al. 2017), grain crops (Avni et al. 2017; Mascher et al. 2017), oil crops (Yin et al. 2018), and fruit crops (Tang et al. 2019). We therefore constructed a contiguous genome assembly of C. mollissima, using a combination of PacBio long reads, and Hi-C data which provides a valuable source of genomic data for future molecular and breeding researches of the Chinese chestnut.

The burrs of chestnut fruits have very sharp spines (Zhang et al. 2005), which often harm chestnut farmers. Indeed, there are many reports of chestnut fruits stuck on farmers’ eyes (Zhang et al. 2013; García-García et al. 2016). Also, the varieties with thin burr are popular in chestnut production areas for their high productivity. Although Zhang once reported natural varieties with no spines or degenerated spines on burr (Zhang et al. 2005), we have been unable to locate these natural varieties in the described region. Thus, we hope that based on the assembly, genes controlling Burr development could be identified in order to promote burr trait improvement.

MATERIALS AND METHODS
Sample collection, library construction and sequencing
In order to get the common genome characteristic of C. mollissima, and eliminate interference from rootstocks with different genotypes, a seedling cultivar line N11-1 was selected as the sequencing sample. This line exhibits not only the common phenotype of C. mollissima, but also excellent characteristics such as high yield and high sugar content. Thus, it was selected as a potential variety, and further studies will be carried out on its excellent traits and pedigree. It is now conserved at the Wanjishan Experimental and Demonstration Station of Shandong Institute of Pomology, Tai’an, Shandong, China (36.206 E, 117.084 N). Genomic DNA was extracted from the leaves using the cetyltrimethyl ammonium bromide (CTAB) method, as described in the PacBio shared protocol (Rhoads and Au 2015). The quality of the extracted genomic DNA was assessed using 1% agarose gel electrophoresis.

All sequencing was performed by Biomarker Technologies Corporation (Beijing, China). For Illumina sequencing, paired-end (PE) library with insert sizes of ~350 kb was constructed following the protocol provided by Illumina Nova seq 6000 (San Diego, CA, USA). In total, 54.55 Gb of cleaned PE sequences were generated (Supplementary Table S1). The cleaned sequences were used for genome feature characterization (including genome size and heterozygosity level), assembly correction, and assembly evaluation.

Long read sequencing was performed on a PacBio Sequel sequencer (Pacific Biosciences, Menlo Park, CA, USA) following the manufacturer’s instructions (Rao et al. 2014). Genomic DNA were mechanically sheared and polished with DNA-damage repair and end-repair enzymes, followed by blunt-end ligation and exonuclease treatment. Fragments >10 kb were selected using Blue Pippin (Sage Science, Inc., Beverly, MA, USA), and used to generate the sequence library. A total of six SMRT (Single Molecule Real-Time) cells and 66.98 Gb of filtered subreads longer than 12 kb were used for assembly (Supplementary Table S2, Supplementary Figure S1).

We constructed Hi-C fragment libraries with insert sizes of 300-700 bp as illustrated in Rao et al. (2014). In brief, nuclear DNA was cross-linked in situ, extracted, and then digested with DpnII. The sticky ends of the digested fragments were biotinylated, diluted, and then randomly ligated to one another. Biotinylated DNA fragments were enriched, re-sheared, and then used to prepare the sequencing library, which was sequenced on Illumina Nova seq 6000 (San Diego, CA, USA).

RNA for transcriptome sequencing was extracted from C. mollissima tissues including leaves, stems, flowers (inflorescences), fruits, and roots, using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer’s instructions. Pools of total RNA from all sampled tissues were used to prepare RNA-Seq libraries using TruSeq RNA Sample Preparation Kits (Illumina, San Diego, CA, USA) following the manufacturer’s instructions. The prepared libraries were sequenced on an Illumina HiSeq 2500 platform (PE, 100-bp reads), generating approximately 10.3 Gb of data. These RNA-Seq data were used for genome annotation and to assess the completeness of the genome assembly.

Genome assembly
C. mollissima genome size (G) was initially estimated based on k-mer frequency (Kim et al. 2011), using the formula $G = (T_k - A_k)/D_k$, where $T_k$ was the total number of $k$-mers, $A_k$ was the number of abnormal $k$-mers (those either too frequent or too infrequent), and $D_k$ was the average $k$-mer depth. The sequencing depth was estimated by determining the highest peak value of the frequency curve of the $k$-mer occurrence distribution.

The PacBio reads were first corrected using the error correction module of Canu v1.5 (https://github.com/marbl/canu). In this correction step, Canu was used to select longer seed reads with the settings
“genomeSize = 700000000” and “corOutCoverage = 60,” and overlapping raw reads were detected using the highly sensitive overlap identifier MHAP (mhap v2.1.2; https://github.com/marbl/MHAP.git), with the option corMhapSensitivity set to “low/normal/high.” Error correction was then performed with Canu, using the falcon_sense method with correctedErrorRate set to 0.025. The error-corrected reads were trimmed by removing unsupported bases and hairpin adapters to obtain the longest supported range, using default parameters. The final cleaned reads were then assembled using Canu.

The PacBio raw reads were also de novo assembled using wtdbg v1.2.8 (https://github.com/ruanjue/wtdbg), with the command “wtdbg -i pbreads.fasta -t 64 -H -k 21 -S 1.02 -e 3 -o wtdbg.” We used the error-corrected reads from Canu to improve the assembly. Finally, a consensus assembly was generated using the command “wtdbg-cns -t 64 -i wtdbg.ctg.lay -o wtdbg.ctg.lay.fa -k 15.”

We merged the two assemblies generated by Canu and wtdbg using Quikmerge (Chakraborty et al. 2016, https://github.com/mahulchak/quickmerge.git), in order to integrate the advantages of the two different assemblers. In brief, we aligned the Canu-generated contigs to the wtdbg-generated contigs using MUMmer v4.0.0 (https://github.com/mummer4/mummer), with the nucmer parameter set “-b 500 -c 100 -l 200 -t 12” and the delta-filter parameter set “-i 90 -r -q.” Based on these alignments, the two set of contigs were merged using Quikmerge, with the parameter set “-hco 5.0 -c 1.5 -l 100000 -ml 5000.”

The merged assembly was polished with the Illumina reads using Pilon v1.22 (Pilon, RRID: SCR 014731), with the parameter set “-mindepth 10 -changes -threads 4 -fix bases.”

**Assembly revision and validation**

We used Hi-C data to correct and optimize our initial assembly. The clean Hi-C reads were first truncated at the putative Hi-C junctions and then the resulting trimmed reads were aligned to the assembly results with BWA v0.7.10 (BWA, RRID: SCR 010910). Only uniquely alignable paired reads, with mapping quality >20, were retained for further analysis. Invalid read pairs, including dangling-end, self-cycle, re-ligation and dumped products, were filtered by Hi-C-Pro v2.8.1 (https://github.com/nservant/HiC-Pro). The valid interaction pairs were used to correct the scaffolds. Scaffolds were clustered, ordered and oriented onto chromosomes by using LACHESIS (http://shendurelab.github.io/LACHESIS/).

Before chromosome assembly, we preassembled scaffolds to correct errors, which required splitting the scaffolds into segments average of 50 kb long. The Hi-C data were mapped to these segments using BWA. The uniquely mapped data were retained and assembled using LACHESIS (Burton et al. 2013). Placement and orientation errors exhibiting obvious discrete chromatin interaction patterns indicate misassemblies and were manually adjusted. We examined the interaction map for each contig and broke 173 that were possibly misassembled. The corrected scaffolds were assembled with LACHESIS using the following parameters: CLUSTER MIN RE SITES = 52, CLUSTER MAX LINK DENSITY = 2, CLUSTER NONINFORMA TIVE RATIO = 2, ORDER MIN N RES IN TRUN = 46, and ORDER MIN N RES IN SHREDS = 42. The Hi-C heatmap of *C. mollissima* was generated using HiCplotter (Akdemir and Chin 2015), at a resolution of 100-kb.

We assessed the completeness of the *C. mollissima* assembly using the Benchmarking Universal Single-Copy Orthologs (BUSCO) (BUSCO, RRID: SCR 015008), against the embryophyta_odb 10 data-set. We also evaluated the completeness of the assembly by mapping Illumina PE short reads back to the assembled genome using BWA.

**Genome annotation**

Protein-coding genes in the *C. mollissima* genome assembly were predicted using a combination of *ab initio*, homolog, and RNA-Seq gene prediction approaches. *Ab initio* predictions were performed using a combination of Genscan (Genscan, RRID: SCR 012902), Augustus v2.4 (Augustus, RRID: SCR 008417), GlimmerHMM v3.0.4 (GlimmerHMM, RRID: SCR 002654), GeneID (v1.4), and SNAP v2006-07-28 (SNAP, RRID: SCR 002127), using parameters trained against *Arabidopsis* gene models. Homolog-based gene prediction was performed with GeMoMa (v1.3.1; Keilwagen et al. 2016), using the protein databases of several plant species: *Arabidopsis thaliana* (GenBank accession no. GCA_000001735.2), *Oryza sativa* (GenBank accession no. GCA_001433935.1), and *Fraxinus excelsior* (GenBank accession no. GCA_900149125.1), and *Quercus suber*.
To predict protein-coding genes with RNA-Seq, we aligned the RNA-Seq data to the *C. mollisima* genome using Hisat v2.0.4, and assembled the genes into transcripts using Stringtie v1.2.3. TransDecoder v2.0, GeneMarkS-T v5.1 (RRID: SCR 011930), and PASA (PASA, RRID SCR 014656) were then used to identify protein-coding genes. Finally, we integrated the protein-coding genes predicted by each of the three methods (*ab initio*, homology, and RNA-Seq) using EVM v1.1.1 (RRID: SCR 014659).

The protein-coding genes were annotated against several databases, including GenBank non-redundant protein database (Nr), eukaryotic orthologous groups of proteins (KOG), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (KEGG, RRID: SCR 001120), and TrEMBL, using the Basic Local Alignment Search Tool (BLAST v2.2.31; Altschul et al. 1990) with an e-value cutoff of 1E-5. Gene Ontology (GO) terms were assigned to the protein-coding genes using the BLAST2GO pipeline (Blast2GO, RRID:SCR 005828).

To predict pseudogenes, we used GenBlastA (https://github.com/pvanheus/genblastA_to_gff.git) to scan the *C. mollisima* genome for homologous sequences and to identify known protein-coding genes. Within those homologous sequences, we used GeneWise (GeneWise, RRID: SCR 015054) to search for frameshift mutations or premature stop codons. Gene sequences containing frameshift mutations or premature stop codons were considered pseudogenes.

A *de novo* repeat library was constructed based on the *C. mollisima* assembly using three software packages: LTR-FINDER (v1.0.5; https://github.com/xzhub/LTR_Finder.git), RepeatScout (v1.0.5; https://github.com/mmccco/RepeatScout.git), and PILER-DF v2.4 (Edger and Myers 2005). The predicted repeats were classified using PASTEClassifier v1.0 (Hoede et al. 2014) and merged with Rebase (Bao et al. 2015). Finally, using the resulting repeat database as the repeat library, we identified repetitive sequences in the *C. mollisima* genome using RepeatMasker v4.0.5 (RepeatMasker, RRID: SCR 012954) with the following parameters: `-nolow -nois -norna -engine wublast`.

Figure 2 Distribution of the interaction frequencies among chromosomes. The distribution is based on the 170x high-throughput chromosome conformation capture (Hi-C) links.
Table 3 Comparison of assembly quality in three genomes of *C. mollissima*

| Parameter                       | Castanea mollissima genome assembly |
|--------------------------------|-------------------------------------|
|                                | I     | II     | III    |
| Total sequence length (Mb)     |       | 689.98 | 785.5  | 725.2  |
| Contigs                         | 671   | 2,707  | 15,892 |
| Total contig No.                |       |        |        |
| Contig N50 (kb)                 | 2,828.6 | 944.5  | 96.7   |
| Contig N90 (kb)                 | 455.88 | 133.7  |        |
| Longest contig length (Mb)     | 19.3  | 6.58   |        |
| Scaffolds                       |       |        |        |
| Total scaffold No.              | 112   |        | 14,110 |
| Total scaffold length (Mb)      | 689.98|        |        |
| Scaffold N50 (kb)               | 57,343.43 |        |        |
| Scaffold N90 (kb)               | 4,301.26 |        |        |
| Longest scaffold length (Mb)    | 90.2  |        |        |
| Anchored onto chromosome (Mb/%)| 687.3/99.75 |        |        |
| Anchored with order and orientation (Mb/%) | 667.1/97.07 |        |        |

Footnotes: I: Present assembly; II: Assembly published by Xing et al.; III: Assembly submitted by Clemson University Genomics Institute. The assembly II has no scaffold chromosome-level information, so the detailed comparisons were mainly on contig-level.

Comparative genomic analysis

We identified gene family clusters in the complete gene sets of *C. mollissima* and eight other plant species (*O. sativa, Solanum tuberosum, A. thaliana, Quercus robur, Casuarina glauca, Juglans regia, Populus trichocarpa, and Populus euphratica*) using OrthoMCL (v2.0.9; Li et al. 2003) Gene family clusters were identified by first eliminating all genes encoding proteins less than 50 aa long in the *C. mollissima* gene sets. Second, similar proteins were identified in the genomes of the nine plant species listed above. Third, identified genes were clustered using OrthoMCL to obtain single-copy gene families and species-specific gene families. A species-level maximum likelihood phylogeny was constructed based on the alignment of 863 concatenated single-copy family genes from the nine plant species using Phyml release 20151210 (Kimura 1981), with the bootstrap set to 1000. Simultaneously, the *C. mollissima* proteins identified in step one were searched against the *Q. robur* dataset using BlastP (with an evalue cutoff of 1E-10). After identifying the chromosome carrying of each gene, gene collinearity between the two genomes was determined using MCScanX (Wang et al. 2012) with the parameters “-s 10 -b 2.” The divergence time was estimated by MCMCtree in the PAML (4.7a package; Yang 1997) using the known divergence time of *C. glauca* and *J. regia*, and *O. sativa* and *A. thaliana* from the Time-Tree database. In addition, we inferred gene family expansion and contraction using CAFE V4.2 (Kim et al. 2011).

RNA-Seq analysis of tissue-specific expression

We analyzed the RNA sequences of several tissues, including the root, stem, leaf, flower (inflorescence) and fruit. To identify genes associated with burr development, we analyzed both the entire fruit and fruit without the burr. RNA sequences were analyzed using BMKCloud (www.biocloud.net). RNA-seq short reads were aligned to the *C. mollissima* genome assembled in this study using HISAT2 v2.0.4 software, with one mapped location being selected randomly for reads mapped to multiple locations. Gene expression levels in terms of fragments per kilobase of transcript per million fragments mapped reads (FPKM) values (Florea et al. 2013), were computed with Stringtie v1.2.33. Genes were considered expressed if the FPKM was >0. Differentially expressed genes (DEGs) analysis were identified using DESeq2 (Love et al. 2014) with the FDR = 0.01 and fold change set to ≥ 2. GO annotations and KEGG pathway enrichment analyses were performed for the tissue-specific DEG using the Database for Annotation, Visualization and Integrated Discovery (DAVID), as implemented in BMKCloud. We also determined the GO annotations and KEGG pathway enrichments of genes expressed only in the whole fruit.

Data availability

Raw data and assembled sequence data for *C. mollissima* are available via NCBI (Bioproject accession PRJNA559042). The assembly and annotation data of *C. mollissima* are in the Genome Warehouse in BIG Data Center under accession number GWHANWH0000000, which are accessible at https://bigd.big.ac.cn/gwh. Supplemental material available at figshare: https://doi.org/10.25387/g3.12858206.

RESULTS

Genome size, heterozygosity and assembly

Based on the distribution of 19-mers among the Illumina HiSeq reads (i.e., the 54.55 Gb cleaned reads from the ~350 bp insert library). The genome (G) of *C. mollissima* was estimated to be 655.18 Mb, with...
approximately 0.78% heterozygosity. The k-mer distribution curve peaked at a depth of 70, with a k-mer number of 46,432,321,425 (Figure S2). The peak at a k-mer depth of 141 was a repetitive peak (i.e., k-mers duplicated because of repetition). The data used for the 19-mer analysis represented approximately 83× coverage of the genome.

The 66.98 Gb PacBio long reads used for de novo assembly represented 80-fold coverage of the estimated C. mollissima genome. The average length of these PacBio reads was 13,408 bp; the longest read was 90,903 bp and the N50 was 22,633 bp (Supplementary Table S3). Using Canu v1.5, we generated a draft assembly of 994,461,872 bp (Supplementary Table S4); using wtdbg v1.2.8, the assembly was 782,890,668 bp. Subsequently, by Quickmerge, we got the initial assembly of 793.80 Mb, with 1,381 contigs and a contig N50 of 3.38 Mb (Supplementary Table S4), which was much larger than k-mer estimation (655.18 Mb).

Genome assembly correction and validation based on chromatin interactions

Our Hi-C sequencing generated a total of 110.83 Gb cleaned data, representing ∼170-fold coverage of the C. mollissima genome, with 91.82% high-quality (Q30) bases (Supplementary Table S5). Approximately 89.63% of the Hi-C reads were mapped to the initial assembly, and 59.63% of the mapped read pairs were uniquely mapped (Supplementary Table S6). Of the 210.65 million unique paired alignments, 161.13 million (76.49%) were valid interaction pairs (Supplementary Table S7). Thus, the Hi-C sequences were sufficient for the refinement of the initial assembly.

After performing error correction using Hi-C sequence data, we generated a final assembly of the C. mollissima genome. The assembly was 688,929,566 bp long, with a contig N50 of 2.83 Mb (Table 1). In this assembly, 652 contigs (687,278,892 bp; 99.75%) were clustered onto 12 pseudochromosomes, corresponding to the 12 chestnut chromosomes. A total of 571 of the clustered contigs with a total length of 667,130,435 bp (97.07%) were correctly ordered and oriented (Table 2). The Hi-C heatmap of C. mollissima, generated by HiCplotter indicated that the frequency of intra-chromosome interactions decreased rapidly with linear distance (Figure 2). Compared with previously published genomes of C. mollissima, the additional mapping of the Hi-C data to the genome yielded a much more continuous, chromosome-level assembly (Table 3).

BUSCO analysis indicated that 92.44% of the core eukaryotic genes (1492 of 1614) were captured by the C. mollissima genome assembly, and that 88.79% (1,433 of 1614) were complete (Supplementary Table S8). We found that 99% of the Illumina PE short reads could be mapped, and more than 95% were properly mapped in pairs (Supplementary Table S9).

Genome annotation

Using a combination of ab initio, homology, and RNA-Seq gene prediction approaches, we predicted 33,638 genes in the C. mollissima genome assembly. A total of 33,074 of the predicted genes were based on homology, and RNA-Seq predictions (Supplementary Tables S10, S11 and Figure S3), 97.73% were classified into Annotation database (Table S12). We identified 366,839,546 bp of repetitive sequences, accounting for 53.24% of the assembly, including 34.24% retrotransposons and 5.52% DNA transposons. Long terminal repeat (LTR) transposons (24.17%) were the largest family of repeats (Supplementary Table S13).

Comparative genomic analysis of C. mollissima and other plant species

We determined gene family clusters in the complete gene sets of C. mollissima and eight other plant species (O. sativa, S. tuberosum, A. thaliana, Q. robur, C. glauca, J. regia, P. trichocarpa, and P. euphratica). Of the 33,638 genes in the C. mollissima genome, 30,192 were grouped into 15,367 gene clusters; 508 of these clusters were specific to C. mollissima (Table 4). The Chinese chestnut-specific gene families contained 3,201
genes. Of these, four genes (EVM0002617, EVM0020045, EVM0029632 and EVM0032868) were associated with the plant-pathogen interaction pathway; three genes (EVM0008748, EVM0019137 and EVM0030327) were associated with vitamin B6 metabolism; two genes (EVM0007389 and EVM0020714) were associated with starch and sucrose metabolism; and one gene (EVM0002118) was associated with mismatch repair. An additional two genes (EVM0004145 and EVM0007164) were functionally categorized in plant self-incompatibility protein S1 family.

The species-level maximum likelihood phylogeny, based on alignment of 863 concatenated single-copy family genes from nine plant species, recovered a sister relationship between C. mollissima and Q. robur (Figure 3). Next, the divergence time of C. mollissima and Q. robur was estimated to be \(~18.3\) million years ago (Mya) (Figure 3). We identified 1,057 gene families that were expanded in the Chinese chestnut compared to other species (Figure 3).

As the assembly was finely anchored and oriented onto the 12 pseudochromosomes with the aid of Hi-C sequence data, MCScanX identified long syntenic blocks shared between the genomes of C. mollissima and Q. robur (Figure 4). There were 18,399 collinear genes, accounting for 30.95% of the total genes.

Figure 4 Genomic synteny between the Chinese chestnut and the European oak. European oak chromosomes are labeled “Qrob_chr”; Chinese chestnut chromosomes are labeled “LG”.
Transcriptome analysis of tissue-specific expression

We identified 2,533 tissue-specific genes: 569 expressed in young fruit, 775 expressed in the root, 247 expressed in the shoot, 298 expressed in the leaf, and 644 expressed in the flower (Figure 5A). KEGG enrichment analyses of these tissue-specific genes showed that gene functions correlate well with the known biological roles of each tissue. That is, the pathways photosynthesis-antenna proteins (ko00196), photosynthesis (ko00195) and porphyrin and chlorophyll metabolism (ko00860) were significantly enriched in the leaf (Rich factors of 17.55, 7.45, and 7.41 respectively) (Figure 5B), while the starch and sucrose metabolism (ko00500, P = 3.2 × 10^{-4}) and pentose and glucuronate interconversions (ko00040, P = 1.8 × 10^{-8}) pathways were significantly enriched in flowers (Figure 5C). Similarly, the phenylpropanoid metabolism (ko00940, P = 1.62 × 10^{-12}) pathway, which is required for the lignin biosynthesis (Boerjan et al. 2003) was the most enriched pathway in the root. In the young fruit, amino sugar and nucleotide sugar metabolism (ANM) pathway was highly enriched (Figure 5D). ANM products have many roles in living organisms, in particular, these products are important for maintaining and repairing cell walls (Lee et al. 2016) in starch storage organs (Zuo et al. 2010), which is consistent with the high starch content of the Chinese chestnut. Three genes (EVM0005986, EVM0008813 and EVM0017354) were expressed only in the entire fruit (which included the burr).

DISCUSSION

As a perennial tree, the Chinese chestnut is highly heterozygous, meaning that genome assemblies for this species are typically highly fragmented, incomplete, and larger than they should be. Long read technologies can improve the resolution of highly repetitive regions (Waterston et al. 2002; Eid et al. 2009; Gnerre et al. 2011; Ono et al. 2013; Ghurye et al. 2017; Jiao et al. 2017; Staton et al. 2019), and Hi-C techniques can capture interactions over much larger genomic distances and produce scaffolds spanning a complete chromosome arm (Lajoie et al. 2015; Putnam et al. 2016; Jia et al. 2019; Liang et al. 2019). Here, combining the advantages of both technologies coupled with high sequencing depth, we got the final genome assembly of 688.93 Mb, which was 104.87 Mb smaller than initial SMRT assembly. Compared with previous published genomes (Staton et al. 2019; Xing et al. 2019) (Table 3), the genomic continuity has been greatly improved.
improved, ex. contig N50 length were three times longer (2.83 Mb vs. 944 Kb); the contig number reduced more than three times (671 vs. 2,707). The total scaffold number was 112, and 687.3 Mb (99.75%) sequences were anchored onto chromosomes. The previous published genome assembly lacked correction based on Hi-C data, thus no chromosome location information, and the size was 785.5 Mb, which is almost the same as our initial SMRT assembly (793.80 Mb). Collectively, we believe that the assembly in this study is the most contiguous and highest quality available for *C. mollissima* so far.

According to comparative genomic analyses of *C. mollissima* and eight other plant species (*O. sativa*, *S. tuberosum*, *A. thaliana*, *Q. robur*, *C. glauca*, *J. regia*, *P. trichocarpa*, and *P. euphratica*), the specific genes of *C. mollissima*, such as self-incompatibility, plant-pathogen interaction pathway, starch and sucrose metabolism will provide clues for understanding the adaptive evolution and nutritive value of Chinese chestnut. Phylogenetic tree represents a close relationship between *C. mollissima* and *Q. robur*, which was consistent with previous results (Staton et al. 2019; Xing et al. 2019). Due to the different species selected, the estimated divergence time would be different, and the time would be larger when *A. thaliana* and *O. sativa* were simultaneously used to estimate the divergence time. In this study, the divergence times between the distant species are similar with Zhang et al. (2017), and of *C. mollissima* and *Q. robur*, the time is slightly different (18.3 Mya vs. 13.62 Mya) with the result of Xing et al. (2019). The Circos plot in Figure 4 illustrates the high degree of collinearity at the whole chromosome level between *C. mollissima* and *Q. robur*, thus, the gene/trait information can potentially be shared between the two species.

We performed RNA-Seq analysis of Chinese chestnut tissues to identify genes specifically expressed in certain tissues, including the root, stem, leaf, flower (inflorescence) and fruit. Most genes were found to be highly expressed in all evaluated tissues, whereas the others only expressed in one or a few tissues, indicating they may play different roles in the regulation of related pathways (Mascher et al. 2017; Zhang et al. 2017; Jia et al. 2019; Liang et al. 2019). We identified three genes (*EVM0005986, EVM0008813* and *EVM0017354*) that were expressed in the complete fruit but not in the root, shoot, leaf, flower or peeled fruit. Therefore, we assumed that these genes were expressed only in the burrs. In the SwissProt database, *EVM0008813* is annotated as root meristem growth factor 3 in *Arabidopsis thaliana*. Thus, this gene may be associated with burr thickness or spine development. In future studies, we will further characterize these genes in order to better understand burr trait development in the Chinese chestnut.

**CONCLUSION**

In this study, we used an integrated PacBio, Illumina and Hi-C sequencing approach to assemble the most contiguous and high-quality reference genome sequence for *C. mollissima* known to date. The assembly constructed herein will be useful for studies aiming at deciphering the structure and evolution of complex genomes, including *C. mollissima* and other related plant species. In addition, specific gene expression patterns in *C. mollissima* will support in-depth genetic analyses of traits of interest in this species, such as disease resistance, burr development, tolerance to adverse growing conditions, and production of highly nutritious nuts.

**ACKNOWLEDGMENTS**

The authors are grateful to Prof. Zhangjun Fei for critically reading of this manuscript. We thank Letpub (www.letpub.com) for linguistic assistance during the preparation of this manuscript. This work was financially supported by grants from Agricultural science and technology innovation project of Shandong Academy of Agricultural Science (CXGC2016B07, CXGC2018F03, CXGC2016A03), Shandong Provincial Key Laboratory for Fruit Biotechnology Breeding (2018KF03), Shandong Provincial Seed Improvement Project (2016LZGC012).

**LITERATURE CITED**

Akdemir, K. C., and L. Chin, 2015 Hicplotter integrates genomic data with interaction matrices. Genome Biol. 16: 198. https://doi.org/10.1186/s13059-015-0767-1

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, 1990 Basic local alignment search tool. J. Mol. Biol. 215: 403–410. https://doi.org/10.1016/0022-2836(90)90570-K

Anagnostakis, S. L., 2012 Chestnut breeding in the United States for disease and insect resistance. Plant Dis. 96: 1392–1403. https://doi.org/10.1094/PDIS-04-12-0350-FE

Avni, R., M. Nave, O. Barad, K. Baruch, S. O. Twardzik et al., 2017 Wild emmer genome architecture and diversity elucidate wheat evolution and domestication. Science 357: 93–97. https://doi.org/10.1126/science.aan0032

Bickhart, D. M., B. D. Rosen, S. Koren, B. L. Sayre, A. R. Haste et al., 2017 Single-molecule sequencing and chromatin conformation capture enable de novo reference assembly of the domestic goat genome. Nat. Genet. 49: 643–650. https://doi.org/10.1038/ng.3802

Bao, W., K. K. Kojiman, and O. Kohany, 2015 Repbase Update, a database of repetitive elements in eukaryotic genomes. Mob. DNA 6: 11. https://doi.org/10.1186/s13100-015-0041-9

Boerjan, W., J. Ralph, and M. Baucher, 2003 Lignin biosynthesis. Annu. Rev. Plant Biol. 54: 519–546. https://doi.org/10.1146/annurev.arplant.54.031902.134938

Burton, J. N., A. Adey, R. P. Patwardhan, R. Qiu, J. O. Kitzman et al., 2013 Chromosome-scale scaffolding of de novo genome assemblies based on chromatin interactions. Nat. Biotechnol. 31: 1119–1125. https://doi.org/10.1038/nbt.2727

Chakraborty, M., J. G. Baldwin-Brown, A. D. Long, and J. J. Emerson, 2016 Contiguous and accurate de novo assembly of metazoa genomes with modest read coverage. Nucleic Acids Res. 44: e147.

Hoede, C., S. Arnoux, M. Moisset, T. Chaumier, O. Inizan et al., 2014 Pastec: an automatic transposable element classification tool. PLoS One 9: e91929. https://doi.org/10.1371/journal.pone.0091929

Eid, J., A. Fehr, J. Gray, K. Luong, J. Lyle et al., 2009 Real-time DNA sequencing from single polymerase molecules. Science 323: 133–138. https://doi.org/10.1126/science.1162896

Edgar, R. C., and E. W. Myers, 2005 PILEUR: Identification and classification of genomic repeats. Bioinformatics 21:i512–i518. https://doi.org/10.1093/bioinformatics/bti1003

Fang, G. C., B. P. Blackmon, M. E. Staton, T. Zhebentyayeva, C. Smith et al., 2013 A physical map of the Chinese chestnut (*Castanea mollissima*) genome and its integration with the genetic map. Tree Genet. Genomes 9: 525–537. https://doi.org/10.1007/s11295-012-0576-6

Florea, L., L. Song, and S. L. Salzberg, 2013 Thousands of exon skipping events differentiate among splicing patterns in sixteen human tissues. PLoS Res. 2: 188. https://doi.org/10.1368/f1000research.2-188.v1

García-García, G. P., N. C. Esaa-Caride, N. D. Jurado-Guano, and L. Muñoz-Bellido, 2016 Ocular Injury With Chestnut Burr: Our Experience. Cornea 35: 1315–1319. https://doi.org/10.1097/ICO.0000000000000952

Gnerre, S., I. Macallum, D. Przybylski, F. J. Ribeiro, J. N. Burton et al., 2011 High-quality draft assemblies of mammalian genomes from massively parallel sequence data. Proc. Natl. Acad. Sci. USA 108: 1513–1518. https://doi.org/10.1073/pnas.1017351108

Hong, Y., and L. Feng, 2017 Actual effects of subsidy policy for woody grain and oil plants and their economic implication. Scientia Silvae Sinicae 53: 113–119. https://doi.org/10.11707/j.1001-7488.20170813

Huang, C., Y. Zhang, and B. Bartholo, 1999 Fagaceae Dumortier, pp. 314–400 in Flora of China, Wu, Z. Y., and P. H. Raven, eds. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis, MI.

**Volume 10 October 2020 | Genome Assembly of the Chinese Chestnut | 3573**
