A New High-Quality Draft Genome Assembly of the Chinese Cordyceps *Ophiocordyceps sinensis*

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Accepted: 21 May 2020

Data deposition: The data generated in this study have been deposited at GenBank under the accessions JAAVMX000000000, PRJNA608258, and PRJNA615047. Raw reads and genome assembly are also publicly available from the Genome Warehouse in BIG Data Center (2018), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession number GWHAAFH00000000 that is publicly accessible at https:// bigd. big. ac. cn/ gwh.

Abstract

Ophiocordyceps sinensis (Berk.) is an entomopathogenic fungus endemic to the Qinghai-Tibet Plateau. It parasitizes and mummifies the underground ghost moth larvae, then produces a fruiting body. The fungus-insect complex, called Chinese cordyceps or DongChongXiaCao, is not only a valuable traditional Chinese medicine, but also a major source of income for numerous Himalayan residents. Here, taking advantage of rapid advances in single-molecule sequencing, we assembled a highly contiguous genome assembly of *O. sinensis*. The assembly of 23 contigs was ~110.8 Mb with a N50 length of 18.2 Mb. We used RNA-seq and homologous protein sequences to identify 8,916 protein-coding genes in the IOZ07 assembly. Moreover, 63 secondary metabolite gene clusters were identified in the improved assembly. The improved assembly and genome features described in this study will further inform the evolutionary study and resource utilization of Chinese cordyceps.

Key words: PacBio sequencing, de novo assembly, genome annotation, secondary metabolite, gene cluster.

Introduction

Chinese cordyceps, called yartsa gunbu or DongChongXiaCao, is a complex of the entomopathogenic fungus *Ophiocordyceps sinensis* (Ascomycetes) parasitizing soil-borne larvae of the ghost moths (belonging to the family Hepialidae) (Dong and Yao 2005). This resource is exclusively distributed in the alpine meadows of the Qinghai-Tibet Plateau (QTP) at altitudes between 3,000 and 5,000 m (Lo et al. 2013). *Ophiocordyceps sinensis* is one of the most famous and perhaps the most expensive fungal species in the world (Zhang et al. 2012). The excavated cordyceps has been used as a traditional Chinese medicine for centuries, with an extremely high market price (reaching $60,000/kg in 2015) (Lei et al. 2015). Former studies have isolated diverse bioactive components and found their corresponding pharmacological actions in *O. sinensis* (Holliday and Cleaver 2008; Liu et al. 2015). Harvesting of the natural *O. sinensis* specimens has become a primary source of income for hundreds of thousands of collectors (Pouliot et al. 2018). In addition to the medicinal and economic values of *O. sinensis*, the fungus is also ecologically important and considered as a flagship species for its ecosystem (Zhang et al. 2012). Over-harvest of the fungus poses a threat to the fragile ecosystem of QTP (Hopping et al. 2018). Due to challenges by overexploitation and climate change, the increasing popularity and demand for this fungus is unsustainable from natural sources and endangers this interesting entomopathogen (Yan et al. 2017; Hopping et al. 2018). Artificial cultivation of this fungus may alleviate the contradiction between supply and demand, but still faces many obscures (Qin et al. 2018). A high-quality reference genome of *O. sinensis* should be of help for an understanding of its pathogenesis and medicinal potential.

Since 2013, at least four isolates from different *O. sinensis* strains have been sequenced to achieve their genome assemblies (Xiao et al. 2013; Li et al. 2016; Xia et al. 2017). However, due to limitations of sequencing techniques and data analysis methods, the 2013 genome project (Xiao et al. 2013) was unable to produce a highly continuous assembly (contig N50, 5.3 kb) for *O. sinensis* strain Co18. A more recently assembly OSIN (Xia et al. 2017) produced by Roche 454 and Illumina sequencing was much more contiguous, but still suffered from short contigs (contig N50, 21.4 kb) and a correspondingly large number of gaps. Previous assemblies showed that the genome of *O. sinensis* was highly repetitive and much larger than those of other fungi (Li et al. 2016), indicating this fungal genome had undergone unique evolutionary events.

Here, we used single-molecule sequencing technology to acquire a high-quality de novo assembly of a strain of *O. sinensis*, IOZ07, providing the community a useful genetic resource for the understanding of mechanism in comparative genomic and evolutionary biology studies. Moreover, our improved annotation allows for better insights to the gene clusters involved in biosynthesis of secondary metabolites, which should be of help to investigate the medical potential of *O. sinensis*.

Materials and Methods

Sample Collection and Sequencing

Strain IOZ07 was initially isolated from a sclerotium of *O. sinensis* (one piece of Chinese cordyceps) gathered in Xiaojin county, Sichuan Province, China. The strain was characterized at both the morphologic and ultrastructural levels before (Li et al. 2020). IOZ07 was cultured in yeast extract peptone dextrose (YPD) medium for 20 days at 18˚ 1 °C, under shaking (100 rpm). The mycelium recollected from the culture was prepared for PacBio and Illumina whole-genome sequencing. The cultured mycelia, sclerotium hyphae, and blastospores extracted from the host larva were used for the Illumina transcriptome sequencing (supplementary table S1, Supplementary Material online).

Genomic DNA extraction, library preparation and sequencing were carried out by an external service (Novogene Co., Ltd., Beijing, China). In brief, the genomic DNA was extracted from mycelia with a standard CTAB extraction method, and the PacBio Sequel platform with P6C4 chemistry was deployed to sequence the genomic library. Single Molecule Real-Time (SMRT) sequencing achieved ~12.6 Gb clean data (~100× coverage, N50 16,804 bp). The IOZ07 genomic DNA was also sequenced at ~80× coverage with the Illumina HiSeq X system, providing 2 × 150 bp paired-end reads with 500 bp insert size.

For RNA-seq, we generated samples from diverse *O. sinensis* tissues (cultured mycelia, blastospores, and sclerotium hyphae) as described before (Li et al. 2020). The RNA-seq libraries were prepared and sequenced by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). Each sample was sequenced as a separated library with ~20 million paired reads. The resulting pair-end libraries (PE150 with 350 bp insert size, supplementary table S1, Supplementary Material online) were sequenced via the Illumina HiSeq X system.

Genome Assembly

The obtained PacBio Sequel reads were trimmed, corrected, and assembled using the Canu assembler (version 1.7) (Koren et al. 2017) with correctedErrorRate = 0.035 read correction parameter to limit the error rate. The resulting contigs were further polished with PacBio long reads using three rounds of Arrow (Chin et al. 2013). The assembly was further polished using Pilon (Walker et al. 2014) with Illumina short reads. The mitochondrial genome of *O. sinensis* was identified using BLAST+ BlastN (Camacho et al. 2009) compared with the reported mitochondrial genome (GenBank accession number: KY622006) (Kang et al. 2017), and the remaining contigs were ordered by length.

Genome Assessment

The continuity of IOZ07 assembly was compared with those of previous assemblies (Co18 and OSIN) built by second-generation sequencing technology. Sequences corresponding to Co18 and OSIN gene sets were downloaded from corresponding databases (Xiao et al. 2013; Xia et al. 2017). Reference proteome of *Neurospora crassa* was mapped to the hypothetical transcripts corresponding to each gene set annotation by BLAST+ BLASTp (Camacho et al. 2009), setting the *e*-value cutoff at 10^{-3}. All the RNA-seq libraries of *O. sinensis* acquired in this study and public databases (supplementary table S2, Supplementary Material online) were mapped to the assemblies by Salmon (version 0.12.0) (Patro et al. 2017) with the default parameters. The output read count tables were used to analysis the expression of predicted genes using DESeq2 (Love et al. 2014). In addition, we evaluated the completeness of IOZ07 assembly based on the 3,725 BUSCO (Waterhouse et al. 2018) groups in Sordariomyceta_odb9 lineage data set with the default parameters.

Repeats Annotation

Transposable elements (TEs) were annotated by RepeatMasker (Smit, AFA, Hubley, R & Green, P. *RepeatMasker Open-4.0*; 2013—2015 at http:// www. repeatmasker. org; last accessed February 22, 2020) and RepeatModeler (Chen 2004). RepeatMasker was conducted to identify known TEs using the TEfam and Repbase (Bao et al. 2015) databases. Then, we searched for repeat families and consensus

Table 1
Summary Statistics for the De Novo Genome Assembly Results of *Ophiocordyceps sinensis* Strains Co18 (PRJNA59569), OSIN (PRJNA382001), and IOZ07 (This Study)

Feature	Assembly				
	Co18 (Contigs)[a]	Co18 (Scaffolds)[a]	OSIN (Contigs)[b]	OSIN (Scaffolds)[b]	IOZ07[c]
Number of sequences	26,538	10,603	9,687	1,141	23
Minimum length	121	670	101	500	1,768
First quartile	725	2,300	2,408	635	56,774
Median	1,485	4,656	7,216	877	652,132
Mean	2,788	7,405	11,567	102,036	4,820,913
Third quartile	3,279	9,274	15,957	1,322	5,297,554
Maximum length	87,316	130,882	120,072	9,561,753	23,284,981
Total	73,982,473	78,515,811	112,046,919	116,423,023	110,880,992
N50	5,252	11,986	21,373	2,999,605	18,163,664
N90	1,230	3,431	6,174	679,076	3,914,182
Repeat content (%)	37.98	37.98	74.67	74.67	68.07
GC content (%)	46.19	46.19	44.77	44.77	45.10

[a]Genome version published by Xiao et al. (2013), contigs shorter than 100 bp were filtered out.
[b]Genome version published by Xia et al. (2017), contigs shorter than 100 bp were filtered out.
[c]Genome version generated in this study.

sequences using the de novo repeat prediction tool RepeatModeler with the default parameters with RECON and RepeatScout as core programs. Telomeric repeats were annotated using SERF (Somanathan and Baysdorfer 2018) and Tandem Repeat Finder (Benson 1999) with adjust settings (1 1 2 80 5 200 2000 -d -h).

Gene Annotation

Genome annotation was performed with the GETA pipeline (https:// github. com/ chenlianfu/ geta; last accessed February 22, 2020) by integrating ab initio, homology-based and transcriptome-based evidence. Illumina RNA-seq reads from different tissues (supplementary table S1, Supplementary Material online) were mapped to the assembly using Hisat2 (Kim et al. 2019). The homologies from other organisms in the *Ophiocordyceps* genus (supplementary table S3, Supplementary Material online) were used as protein evidence to predict the gene set with GeneWise (Birney et al. 2004). Ab initio gene predictions were performed with Augustus (Stanke et al. 2004), trained by the RNA-seq data. All the evidences above were integrated by the GETA pipeline. The predicted gene sets were functionally annotated using eggNOGmapper (Huerta-Cepas et al. 2019) and InterProScan (Jones et al. 2014) with the default parameters. The noncoding RNA was predicted using Infernal (version 1.11) (Nawrocki and Eddy 2013) with the Rfam 14.1 database (Kalvari et al. 2018).

Gene Clusters Involved in Secondary Metabolism

To capture the secondary metabolite potential of *O. sinensis*, two gene cluster predictors, antiSMASH (fungal version 4.2.0) (Blin et al. 2017) and SMURF (Khaldi et al. 2010), were used to identify secondary metabolite gene clusters in the *O. sinensis* assemblies. Gene clusters from different assemblies were compared using LASTAL (Kiełbasa et al. 2011) and visualized by Jcvi (Tang et al. 2008). The polymerase chain reaction (PCR) gel electrophoresis was conducted to verify the diverged regions between different assembles. The PCR primers specific to the object regions were designed with Primer-BLAST (Ye et al. 2012).

Results and Discussion

Genome Assembly and Annotation

We applied ~12.6 Gb of PacBio long-read clean data (~100-fold coverage for a 120-Mb genome) from two Sequel cells to generate a primary assembly. The Canu assembly resulted in 24 contigs and a significant increased N50 (18.16 Mb). The assembly was polished to remove erroneous base calls and insertion/deletions (indels) using the PacBio reads and Illumina reads. By comparing to the reported mitochondrial genome (KY622006) of *O. sinensis*, we identified one circular contig as mitochondrial in the primary assembly. The remaining contigs were ordered by length. The unscaffolded assembly consisted of 23 contigs (excluding the circular mitochondrial genome) and a total size of ~110.88 Mb (table 1). The longest contig exceeded 23 Mb. Eleven of these contigs (supplementary table S4, Supplementary Material online) contained characteristic $(TTAGGG)_n$ telomeric repeats on either the 5′ or 3′ end. No contig with telomeric repeats on both ends was found.

The contiguity of IOZ07 was greatly improved compared with those of previous assemblies (table 1). The 23 assembled contigs of IOZ07 were extremely less than those of Co18 and OSIN (9,687 and 26,538, respectively), indicating that a more complete assembly was carried out in this study (table 1, fig. 1*a*—*c*). The assembly improvements mainly benefited

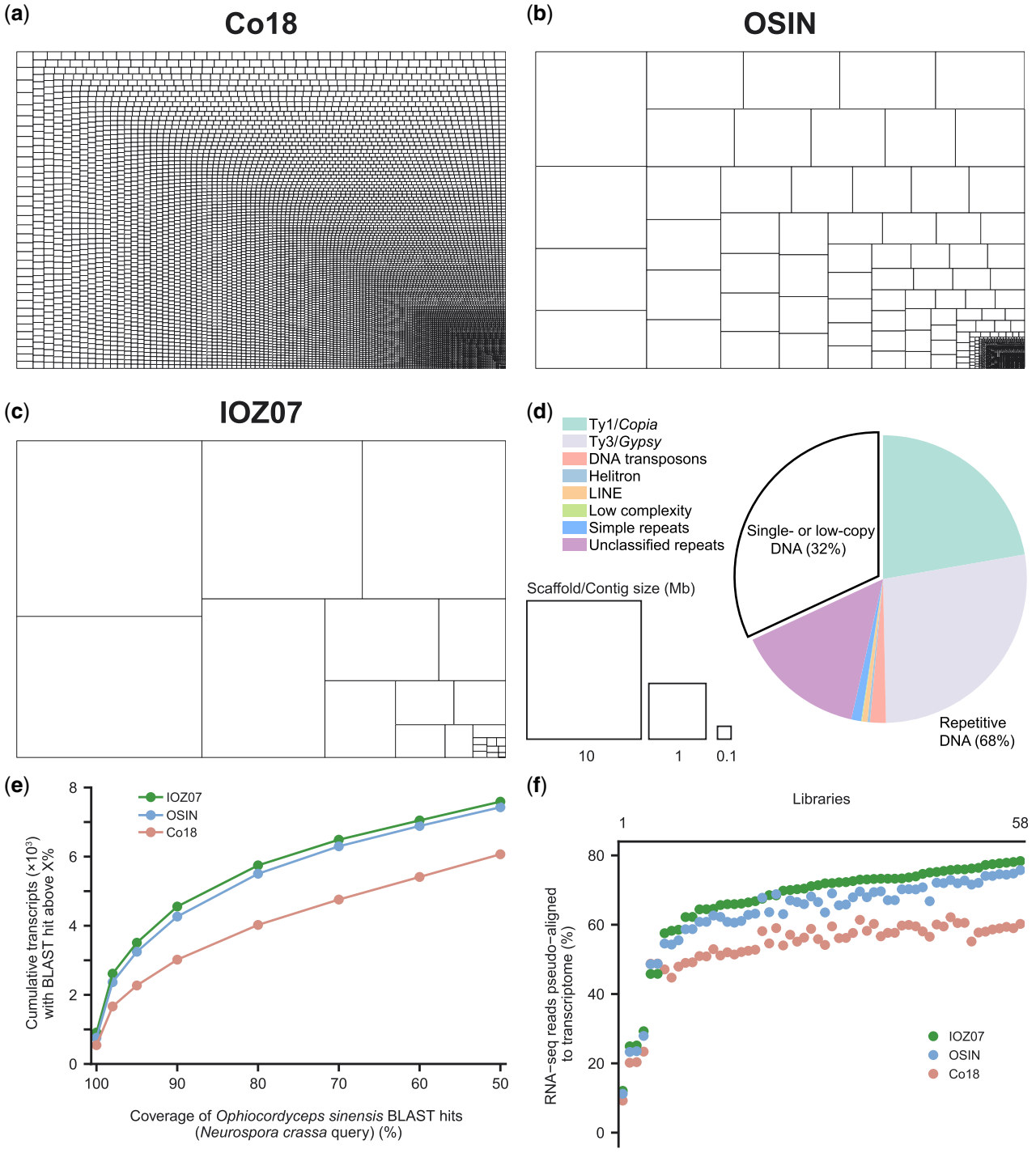

Fig. 1.—IOZ07 assembly statistics and annotation. (*a*, *b*) Treemaps of Co18 (*a*) and OSIN (*b*) scaffolds scaled by length. (*c*) Treemap of IOZ07 contigs scaled by length. (*d*) Genome composition of *Ophiocordyceps sinensis*. Ty1/*Copia*, *Copia* elements of long terminal repeat retrotransposons; Ty3/*Gypsy*, *Gypsy* elements of long terminal repeat retrotransposons; LINE, long interspersed nuclear elements. (*e*) Gene set alignment BLASTp coverage compared with previous assemblies by *Neurospora crassa* protein queries. (*f*) Alignment of 58 RNA-seq libraries to the IOZ07, OSIN, and Co18 gene set annotations.

from the usage of SMRT, a long reads sequencing technique, especially for the highly repetitive *O. sinensis* genome (fig. 1*d*). Approximately 68.07% of the *O. sinensis* whole-genome sequence is composed of TEs or other repetitive sequences (table 1, fig. 1*d*), which is much higher than the repeat rate of Co18 assembly (37.98%). Long terminal repeat

retrotransposon (LTR-RT) is the most abundant type of TE in the *O. sinensis* genome.

Genome Properties

In the IOZ07 assembly, a total of 8,916 protein-coding genes were identified by the GETA genome annotation pipeline, of which 8,160 genes were functional annotated (supplementary table S5, Supplementary Material online). The IOZ07 gene set was markedly more complete than previous assemblies. When compared with reference proteome of *N. crassa*, more genes were found at a given protein coverage (compared with Co18, 1,724 more genes with >80% coverage, a 19.3% increase; compared with OSIN, 243 more genes with >80% coverage, a 2.71% increase, fig. 1*e*). When mapping the transcriptome libraries to the assemblies, ~12% more RNA-seq reads mapped to the IOZ07 gene set annotation than to Co18 on average (fig. 1*f*). We mapped the BUSCO conserved genes from the Sordariomyceta lineage to our genome assembly. The result demonstrated a sharp increase in complete orthologs (96.24%) and a reduction in fragmented (1.88%) and missing (1.88%) genes compared with previous assemblies (supplementary fig. S1, Supplementary Material online). In addition, we predicted a total of 106 tRNAs and 65 rRNAs in the *O. sinensis* genome (supplementary table S6, Supplementary Material online).

Secondary Metabolite Potential

The genomes of entomopathogenic fungi sequenced to date are replete with secondary metabolite gene clusters (Gibson et al. 2014). The improved IOZ07 assembly has great potential to find novel secondary metabolism. Here, the IOZ07 assembly was used to capture the secondary metabolite potential of *O. sinensis*. Eleven of the 23 contigs in the *O. sinensis* genome were profiled for the presence of secondary metabolite gene clusters by two gene cluster predictors, antiSMASH and SMURF. In total, 63 secondary metabolite gene clusters (supplementary table S7, Supplementary Material online) were identified in the IOZ07 assembly, 29 more than previous ones. The 63 metabolites producing gene clusters were from a variety of classes, including sixteen type 1 polyketide synthases, eight nonribosomal peptide synthetases, nine terpenes, and one indole. Many of them have no identified specific products and are unique to the group of entomopathogens. Only 16 secondary metabolite gene clusters shared limited similarity (>50% genes in the cluster) with those of other insect pathogens (supplementary fig. S2, Supplementary Material online). Given that *O. sinensis* is obligate to the underground larvae of Hepialidae (Dong and Yao 2005), it was not surprising to find so many specific secondary metabolite gene clusters in the IOZ07 assembly. As each secondary metabolism related gene cluster could be involved in the biosynthesis of a specific metabolite (Keller and Hohn 1997), the secondary metabolite gene clusters found in the IOZ07 assembly could represent potential unity in medicine and key roles in the entomopathogenesis of *O. sinensis*.

We identified a NRPS gene cluster with 18 destruxin-like biosynthetic genes in the IOZ07 assembly. These genes display high amino acid sequence similarity to those of *Hirsutella minnesotesis* (*O. sinensiss* nearest sequenced relative) (Lai et al. 2014) and occur in the same order (supplementary fig. S3, Supplementary Material online). However, this gene cluster was split into two scaffolds in the Co18 assembly (supplementary fig. S3, Supplementary Material online). The size of the gene cluster is ~89 kb, including an ~12-kb gap between different scaffolds in the Co18 assembly. To validate the contiguity of the IOZ07 assembly, we designed two pairs of primers (supplementary table S8, Supplementary Material online) for the divergent regions (regions A and B in supplementary fig. S3, Supplementary Material online) between IOZ07 and Co18. The PCR gel electrophoresis image showed that regions A and B were both consecutive (supplementary fig. S3, Supplementary Material online), supporting the integrity of the destruxin-like gene cluster in the IOZ07 assembly. From the RNA-seq data, we also found this gene cluster was actively transcribed in diverse tissues (supplementary fig. S3, Supplementary Material online). The genes in this cluster are highly expressed during the sclerotium hypha period, when the fungus switches to sexual development.

Conclusion

With a combination of PacBio and Illumina reads, we present a new high-quality draft assembly for *O. sinensis* in terms of contiguity, gap-free sequences, and annotation. With the improved continuity of the IOZ07 assembly, more TEs and protein-coding genes were found and annotated. This newly assembled genome will promote the discovery and identification of novel secondary metabolism gene clusters, which possesses great medicinal prospects.

Overall, our improved assembly, IOZ07, provides better insights into the genome biology of *O. sinensis* and a solid foundation for further genomic and evolutionary studies within the *Ophiocordyceps* genus, which contains many entomopathogenic fungi of great pest control application and medicinal values.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (31872297, 31772525); and China Postdoctoral Science Foundation (2017M610988). The authors extend their sincere gratitude to Qiang Gao from

BGI Beijing Center and Lianfu Chen from Huazhong Agricultural University. They offered many constructive suggestions for the genome assembly and annotation reported in this work.

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Associate editor: Jason Stajich