A high-quality genome assembly of *Lactarius hatsudake* strain JH5

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Abstract

*Lactarius hatsudake* is a species of *Lactarius* commonly found in pine forests, is edible with a delicious and nutritious fruiting body, and exhibits medicinal properties. It is an ideal natural multifunctional food with bioactive components including fungal polysaccharides, crude fiber, unsaturated fatty acids, nucleic acid derivatives, various amino acids, and vitamins. However, biological and genomic analyses of this mycorrhizal mushroom are sparse, thereby hindering large-scale cultivation. Previously, we isolated and screened *L. hatsudake* JH5 strains and have applied our garnered knowledge to the large-scale cultivation of mycorrhizal seedlings. In this study, we produced a high-quality genome assembly of *L. hatsudake* JH5 by combining Illumina paired-end and PacBio single molecule real-time sequencing, resulting in PacBio single molecule real-time reads of 7.67 Gb and Illumina Pair-End reads of 1,560 Mb. Based on the distribution of k-mer frequencies, the genome size of this strain was estimated to be 63.84 Mb (1.14% heterozygosity). Based on de novo genome assembly, the final genome size was determined to be 76.7 Mb, with scaffold N50 of 223.2 kb and N90 of 54.5 kb, and a GC content of 54.38%. BUSCO assessment showed that genome completeness was 89.0%. The N50 length of the JH5 genome was 43.6% longer than that of the previously published *L. hatsudake* MG20 genome. This high-quality *L. hatsudake* genome assembly will facilitate research on the functional genome, molecular breeding, yield enhancement, and sustainability of *L. hatsudake* cultivation.

Keywords: *Lactarius hatsudake*; Illumina; PacBio; genome

Introduction

*Lactarius hatsudake* Tanaka, also known as red milk mushroom, is a high-quality wild edible and medicinal mycorrhizal fungus that is symbiotic with Pinaceae or Quercus. It is principally distributed in North America, Europe, and Southeast Asia within China, Korea, Thailand, and Japan (Fang et al. 2006). The fruiting bodies of *L. hatsudake* are nutritious and rich in bioactive components such as fungal polysaccharides, crude fiber, unsaturated fatty acids, nucleic acid derivatives, various amino acids, and vitamins (Miayazawa et al. 2010; Wang et al. 2016). Previous studies have shown that *L. hatsudake* is able to alleviate symptoms of Diabetes patients, improve immune responses, and inhibit pathogenic bacteria (Zhang et al. 2007; Tako et al. 2012), thus serving as an ideal natural multifunctional high-grade food source. *Lactarius hatsudake* is also known as cold fungus, wild goose fungus, gongo fungus, silk fungus, and purple flower fungus and has become a major species in the wild edible mushroom trade in southeastern China (Li et al. 2018) with popularity extending to Japan, South Korea, Thailand, among other locations (Miayazawa et al. 2010; Tako et al. 2012). The mushroom can be consumed fresh, frozen, or processed into mushroom oil.

Successful cultivation of *L. hatsudake* has been a long-standing desire within production regions. Initially, *L. hatsudake*–P. massoniana Lamb was obtained by tissue isolation and mycorrhizal synthesis techniques (Tan 2005, 2006; Tan et al. 2007). Currently, the red milk mushroom plantation in Hunan Province covers 118 hectares with the fruiting bodies produced in 3–4 years after mycorrhizal seeding. Successful cultivation of *L. hatsudake* has led to a novel forest economy with associated ecological and economic benefits. However, due to the extended cultivation time and a need for extensive plantation management, it has been difficult to reliably achieve stable and high yields. A lack of comprehensive whole-genome analysis for *L. hatsudake* has also served to limit mechanistic analysis. What is currently known is that, based on whole-genome sequencing, sexual reproduction was found to be heterotypical coordinated in *Tuber melanosporum* (Martin et al. 2010), while *Laccaria bicolor* was found to possess a family of genes associated with symbiosis, called the "symbiosis..."
Materials and methods

Fungal strain and DNA extraction

Lactarius hatsudake strain JHS was obtained from the production and promotion base established by the project team in Pumen Village, Jiah County, Chenzhou City, Hunan Province. Lactarius hatsudake JHS was selected which was stored in the General Microbiology Center of China microbial species Preservation and Administration Committee, registration number CGMCCNo19369. Mycelia of JHS was grown in 100-ml biotin-aneurine-folic acid liquid medium at 22°C, 120 rpm for 14 days in darkness. Then JHS mycelia were separated from the culture medium, frozen in liquid nitrogen and ground to a fine powder and subjected to genome sequencing. DNA was extracted using the DNA extraction kit from DNAeasy plant mini kit (Qiagen). DNA quality and concentration were assessed using the Biorad spectrophotometer.

Genome sequencing and assembly

The genome of L. hatsudake JHS was de novo sequenced using high-throughput Illumina Hiseq X-Ten and PacBio RSII long-read sequencing platforms (PacBio P6-C4) at Beijing Novogene Technology. DNA libraries with 350-bp inserts were constructed and sequenced on the Illumina Hiseq-X-Ten platform. For the PacBio RSII platform, a 20-kb library was generated and sequenced. The genome size of L. hatsudake JHS was estimated by the k-mer method using sequencing data from the Illumina DNA library. A 15-mer frequency distribution analysis of the quality-filtered reads was performed using Jellyfish v2.2.10 (Marcia and Kingsford 2011). Genome size, heterozygosity, and repeat content were then estimated by the Genome Scope web tool (Vurture et al. 2017).

The genome of JHS was de novo assembled in 3 steps. Assembly of contigs was performed with FALCON (version 0.7.0; Chin et al. 2016). In brief, the longest 40× reads were selected as “seed” reads for error correction (“pre-assembly”). Preassembly in FALCON uses DAAligner to perform all-by-all alignments of the raw reads. The FALCON assembly resulted in 312 primary contigs. The initial polishing was performed with Arrow (included in the FALCON-Unzip) exclusively using PacBio (https://www.pacb.com/support/software-downloads/) long reads, and then SSPACE-LongReads was implemented to scaffold the contigs. Finally, Pilon (v 1.23; Walker et al. 2014) was utilized to further correct the PacBio-corrected contigs with accurate Illumina short reads and generate the genome assembly of L. hatsudake JHS. The completeness of the JHS genome assembly was evaluated using BUSCO 3.1.0 (Benchmarking Universal Single-Copy Orthologs) with comparison to lineage dataset fungi_odb9 (Creation date: 2016 October 21, number of species: 85, number of BUSCOs: 290; Simao et al. 2015).

Genome annotation

To annotate the assembled JHS genome, we used funannotate (v1.5.2; Love et al. 2019) with the pipeline described in https://funannotate.readthedocs.io/en/latest/tutorials.html with the following commands: funannotate mask, to softmask the genome, funannotate training, and funannotate predict to generate preliminary gene models, and consensus gene models (using AUGUSTUS (Stanke and Waack 2003), GeneMark (Borodovsky and McIninch 1993), and EvidenceModeler (Haas et al. 2008)). funannotate annotate to add functional annotation, in addition, protein-coding gene (PCG) models also were identified according to our 12 transcriptome data (PRJNA841037). The rRNA was predicted by using RNAmmer v1.2 (Lagensen et al. 2007), and tRNAs were identified with tRNAscan-SE v1.4 (Lowe and Eddy 1997). The sRNA was identified by comparing with the Rfam database (Gardner et al. 2009). The functional annotation obtained with funannotate includes Interpro terms, Pfam domains, CAZYmes (CAZY_DB: 201604), secreted proteins, proteases (MEROPS), BUSCO groups, EggnoG annotations, Clusters of Orthologous Groups (COGs), GO ontology, secretion of signal peptides, and transmembrane domains (the full annotation is available in Supplementary Table 1).

Results and discussion

High-quality genome assembly of L. hatsudake JHS

To construct a high-quality reference genome of L. hatsudake JHS, a total of 7.67 Gb PacBio single molecule real-time (SMRT) reads and 1,560 Mb Illumina pair-end reads were generated in this study. The PacBio read lengths ranged from 200 to 50,000 bp with an average read length of 7,418 bp (Fig. 1a). We estimated the genome size of L. hatsudake JHS as 63.84 Mb with a heterozygosity rate of 1.14% via the distribution of k-mer frequency using Illumina paired-end (PE) reads (Fig. 1b). High-quality PacBio SMRT reads were used to assemble the L. hatsudake genome. The contigs were then polished using Illumina PE reads, which yielded a draft genome assembly of 76.7 Mb, with contig N50 of 223.2 kb, N90 of 5.0 kb, GC content of 54.4%, and a BUSCO result of 89.0% (Table 1). We annotated 19,616 genes with an average gene length of 1,765 bp. The cumulative length of genes was 34.6 Mb, which accounted for 45.14% of the whole JHS genome (Tables 1 and 2). The size of contig N50 and BUSCO results was higher than that of the previously published L. hatsudake MG20 genome (contig N50: 5,268 bp, BUSCO result: 84.5%; Tables 1 and 2).
Compared with the N50 obtained only by Illumina sequencing, the N50 length was 44.6 times higher and the number of genes increased by 1.06 times. The Scaffold N(x) length distribution in *L. hatsudake* JH5 was also significantly higher than the length of Scaffold N(x) distribution in *L. hatsudake* MG20 (Fig. 2).

In total, this *L. hatsudake* genome assembly represents a significant improvement than that of other previously released *Lactarius* genomes (contig N50: 5.0–261.3 kb; Table 1; Saier et al. 2014; Li et al. 2018; Lebreton et al. 2022). Compared with *L. hatsudake* 109 genome assembly, JH5 genome show the considerable N50 length (223.2 kb vs 261.3 kb), and BUSCO results (89.0% vs 87.6%). However, the number of scaffolds of JH5 genome was far fewer than that of *L. hatsudake* 109 genome (312 vs 815), which indicated JH5 genome presented here was less fragmented. Furthermore, 2,785 additional genes were predicted compared to *L. hatsudake* 109 genome (Tables 1 and 2).

### Identification of repetitive sequences

In this study, a total of 33,787 repeat sequences were predicted within the *L. hatsudake* JH5 genome. Among these, the number of
long terminal repeat sequences was 12,436, which accounted for 10.18%, at an average length of 651 bp. This was followed by tandem repeat sequences (TR), which consisted of 10,801 sequences (1.57% of total bases), long interspersed nuclear elements (0.19%), short interspersed nuclear elements (0.13%), minisatellite DNA (0.57%), and microsatellite DNA (0.09%) (Table 3).

**Table 2.** Comparison of JH5, MG20, and 109 genome assembly and annotation.

| Sample ID   | JH5 | MG20 | 109 |
|-------------|-----|------|-----|
| Scaffolds   | 312 | 36,963 | 815 |
| Contigs     | 312 | 37,440 | 815 |
| Max length of contigs (bp) | 3,148,306 | 85,124 | 1,836,981 |
| N50 length of contigs (bp) | 223,180 | 5,268 | 261,250 |
| N90 length of contigs (bp) | 54,521 | 519 | 51,284 |
| Total length (Mb) | 76.7 | 73.8 | 95.5 |
| GC (%)      | 54.4 | 51.9 | 52.1 |
| Gene number (#) | 19,616 | 18,513 | 16831 |
| Gene total length (Mb) | 34.6 | 19.0 | 29.0 |
| Gene average length (bp) | 1,765 | 1,025 | 1,724 |
| Gene length/genome (%) | 45.1 | 26.0 | 30.4 |

**Identification of noncoding RNAs**
Noncoding RNAs are a type of RNA that has been found to perform a variety of biological functions. It does not carry information that is translated into proteins though it still directly plays a role in activities at the RNA level (Bracher et al. 2020). Among microbes, sRNA, rRNA, and tRNA are the most commonly studied. For *L. hatsudake* JH5, tRNAs were found to be the most abundant, with a total length of 15.97 kb. This was followed by 5S rRNA, 18 and 28 s, for a total of 9, with a total length of 8.0 kb. In addition, there were 19 snRNAs, with an average length of 121 bp and a total length of 2.3 kb (Table 4).

**Gene function analysis**
The EVM pipeline was used to predict the PCGs of the *L. hatsudake* JH5 genome, with a total of 19,616 gene models identified and...
spp. and several other basidiomycetes.

| Type       | Number (#) | Average length (bp) | Total length (kb) |
|------------|------------|---------------------|-------------------|
| tRNA       | 204        | 78                  | 16.0              |
| 5s (de novo)| 6          | 114                 | 0.7               |
| 18s (de novo)| 2         | 1,644               | 3.3               |
| 28s (de novo)| 1         | 3,966               | 4.0               |
| snRNA      | 19         | 121                 | 2.3               |

| Sample ID | CBM | CE | GH | GT | PL | AA | TOTAL |
|-----------|-----|----|----|----|----|----|-------|
| L. hatsudake (JH5) | 24  | 12 | 110 | 69 | 1  | 50 | 266  |
| L. hatsudake (MG20)a | 12  | 10 | 27  | 12 | 2  | 9  | 72   |
| L. deliciosus (MG9)b | 9   | 12 | 34  | 16 | 1  | 9  | 81   |
| L. echinatus (MG122)a | 11  | 10 | 23  | 20 | 2  | 17 | 83   |
| L. hygrophoroides (MG19)a | 9   | 9  | 24  | 16 | 0  | 14 | 72   |
| L. indigo (MG109)a | 11  | 9  | 30  | 29 | 3  | 14 | 96   |
| L. pinguis (MG27)a | 5   | 16 | 26  | 18 | 0  | 17 | 82   |
| L. piperatus (MG49)a | 7   | 10 | 23  | 13 | 0  | 16 | 69   |
| L. rugatus (MG108)b | 1  | 9  | 18  | 12 | 2  | 10 | 52   |
| L. solemorus (MG8) | 5   | 13 | 26  | 19 | 2  | 15 | 80   |
| Boletus edulis (MG6)b | 12  | 16 | 34  | 13 | 2  | 14 | 91   |
| T. calosporum (MG102)a | 9   | 13 | 22  | 19 | 1  | 13 | 77   |
| L. edodesb | 58  | 31 | 245 | 75 | 9  | 85 | 461  |
| P. chrysosporiumb | 61  | 26 | 182 | 68 | 6  | 97 | 397  |
| P. ostreatusb | 85  | 32 | 231 | 65 | 23 | 131| 521  |
| H. erinaceusb | 4   | 26 | 161 | 59 | 7  | 84 | 341  |

| Type          | Number (%) | Average length (bp) | Total length (kb) |
|---------------|------------|---------------------|-------------------|
| GO database   | 14,261     | 72.70%              |                   |
| KEGG database | 22.28%     |                     |                   |
| NR database   | 20.09%     |                     |                   |
| Carbohydrate enzymes database annotation

A database of carbohydrate enzymes was used that included a family of enzymes that can catalyze carbohydrate degradation, modification, and biosynthesis within 5 principal categories: glyco-side hydrolases (GHs), glycosyl transferases (GTs), polysaccha-ride lyases (PLs), carbohydrate esterases (CEs), and auxiliary activities (AAs). The GH family comprised the largest proportion, with 110 annotated genes accounting for 41.35% (Fig. 3d). The GT family annotated 69 genes that contained 25.94% but the PL family annotated only one family that comprised only 0.38%. The largest abundances within the GH family indicates that JHS plays an important role in the formation of monosaccharides, oligosaccharides, or carbohydrate complexes, the synthesis of alky glyco-sides and aromatic glycosides, the glycosylation of amino acids and peptides, and the glycosylation of antibiotics (Fig. 3d). The comparison of CAZy functional classification in Lactarius spp. showed that the annotated gene numbers of JHS were higher than other Lactarius spp. (Table 4). We also found that the number of carbohydrate enzymes of L. hatsudake JHS was lower than saprophytic fungi such as Lentinula edodes, Phanerochaete chrysosporium, Pleurotus ostreatus, and Hericium erinaceus. The results showed that perhaps due to the nature of JHS as symbiotic mycorrhizal fungi, these levels were more in L. hatsudake JHS than the other edible mycorrhizal fungi such as L. hatsu- dake MG20, other Lactarius spp., Tuber calosporum and Boletus edulis. Thus, it appears that the capability of carbohydrate degradation is stronger than that of other mycorrhizal fungi of Lactarius.

NR database annotation

The annotation of the NR database resulted in a total of 11,495 genes, of which JHS exhibited the highest similarity with Heterobasidion irregulare. Here, 2,910 genes were annotated to the species for a total of 25.36% of all genes. This was followed by similarity to Moniliophthora roreri and Stereum hirsutum, with 1,148 (9.99%) and 1,008 (8.77%) genes shared, respectively. These data indicate that the majority of the genetic annotations of L. hatsudake strain JHS are related to “mycorrhiza” and “red juice.” Through comparison of the NR database, this genome was found to exhibit a high degree of similarity with other genomes, likely since the species are closely related and genes have not undergone major sequence differentiation (Fig. 3e).

Conclusion

In order to improve the genome assembly of L. hatsudake, we performed de novo sequencing and assembly of L. hatsudake JHS by combining Illumina and PacBio sequencing. A total sequence length of 76.7 Mb of JHS genome was assembled into 312 scaffolds with an N50 of 223.2 kb, and encoded 19,616 putative predicted genes. Compared with the released Lactarius spp. genomes, JHS genome assembly presented the improved completeness and integrity. Here, the high-quality genome assembly of JHS provides...
important insights into the biology of *L. hatsudake*. In addition, the identified genes may enhance our understanding of predicted gene function, enabling the study of biosynthesis of active compounds. Further research could focus on genes associated with growth and development or the biosynthesis of secondary metabolites. Although incomplete, the basic information provided by the elucidation of the *L. hatsudake* genome in this study is a novel attempt to facilitate biologically and agriculturally based research and thus support future applications of fungal species.

**Data availability**

Sequencing data and genome assembly of JH5 for this project have been deposited in NCBI databases under project accession...
number PRJNA605941 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA841037, 2022/10/01). Transcriptome data of JH5 were deposited into GenBank under the accession numbers of PRJNA841037 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA605941, 2022/10/01).

Supplemental material is available at G3 online.

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**Conflicts of interest**

None declared.

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