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Two high-quality genomes of *Prototheca bovis* strain SH08 and *Prototheca ciferrii* strain SH13

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***Prototheca*, one of the foremost causative agents responsible for opportunistic infections in both humans and animals. Despite the availability of several *Prototheca* genomes, the genomes of *Prototheca bovis* and *Prototheca ciferrii* have remained unsequenced. In this study, we report two high-quality genome assemblies for *P. bovis* strain SH08 and *P. ciferrii* strain SH13, representing two distinct species of the genus *Prototheca*. The final assembled genome sizes were 30.6 Mb for *P. bovis* SH08 and 32.7 Mb for *P. ciferrii* SH13, with contig N50 values of 1.19 Mb and 1.78 Mb, respectively. The repetitive sequences were identified in 14.79% and 14.24% of the assembled genomes, respectively. A total of 5,141 protein-coding genes were predicted for *P. bovis* SH08, while *P. ciferrii* SH13 contained 4,986 protein-coding genes. Functional annotation identified 97.28% and 99.16% of the genes in the genomes, respectively. These two novel high-quality genome assemblies represent valuable resources for advancing research in evolutionary biology, comparative genomics, pathogenicity assessment, diagnostic strategies, and therapeutic interventions in protothecosis.**

Background & Summary

Prototheca is a genus of achlorophyllous green microalgae classified within the phylum Chlorophyta, class Trebouxiophyceae, and family Chlorellaceae. It exhibits wide distribution across diverse environments worldwide[1]. *Prototheca* has been identified as a pathogen capable of infecting humans (primarily caused by *Prototheca wickerhamii*), cattle and dog (mainly caused by *Prototheca bovis* and *Prototheca ciferrii*), and some other reported species caused by *Prototheca cutis*, *Prototheca blaschkeae* and *Prototheca miyajii*[1–3]. The *Prototheca* infection, also known as Protothecosis, has been documented in several hundred cases[4]. Over the past two decades, there has been a significant increase in the prevalence of *Prototheca* as an emerging pathogen[5]. However, several knowledge gaps persist regarding their biological characteristics, particularly concerning their pathogenicity. In particular, the limited genomic and molecular research on *Prototheca* impedes the development of robust diagnostic tools and a comprehensive understanding of its pathogenesis. To enhance public awareness of protothecosis and improve its identification and diagnosis, we have initiated and established the Protothecosis Science Popularization and Monitoring Consortium (PSPMC) and China Prototheca Working Group (CPWG) in collaboration with multiple organizations.

Due to the rapid development and cost reduction of next-generation long-read sequencing technologies, genome sequencing has become more cost-effective[6,7]. For the *Prototheca* genus, several genomes have been assembled, including *P. wickerhamii* strain ATTC16529[4], *P. wickerhamii* strain S1 and S931[8], *P. wickerhamii* strain InPu-22_FZ[9] and two strains of *Prototheca zopfii* Pz20 and Pz23[10]. In addition to genome reference resources, multi-omics approaches such as transcriptomics, proteomics, and metabolomics have been employed to further elucidate the pathogenic mechanisms of *Prototheca*[11]. The diagnosis of *Prototheca* infection is generally determined through blood, body fluid, or tissue culture[12]. However, it poses a significant challenge, particularly in cases where protothecosis is not suspected clinically. The diagnosis cannot be classified as different *Prototheca* without DNA sequencing. The genomic data of different *Prototheca* species not only enhances diagnostic precision but also provides valuable insights for foundational research. A total of eighteen *Prototheca* species have

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been reported, with fifteen being documented in 2019[13], and three newly identified species, *Prototheca fontanea*, *Prototheca lentecrescens*, and *Prototheca vistulensis* were later characterized[14]. However, all the taxonomic samples utilized in the study were obtained from Poland based on the cytochrome B(*CYTB*)-based PCR-restriction fragment length polymorphism (RFLP). *P. bovis* and *P. ciferrii*, previously classified as *P. zopfii* genotype 1 and genotype 2 respectively[5,15], are closely associated with dairy herd environments and remain under investigation. In this study, samples of the two species were collected from China. The species *P. bovis* has been identified as the most pathogenic among dairy cattle[16], while *P. ciferrii* has been reported that it can cause the infections in dogs inducing a more aggressive disease course[17,18]. A high-quality genome serves as the genetic foundation for molecular research and gene diagnostics. Recently, high-throughput long-read sequencing, particularly PacBio HiFi sequencing, has enabled the generation of the highest-quality genomes to date[19]. The genome assembly algorithms have revolutionized the field by significantly improving computational efficiency and reducing costs[19]. These two high-quality genome resources hold significant value for taxonomy identification, evolutionary studies, comprehensive understanding, and even the diagnosis of Protothecosis.

Methods

The process of isolating and obtaining the *Prototheca* strains was approved by the Medical Ethics Committee of Shanghai East Hospital, Tongji University (Approval No. 2024YS-274). An overview of the experimental and bioinformatic workflow used in this study is depicted in Fig. 1. Briefly, the process encompassed sample collection, DNA extraction, multi-platform sequencing, genome assembly, annotation, and comprehensive quality assessment.

Sample collection and extraction. The strain of *Prototheca bovis* SH08 was obtained from fresh cow milk sample in Shanghai, China. *Prototheca ciferrii* SH13 was obtained from skin tissue samples of human patients in Shanghai, China. The two strains were classified as *P. bovis* and *P. ciferrii* based on the previous protocol and analysis of the *CYTB* gene[14]. The samples of *P. bovis* SH08 and *P. ciferrii* SH13 can be available at the biobank of Shanghai East Hospital, Tongji University. The two strains were cultivated in a bacteriological incubator on Sabouraud dextrose agar medium and incubated at 35 °C for 4 days, followed by subsequent harvesting for DNA extraction. The high molecular weight (HMW) genomic DNA of the *P. bovis* SH08 and *P. ciferrii* SH13 were extracted using the CTAB method, following a previously reported protocol[20].

Short library construction, sequencing, and genome survey. The short reads library was generated for *P. bovis* SH08. The library with an insert size of 300–500 bp was subjected to Covaris E220 System for fragmentation and prepared using the MGIEasy Universal DNA Library Prep Set (MGI-Tech). The PE 150 reads were sequenced on the next generation sequencing MGISEQ-2000 platform, generating approximately 5.5 gigabases (Gb) of sequencing data (Table 1), following identical filter parameters as our previous study[10]. The 17-mer and 19-mer frequency were calculated for *P. bovis* SH08 using jellyfish[21]. The estimated genome sizes are approximately 29.06 Mb and 30.1 Mb, with a total of 4,621,095,510 and 4,551,930,609 k-mers, and peak depths of 159 and 151, respectively, as calculated by KmerFreq v5.0[21].

PacBio Library construction, sequencing, and genome assembly. For PacBio HiFi sequencing, two libraries with an approximate insert size of 20 kb were generated for *P. bovis* SH08 and *P. ciferrii* SH13 using the SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences, USA). A total of approximately 1.99 Gb (119,083 reads) and 4.17 Gb (242,272 reads) clean data were generated using PacBio SequelII SMRT cell with HiFi model, respectively (Table 1). The average length of CCS reads was 16,720 bp and 17,214 bp for *P. bovis* SH08 and *P. ciferrii* SH13, respectively (Table 1). Using the HiFi long reads, the nuclear genomes of the *P. bovis* SH08 and *P. ciferrii* SH13 were assembled using hifiasm v0.7 with default parameters[22]. The assembled genome of *P. bovis* SH08 is 30.6 Mb, with a contig N50 of 1.19 Mb and a total number of 53 contigs. Similarly, the assembled genome of *P. ciferrii* SH13 is 32.7 Mb, with a contig N50 of 1.78 Mb and a total number of 96 contigs (Fig. 2 and Table 2). The maximum length of assembled genome of *P. bovis* SH08 and *P. ciferrii* SH13 is 2,899,485 bp and 2,975,726 bp, respectively (Table 1). The GC content of *P. bovis* SH08 and *P. ciferrii* SH13 is 73.6% and 67.8%, respectively (Table 1).

Genome annotation. The repeat annotation was conducted by employing TRF (4.9) with default parameters for the identification of Tandem repeats[23]. Then, the homologous sequences of the *P. bovis* SH08 and *P. ciferrii* SH13 genomes were identified using the software RepeatProteinMask (v 4.0.7)[24] and RepeatMasker (open-4.0.9)[25] based on the Repbase library (http://www.girinst.org/repbase)[26]. The databases of two strains repetitive sequence were generated using RepeatModeler open-1.0.1140 and LTR_FINDER_parallel 1.0.741, followed identified using the *ab initio* method with RepeatMasker (open-4.0.9)[25]. Finally, 14.79% and 14.24% of assembled *P. bovis* SH08 and *P. ciferrii* SH13 genomes were classified as repetitive sequences (Table 2 and Table 3). After repeat identification, three different algorithms, namely homology-based annotation, *ab initio* prediction and RNA-Seq data-based annotation were employed for gene prediction. In homology-based annotation, a total of 10 published homology protein sequences from *Coccomyxa subellipsoidea, Chlorella variabilis, Chlorella desiccate, Chlorella sorokiniana, Auxenochlorella protothecoides*, three strains of *Prototheca wickerhamii* ATTC16529, S1 and S931, and two strains of *Prototheca zopfii* Pz20 and Pz23 were integrated with the MAKER2 pipeline[27]. In the *ab initio* prediction, the *P. bovis* SH08 and *P. ciferrii* SH13 genome sequences, which were masked for repeated elements, were employed to identify coding regions of genes using AUGUSTUS v3.2.3[28] and SNAP[29]. For RNA-based prediction, the relative transcriptome of *P.zopfii* (7.5 Gb) from the published study[30]. The RNA reads were mapped to the genomes of two strains of *P. bovis* SH08 and *P. ciferrii* SH13 genomes using hista2.2.1[31], followed by transcript assembly using stringtie2.1.6[32]. The gene sets were obtained by integrating the three strategies using Maker2 (2.31.10)[27]. Finally, a total of 5,141 and 4,986 protein-coding genes were obtained respectively for *P. bovis* SH08

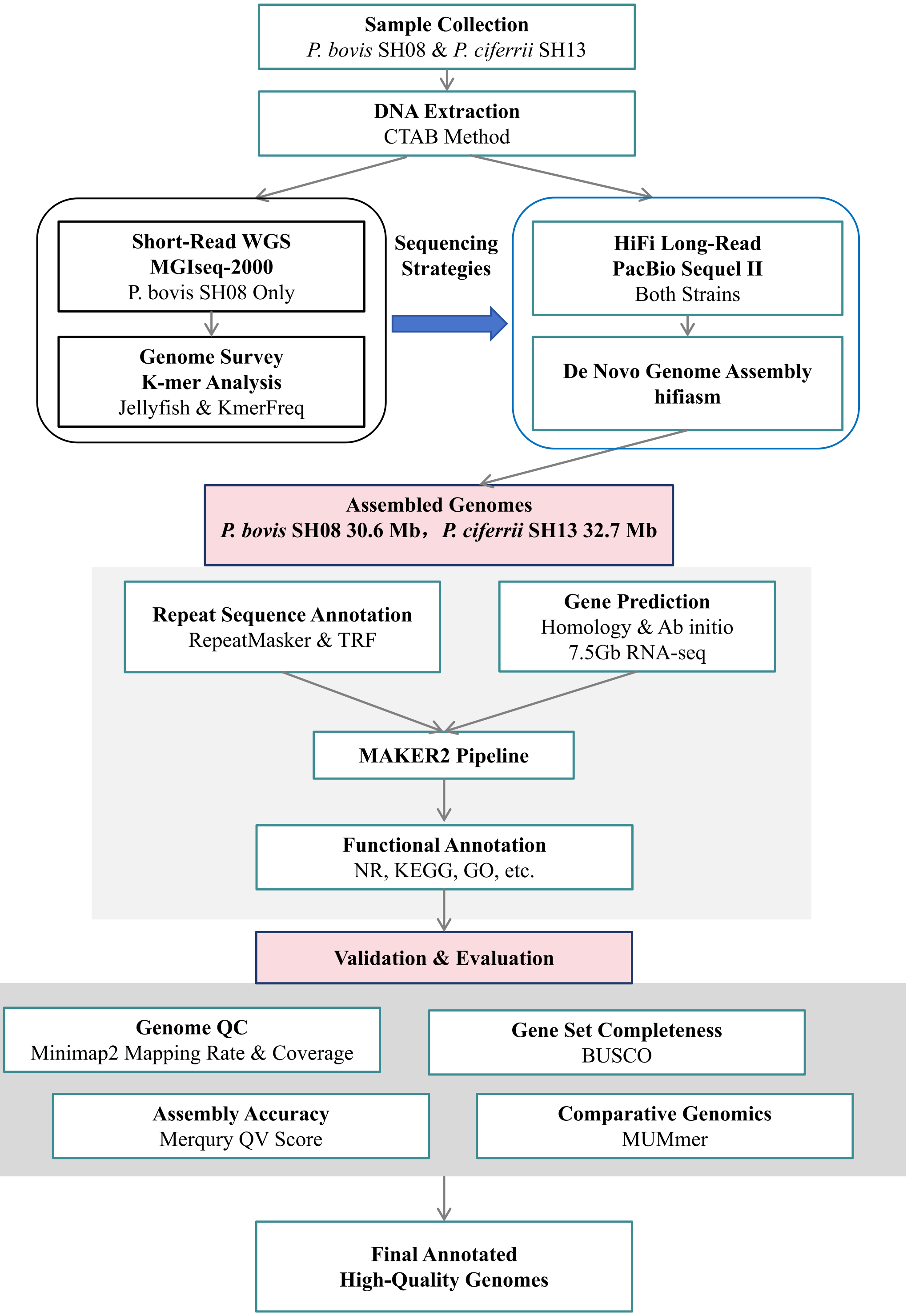


Fig. 1 Workflow of genome sequencing, assembly, and annotation for *Prototheca bovis* SH08 and *Prototheca ciferrii* SH13.

Sequencing technology	Sample	Insert size	Reads Number	Clean data (bp)	Average length (bp)	Depth (×)
WGS	SH08	300–500 bp	36,642,928	5,496,439,200	150	179.5
PacBio	SH08	20 kb	119,083	1,991,061,870	16,720	65.0
PacBio	SH13	20 kb	242,272	4,170,365,657	17,214	127.4

Table 1. The sequencing data statistics for the *P. bovis* SH08 and *P. ciferrii* SH13 genomes.

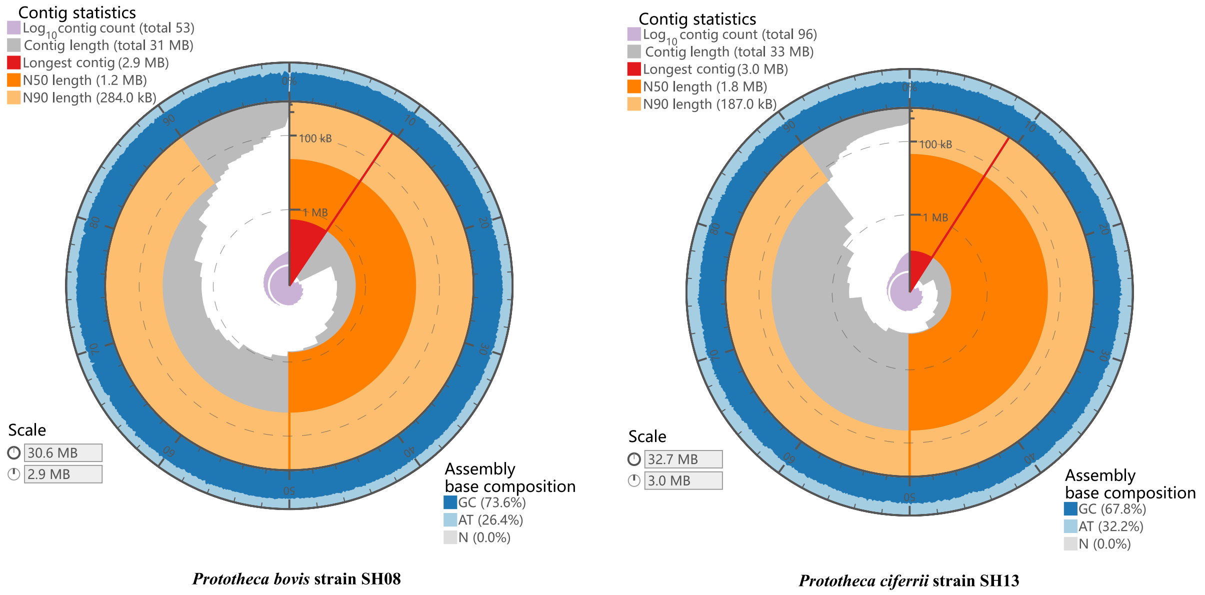


Fig. 2 The genome characteristic of two species *P. bovis* SH08 and *P. ciferrii* SH13.

Characteristics	*P. bovis* SH08	*P. ciferrii* SH13
Total length (bp)	30,622,000	32,731,977
Total number of contig	53	96
Maximum length (bp)	2,899,485	2,975,726
Contig N50 (bp)	1,187,196	1,783,588
Number of Contig N50	9	8
Contig N90 (bp)	283,985	187,002
Number of Contig N90	27	18
GC content	0.736	0.678
Repeat content	14.79%	14.24%
Protein-coding genes	5,141	4,986

Table 2. The genome assembly and annotation statistics for *P. bovis* SH08 and *P. ciferrii* SH13.

	P. bovis SH08		*P. ciferrii* SH13	
Type	Repeat Size	Percent of genome (%)	Repeat Size	Percent of genome (%)
Trf	2,704,306	8.83	1,625,599	4.97
Repeatmasker	267,470	0.87	639,719	1.95
Proteinmask	59,521	0.19	166,057	0.51
De novo	2,090,740	6.83	3,144,973	9.61
Total	4,529,376	14.79	4,660,975	14.24

Table 3. The repetitive sequence statistics in the *P. bovis* SH08 and *P. ciferrii* SH13.

and *P. ciferrii* SH13 genomes (Table 2). The total count of gene models exhibiting an Annotation Edit Distance (AED) score ≤ 0.5 reached 4,957 (96.4%) for *P. bovis* SH08 and 4,805 (96.4%) for *P. ciferrii* SH13, indicating robust support from the available evidence.

Functional annotation. The gene set of the *P. bovis* SH08 and *P. ciferrii* SH13 genomes was functionally annotated based on the seven databases including NR version 2023-04-01 (NCBI nonredundant protein), KEGG version 105.0, 2023-01-01 (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/), KOG version 2023-03-0147, TrEMBL version 2023-03-01 (http://www.uniprot.org), Swiss-Prot version 2023-03-01 (http://www.gpmaw.com/html/swiss-prot.html), InterPro 93.0 and GO Ontology (GO) version 2023-04-01. The functional annotation for *P. bovis* SH08 and *P. ciferrii* SH13 genomes accounted for 97.28% and 99.16% of the annotated genes, respectively (Table 4). A total of 4,344 (84.50%) and 4,175 (83.73%) genes can be functional annotation in KEGG database (Fig. 3). The primary metabolic pathway identified is Carbohydrate metabolism

Species		Total	Nr-Annotated	Swissprot-Annotated	KEGG-Annotated	KOG-Annotated	TrEMBL-Annotated	Interpro-Annotated	GO-Annotated	Overall
***P. bovis* SH08**	Number	5,141	4,972	4,259	4,344	3,842	4,968	4,624	3,328	5,001
	Percentage	100%	96.71%	82.84%	84.50%	74.73%	96.63%	89.94%	64.73%	97.28%
***P. ciferrii* SH13**	Number	4,986	4,930	4,199	4,175	3,814	4,937	4,560	3,355	4,944
	Percentage	100%	98.88%	84.22%	83.73%	76.49%	99.02%	91.46%	67.29%	99.16%

Table 4. The functional annotation statistics of the *P. bovis* SH08 and *P. ciferrii* SH13.

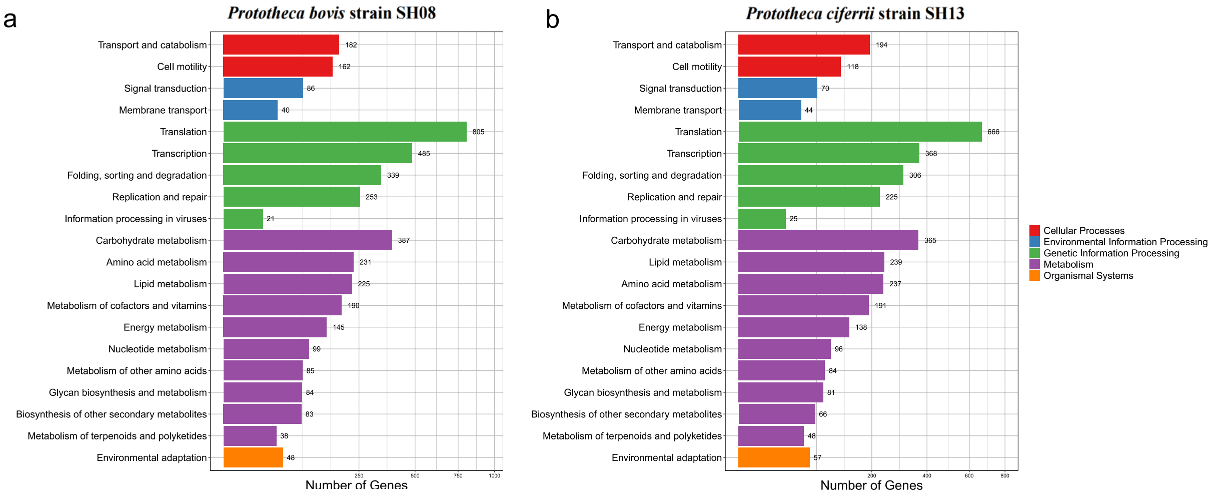
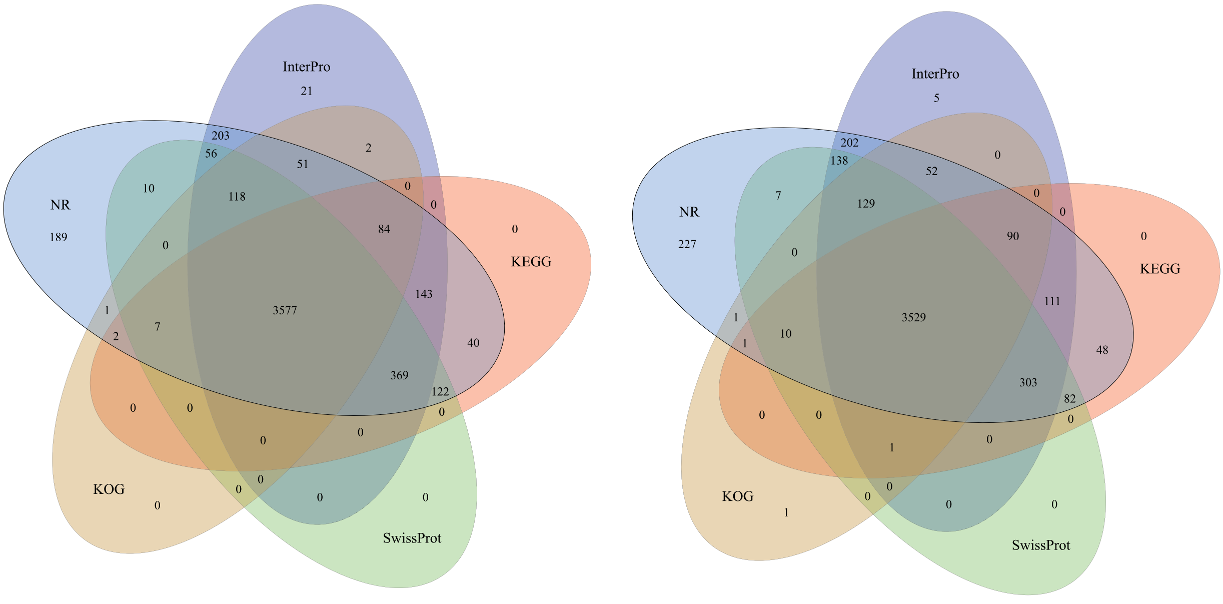


Fig. 3 The KEGG annotation of predicted coding genes in *P. bovis* SH08 and *P. ciferrii* SH13.



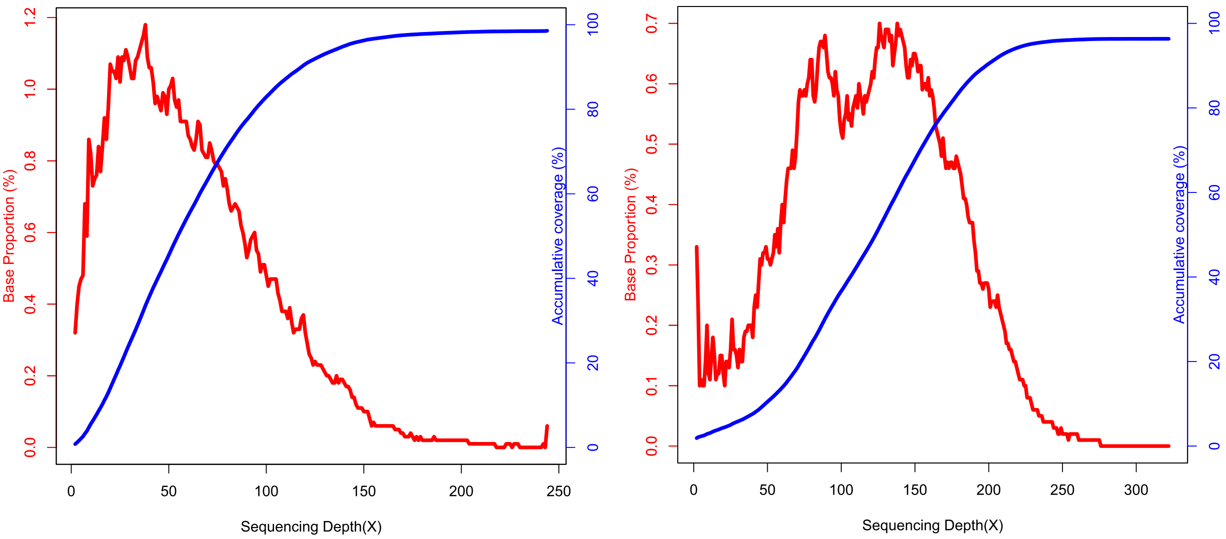
P. bovis strain SH08

P. ciferrii strain SH13

Fig. 4 The Venny picture of functional annotation in five databases (NR, InterPro, KEGG, KOG and Swissport).

(387), with *P. bovis* SH08 and *P. ciferrii* SH13 exhibiting significant involvement in Carbohydrate metabolism (365) as shown in Fig. 3. A total of 3,577 and 3,529 genes can be annotated in all seven databases for *P. bovis* SH08 and *P. ciferrii* SH13, respectively (Fig. 4).

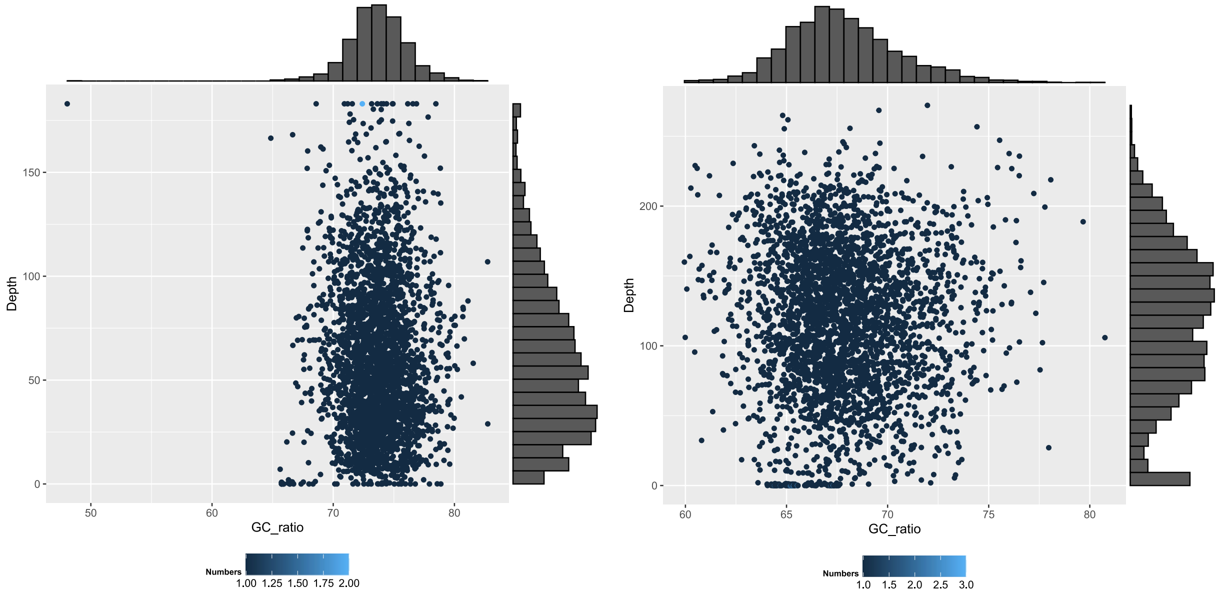
Ethics statement. The Ethics Committee of Shanghai East Hospital, Tongji University approved the study protocol and the sharing of de-identified genomic data. Informed consent was obtained from all participants. The



Prototheca bovis SH08

Prototheca ciferrii SH13

Fig. 5 The plot of sequencing depth and accumulative coverage.



Prototheca bovis SH08

Prototheca ciferrii SH13

Fig. 6 The distribution of GC and depth and accumulative coverage.

ethical approval for this study includes the isolation of Prototheca from the milk of the cows and sample of the patients, and the consent of the dairy farm manager was obtained when collecting the milk samples.

Technical Validation

Evaluation of the genome assembly. The quality assessment of the two strain genomes was conducted by aligning the HiFi reads to a high-quality assembled genome using Minimap2, resulting in a mapping rate and coverage of 96.91% and 98.57% for *P. bovis* SH08, and a mapping rate and coverage of 96.35% and 96.71% for *P. ciferrii* SH13, respectively. The mean depth of mapping coverage is 60.15 × and 114.37 × for *P. bovis* SH08 and *P. ciferrii* SH13, respectively. The distribution of sequencing coverage depth and accumulative coverage showed that the genome assembly is high quality (Fig. 5) with 10 kb window. The accuracy of the final assembly of the two strains was assessed using Merqury with a 19-mer, yielding a quality value (QV) score of 56.4816, indicating an accuracy rate of 99.99977% for SH08 and a QV score of 57.2924, reflecting an accuracy rate of 99.99981% for SH13. The genome sizes of *P. bovis* SH08 and *P. ciferrii* SH13 are comparable to those of *P. zopfii* Pz20 and Pz23 (~31 Mb), while the contig N50 values for *P. bovis* SH08 and *P. ciferrii* SH13 exceed 1 Mb, indicating exceptionally

	P. bovis SH08 (This study)	*P. ciferrii* SH13 (This study)	*P. zopfii* Pz20 (Jian, J., *et al.* 2024)	*P. zopfii* Pz23 (Jian, J., *et al.* 2024)	*P. bovis* (GCA_003612995.1)	*P. cutis* JCM 15793 (GCA_002897115.2)	*P. stagnorum* (GCA_002794665.1)	*P. wickerhamii* S1 (Guo, J., *et al.*[8])
Complete BUSCOs (chlorophyta_odb)	76.70%	78.20%	78.30%	78.40%	73.80%	86.00%	83.10%	86.50%

Table 5. The BUSCO statistics of Prototheca genomes.

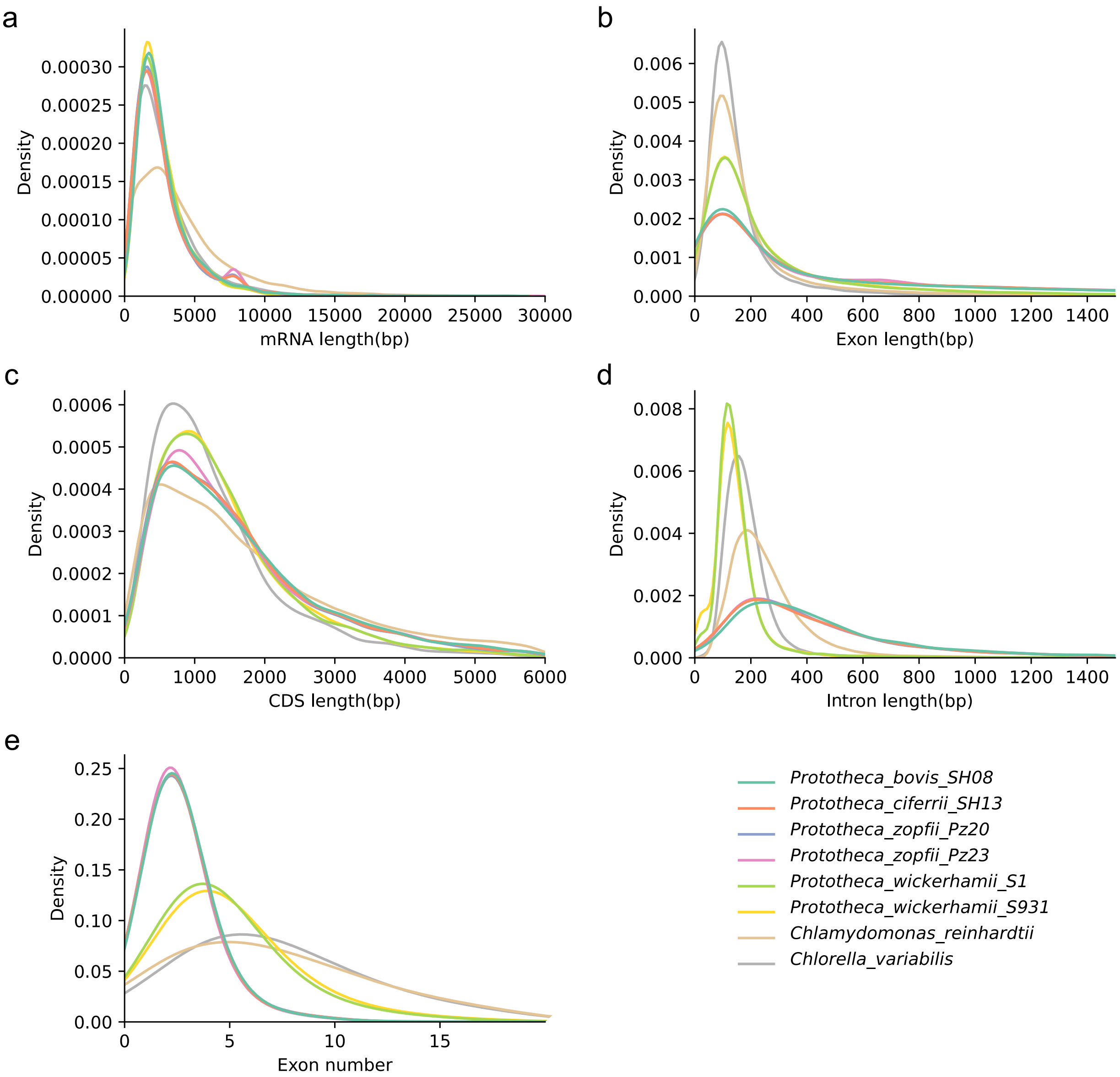


Fig. 7 The gene features in *P. bovis* SH08, *P. ciferrii* SH13 and other related green algal species. (**a**) Distribution of gene(mRNA) length. (**b**) Distribution of exon length. (**c**) Distribution of CDS length. (**d**) Distribution of Intron length. (**e**) Distribution of exon number.

high-quality genome assemblies. The analysis of GC content revealed that the genomes of *P. bovis* SH08 and *P. ciferrii* SH13 exhibit a GC content of 73.6% and 67.8% (Fig. 6), respectively, indicating the absence of exogenous species contamination in both species. To mitigate potential contamination, the HiFi assembled genomes were aligned against the GenBank nucleotide (nt) database, and the available *Prototheca* organelle Refseq genome was retrieved from NCBI. The contigs exhibited no bacterial contamination, and only a limited number of organelle sequences were identified and subsequently excluded from the nuclear genomes. The completeness of the *P. bovis* SH08 and *P. ciferrii* SH13 genome sequences were evaluated using BUSCO, based on the "chlorophyta_odb10" database. The analysis revealed that 76.7% and 78.2% of the 1519 conserved chlorophyta genes were identified as complete (Table 5), which is comparable to other *Prototheca* species.

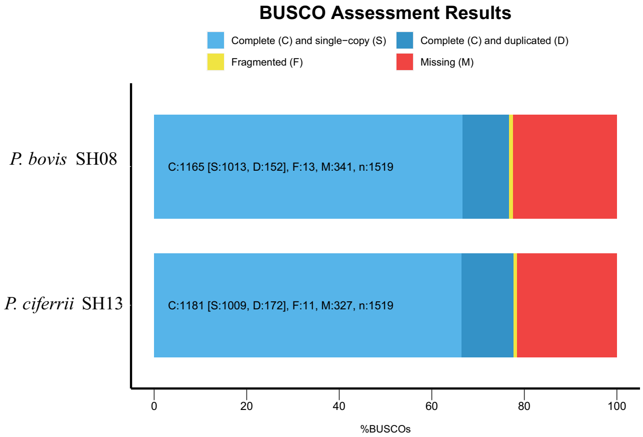


Fig. 8 The BUSCO assessment analysis for gene sets in *P. bovis* SH08 and *P. ciferrii* SH13.

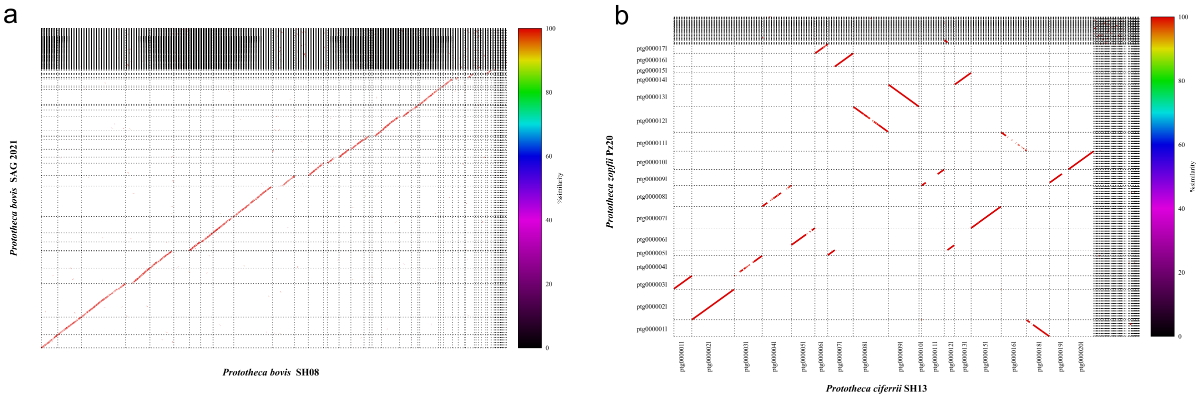


Fig. 9 The genome sequences comparison. (**a**) The sequence comparison between *P. bovis* SH08 and *P. bovis* SAG 2021 genomes. (**b**) The sequence comparison between *P. ciferrii* SH13 and *P. zoffii* Pz20 genomes.

Evaluation of the gene set. Based on the comparison of gene features, the distribution of gene(mRNA) length and coding sequence (CDS) length exhibited remarkable similarity among the eight green algae (Fig. 7a, c). The *P. bovis* SH08 and *P. ciferrii* SH13 exhibited a similar pattern to *P. zopfii* Pz20 and Pz23 in terms of exon length, intron length, and exon number, which is consistent with their comparable genome size and relatedness (Fig. 7b, d, e). Furthermore, the gene features indicated that the gene annotation is of high quality when compared to published data. The completeness of the *P. bovis* SH08 and *P. ciferrii* SH13 gene sets were evaluated using BUSCO, based on the "chlorophyta_odb10" database. The analysis revealed that 76.7% and 77.7% of the 1519 conserved chlorophyta genes were identified as complete (Fig. 8), indicating high-quality gene annotation comparable to other *Prototheca* species (77.3% and 77.6% in *P. zopfii* Pz20 and Pz23)[10].

Genome sequences comparison. To confirm the genome quality, the published *P. bovis* SAG 2021 and *P. zoffii* Pz20 genomes were downloaded. The published *P. bovis* SAG 2021 genome assembly comprised 24,744,895 bp and consisted of 4,555 contigs[15]. The previously fragmented *P. bovis* SAG 2021 genome was scaffolded and patched using the RagTag software[33], based on our newly assembled reference *P. bovis* SH08 genome. A total of 3,815 contigs were placed, with a total length of 21,558,679 bp (87.1% of assembled genome size). Then, two genomes were compared using MUMmer4 with nucmer model (c 2000 -l 400). The mummer analysis of *P. bovis* genomes revealed clearly and high similarity (>97%), and suggested genome assembly was accurate (Fig. 9a). The newly *P. ciferrii* SH13 genome was compared with previous published *P. zoffii* Pz20 genome also using MUMmer4 with nucmer model (c 2000 -l 400). The mummer analysis of genomes revealed nearly one contig to one contig clearly and high similarity (>96%) and suggested genome assembly was correct (Fig. 9b).

Data Records

The *P. bovis* SH08 and *P. ciferrii* SH13 genome sequences and annotations were deposited in Figshare.The dataset had been deposited at PRJNA1184973 in the Sequence Read Archive (SRA)[34]. DNBSeq short-read data of *P. bovis* SH08 was deposited in the SRA at SRR31320872. HiFi long-read data of *P. bovis* SH08 and *P. ciferrii* SH13 were deposited in the SRA at SRR31320874 and SRR31320873. The genome assembly of *P. bovis* SH08 and *P. ciferrii* SH13 had been deposited at GenBank under accession JBJGBU000000000[35] and JBJGBT000000000[36]. The *P. bovis* SH08 and *P. ciferrii* SH13 genome sequences and annotations were deposited in Figshare[37].

Data availability

The dataset of DNB-Seq short-read, HiFi long-read had been deposited in the Sequence Read Archive (SRA) under project number PRJNA1184973[34]. The link of sequencing data was provided below:

DNBSeq short-read data of *P. bovis* SH08 was deposited in the SRA at SRR31320872.

HiFi long-read data of *P. bovis* SH08 was deposited in the SRA at SRR31320874.

HiFi long-read data of *P. ciferrii* SH13 was deposited in the SRA at SRR31320873.

The genome assembly of *P. bovis* SH08 had been deposited at GenBank under accession JBJGBU000000000[35]. The genome assembly of *P. ciferrii* SH13 had been deposited at GenBank under accession JBJGBT000000000[36].

The *P. bovis* SH08 and *P. ciferrii* SH13 genome sequences and annotations were deposited in Figshare[37].

Code availability

This study did not involve the development of any specific code. The data analyses were conducted in accordance with the protocols outlined in the Methods section.

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Author contributions

C. Jiang, W. Wu conceived the study. J. Guo, B. Luo, H. Zhu collected the samples, conducted experiments, Y. Ming, Y. Peng performed bioinformatics analysis. J. Guo, Y. Ming and Y. Xue wrote the manuscript. W. Wu provided suggestion and revised the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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