SCIENTIFIC DATA

DATA DESCRIPTOR

Four high-quality draft genome assemblies of the marine heterotrophic nanoflagellate *Cafeteria roenbergensis*

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The heterotrophic stramenopile *Cafeteria roenbergensis* is a globally distributed marine bacterivorous protist. This unicellular flagellate is host to the giant DNA virus CroV and the virophage mavirus. We sequenced the genomes of four cultured *C. roenbergensis* strains and generated 23.53 Gb of Illumina MiSeq data (99–282 × coverage per strain) and 5.09 Gb of PacBio RSII data (13–45 × coverage). Using the Canu assembler and customized curation procedures, we obtained high-quality draft genome assemblies with a total length of 34–36 Mbp per strain and contig N50 lengths of 148 kbp to 464 kbp. The *C. roenbergensis* genome has a GC content of ~70%, a repeat content of ~28%, and is predicted to contain approximately 7857–8483 protein-coding genes based on a combination of *de novo*, homology-based and transcriptome-supported annotation. These first high-quality genome assemblies of a bicosoecid fill an important gap in sequenced stramenopile representatives and enable a more detailed evolutionary analysis of heterotrophic protists.

Background & Summary

The diversity of eukaryotes lies largely among its unicellular members, the protists. Yet, genomic exploration of eukaryotic microbes lags behind that of animals, plants, and fungi[1]. One of these neglected groups is the Bicosoecida within the Stramenopiles, which contains the widespread marine heterotrophic flagellate, *Cafeteria roenbergensis*[2–6]. The aloricate biflagellated cells lack plastids and feed on bacteria and viruses by phagocytosis[2]. *C. roenbergensis* has been used a model system for many years to study protistan grazing on bacteria[7–9]. The organism appears to be diploid based on sequencing data analysis[10], reproduces by binary fission and has no known sexual cycle. To our knowledge, the only other bicosoecid with a sequenced genome is *Halocafeteria seosinensis*[11], and the most closely related sequenced organisms from other stramenopile groups are the Placidozoa *Blastocystis hominis* and *Incisomonas marina*, as well as members of the Labyrinthulea and the more distant Oomycota and Ochrophyta[12,13]. In addition to these cultured representatives, single-cell genomics of uncultured marine stramenopiles increasingly illuminates the genomic landscape of this diverse group of protistan grazers[14,15]. The phylogenetic position of *Cafeteria* at the base of stramenopiles and the paucity of genomic data among unicellular heterotrophic grazers thus make *C. roenbergensis* an interesting object for genomic studies.

Heterotrophic flagellates of the *Cafeteria* genus are subject to infection by various viruses, including the lytic giant Cafeteria roenbergensis virus (CroV, family *Mimiviridae*) and its associated virophage mavirus (family *Lavidaviridae*)[16–18]. We recently showed that mavirus can exist as an integrated provirophage in *C. roenbergensis* and provide resistance against CroV infection on a host-population level[10]. Genomic studies of *Cafeteria* will reveal new insight into the importance of endogenous viral elements for the evolution and ecology of this group.

Here we present whole-genome shotgun sequencing data and high-quality assemblies of four cultured clonal strains of *C. roenbergensis*: E4-10P, BVI, Cflag and RCC970-E3. The strains were individually isolated from four

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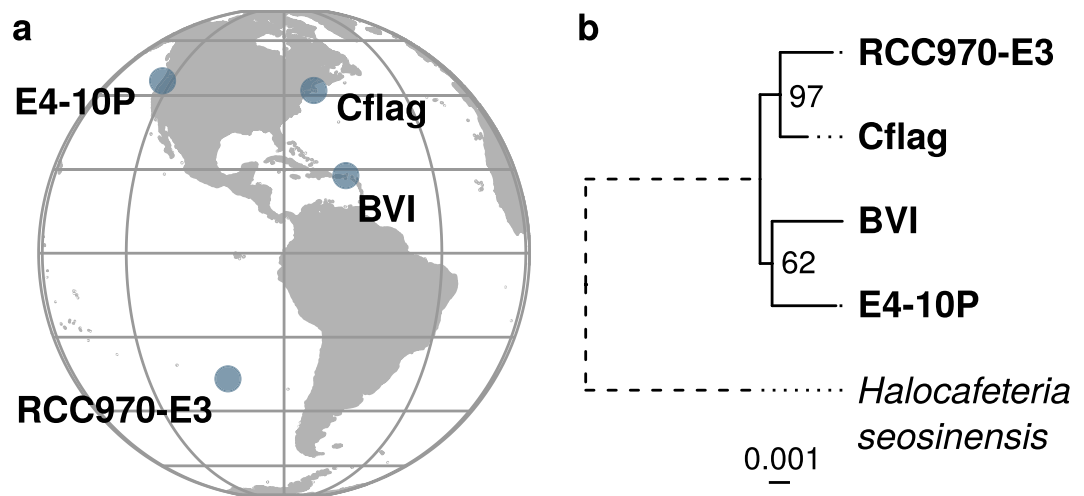
Fig. 1 Sampling locations and phylogenetic relationship of *Cafeteria roenbergensis* strains. (**a**) Map representing the sampling sites of the four *C. roenbergensis* strains around the Americas. (**b**) Maximum likelihood tree reconstructed from a concatenated alignment of 123 shared single-copy core genes for the four *C. roenbergensis* strains and their outgroup *Halocafeteria seosinensis*. Numbers next to internal nodes indicate bootstrap support based on 100 iterations. The branch to the outgroup represented by a dashed line has been shortened for visualization.

Species	Strain	Location	Coordinates	Year	Biosample	Roscoff ID
C. roenbergensis	E4-10P	North Pacific, 5 km west of Yaquina Bay, Oregon	44.62N 124.06W	1989	SAMN12216681	RCC:4624
C. roenbergensis	E4-10M1	North Pacific, 5 km west of Yaquina Bay, Oregon	44.62N 124.06W	1989	SAMN12216695	RCC:4625
C. roenbergensis	BVI	The British Virgin Islands	18.42N 64.61W	2012	SAMN12216698	
C. roenbergensis	Cflag	North Atlantic Ocean, Woods Hole, MA	41.52N 70.67W	1986	SAMN12216699	
C. roenbergensis	RCC970-E3	South Pacific Ocean, 2200 km off the coast of Chile	30.78S 95.43W	2004	SAMN12216700	RCC:4623

Table 1. Strain and sample information.

different locations (Fig. 1a). CrCflag and CrBVI were obtained from coastal waters of the Atlantic Ocean at Woods Hole, MA, USA (1986) and the British Virgin Islands (2012). CrE4-10P was collected from Pacific coastal waters near Yaquina Bay, Oregon, USA (1989). CrRCC970-E3 was obtained from open ocean waters of the South Pacific, collected about 2200 km off the coast of Chile during the BIOSOPE cruise[19] (2004) (Table 1). In addition, we also sequenced strain CrE4-10M1, an isogenic variant of CrE4-10P carrying additional integrated mavirus genomes previously described by Fischer and Hackl[10]. CrE4-10M1 read data was used to support the CrE4-10P genome assembly after mavirus-containing data was removed.

Overall we generated 23.53 Gbp of raw short read data on an Illumina MiSeq platform with 99–282 × coverage per strain, and 5.09 Gbp of raw long read data on a PacBio RS II platform with 13–45 × coverage per strain (Table 2)[20]. Based on 19-mers frequencies, we estimate a haploid genome size for *C. roenbergensis* of approximately 40 Mbp (Fig. 2). We first generated various draft assemblies with different assembly strategies and picked the best drafts for further refinement (see Technical Validation)[21]. After decontamination, assembly curation and polishing, we obtained four improved high-quality draft assemblies with 34–36 Mbp in size and contig N50s of 148–460 kbp (Table 3)[22–25]. The genomes have a GC-content of 70–71%, and 28% of the overall sequences were marked as repetitive.

We annotated 82–84% of universal eukaryotic single-copy marker genes in each genome (Fig. 3). The majority of the missing markers are consistently absent from all four genomes, suggesting poor representation in reference databases or the complete lack of these genes from the group rather than problems with the quality of the underlying assemblies as the most likely explanation. A maximum-likelihood phylogeny reconstructed from a concatenated alignment of 123 shared single-copy markers suggests that CrRCC970-E3 and CrCflag diverged most recently (Fig. 1b). The exact placement of the other two strains within the group could only be determined with low bootstrap support. These observations are consistent with average nucleotide identity (ANI) and comparisons of the ribosomal DNA operon. All four strains are within 99% ANI, with CrCflag and CrRCC970-E3 being most similar (99.67%). All other pairwise comparisons are within 99.05% to 99.22% ANI (Supplementary Fig. 1)[21]. The 18S rDNA sequences of all four strains are identical. For the full operon, we observe small differences in the two more divergent strains CrBVI and CrE4-10P (CrBVI: 1 substitution in the 28S, 2 in the ITS1; CrE4-10P: 1 substitution in the ITS2). We also found the gene copy number of the operon to vary widely amongst the strains. Based on read coverage we estimate that CrCflag carries 18 haploid copies of the operon, CrRCC970-E3 21, CrBVI 63 and CrE4-10P 83. Although variation on this scale has been observed as a common feature of marine eukaryotic plankton[26], it is surprising to find this much variation among such closely related strains. Finally, we identified 4

Cafeteria strain	Instrument	Library layout	# Libraries	Library size (Gbp)	Coverage	SRA study accession	SRA run accession
E4-10P	Illumina MiSeq	paired	2	6.85	171	SRP215872	SRR9724619
E4-10P	PacBio RS II	single	2	0.52	13	SRP215872	SRR9724618
E4-10M1	Illumina MiSeq	paired	2	4.45	111	SRP215872	SRR9724621
E4-10M1	PacBio RS II	single	2	1.3	32	SRP215872	SRR9724620
BVI	Illumina MiSeq	paired	1	4.31	108	SRP215872	SRR9724615
BVI	PacBio RS II	single	3	1.8	45	SRP215872	SRR9724614
Cflag	Illumina MiSeq	paired	1	3.94	99	SRP215872	SRR9724617
Cflag	PacBio RS II	single	2	0.95	24	SRP215872	SRR9724616
RCC970-E3	Illumina MiSeq	paired	1	3.98	100	SRP215872	SRR9724623
RCC970-E3	PacBio RS II	single	2	0.52	13	SRP215872	SRR9724622

Table 2. Sequencing information and library statistics.

Assembly	# Contigs	Total size (bp)	Contig N50 (bp)	% GC	% Repeats	% BUSCOs	# Proteins	Genbank Accession
CrE4-10P	218	35,335,825	402,892	70.5	27.8	83.8	8364	VLTO01000000
CrBVI	170	36,327,047	460,467	70.1	27.7	83.2	8483	VLTN01000000
CrCflag	270	34,521,237	231,394	70.5	27.9	82.8	8018	VLTM01000000
CrRCC970-E3	396	33,988,271	148,311	70.5	27.9	81.8	7857	VLTL01000000

Table 3. Assembly and annotation statistics.
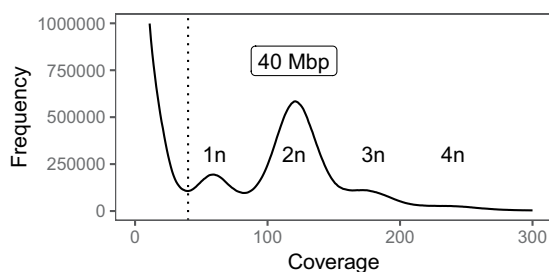
Fig. 2 K-mer frequency distribution and estimated genome size of *Cafeteria roenbergensis* strain E4-10P. Frequency distribution of 19-mers in the quality-trimmed MiSeq read set of CrE4-10P. The major peak at ~$120\times$ coverage corresponds to the majority of homozygous k-mers of the diploid (2n) genome, the smaller peak at half the coverage comprises haplotype-specific (1n) k-mers. Small peaks at 3n and 4n represent regions of higher copy numbers. Low-coverage k-mers derive from sequencing errors and bacterial contamination. Cumulatively, the k-mer distribution suggests an approximate haploid genome size of 40 Mbp.

single nucleotide polymorphisms (SNPs) in the rDNA operon across all four strains; 3 unique to CrBVI, 1 unique in CrE4-10P. These 4 SNPs match the sites of the 4 divergent substitutions described above. Interestingly, at all four sites, we observe that the minor alternative variant with a relative frequency of 26–27% corresponds to the nucleotide found at this position in the other 3 strains.

To analyze the genomic capabilities of *C. roenbergensis*, we annotated 7857–8483 protein-coding genes per strain using a combination of *de novo*, homology- and RNA-seq-supported gene prediction and homology-based functional assignments against UniProtKB/Swiss-Prot and the EggNOG database. The nuclear genomes contained on average 1.67–1.89 introns per gene. In addition, all four assemblies comprise a curated circular mitochondrial genome that was annotated independently using a non-standard genetic code with UGA coding for tryptophan instead of a stop codon[27].

We anticipate that the genomic data presented here will enable detailed studies of *C. roenbergensis* to shed more light on the biology of this ecologically important group of marine grazers. For instance, no sexual modes of reproduction have been described for *C. roenbergensis*, which does not preclude their existence. The *Cafeteria* genome may thus provide insights into possible sexual processes and how its evolution is influenced by mobile genetic elements.

Methods
Strain maintenance, sample preparation, and sequencing.
We selected and sequenced four strains of *C. roenbergensis* isolated from different locations in the Atlantic (Woods Hole, MA, USA; British Virgin Islands) and the Pacific (Yaquina Bay, OR, USA; South Pacific Ocean, 2200 km off the coast of Chile) (Table 1). All flagellate strains were grown in f/2 artificial seawater medium in the presence of either sterilized wheat grains
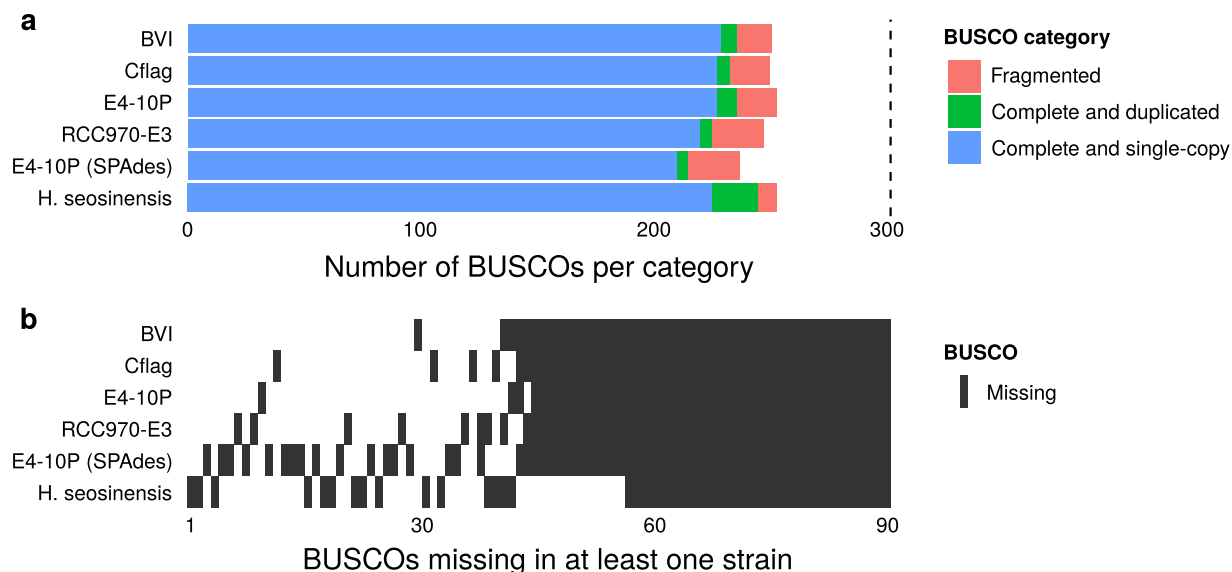
Fig. 3 Completeness assessment of *Cafeteria* genome assemblies based on single-copy orthologs. (**a**) Abundance of 303 single-copy core gene markers (BUSCOs) in different categories and assemblies. (**b**) Distribution of BUSCOs missing in at least one assembly (black tiles).

(stock cultures) or 0.05% (w/v) yeast extract (for rapid growth), to stimulate the growth of a mixed bacterial community, which serve as a food source for *C. roenbergensis*. Each *C. roenbergensis* culture was subject to three consecutive rounds of single-cell dilution to obtain clonal strains as described previously[10]. For genome sequencing, suspension cultures of 2 L for each strain were grown to approximately 1×10^6 cells/mL, then diluted two-fold with antibiotics-containing medium (30 μg/mL Streptomycin, 60 μg/mL Neomycin, 50 μg/mL Kanamycin, 50 μg/mL Ampicillin, 25 μg/mL Chloramphenicol) and incubated for 24 h at 22 °C and 60 rpm shaking to reduce the bacterial load. Cultures were filtered through a 100 μm Nitex mesh to remove larger aggregates and centrifuged in various steps to further remove bacteria. First, the cultures were centrifuged for 40 min at 6000 × g and 20 °C (F9 rotor, Sorvall Lynx centrifuge), and the cell pellets were resuspended in 50 mL of f/2 artificial seawater medium, transferred to 50 mL polycarbonate tubes and centrifuged for 10 min at 4500 × g, 20 °C in an Eppendorf 5804 R centrifuge. The supernatant was discarded and the cell pellet was resuspended in 50 mL of PBS (phosphate-buffered saline) medium. This washing procedure was repeated 10 times until the supernatant was clear, indicating that most bacteria had been removed. In the end, the flagellates were pelleted and resuspended in 2 mL of PBS medium. Genomic DNA from approximately 1×10^9 cells of strains was isolated using the Blood & Cell Culture DNA Midi Kit (Qiagen, Hilden, Germany). The genomes were sequenced on an Illumina MiSeq platform (Illumina, San Diego, California, USA) using the MiSeq reagent kit version 3 at 2 × 300-bp read length configuration. The E4-10P genome was sequenced by GATC Biotech AG (Constance, Germany) with the standard MiSeq protocol. The E4-10M1, BVI, Cflag and RCC970-E3 genomes were prepared and sequenced at the Max Planck Genome Centre (Cologne, Germany) with NEBNext High-Fidelity 2 × PCR Master Mix chemistry and a reduced number of enrichment PCR cycles (six) to reduce AT-bias. We also sequenced genomic DNA of all strains on a Pacific Biosciences RS II platform (2–3 SMRT cells each, Max Planck Genome Centre, Cologne, Germany).

Assembly, decontamination, and refinement. MiSeq reads were trimmed for low-quality bases and adapter contamination using Trimmomatic[28]. PacBio reads were extracted from the raw data files with DEXTRACTOR[29]. Proovread[30] was used for the hybrid correction of the PacBio reads with the respective trimmed MiSeq read sets. The K-mer analysis was carried out with jellyfish[31] and custom R scripts to plot the distribution and estimate the genome size[21]. To determine the best assembly strategy, we assessed draft assemblies generated with different approaches as described in the Technical Validation section. The improved high-quality drafts presented here were assembled using Canu v1.8[32] from raw PacBio reads only for CrE4-10P, CrBVI, and CrCflag, and from raw and Illumina-corrected PacBio reads for CrRCC970-E3. For the latter strain, raw and corrected versions of the same PacBio reads were used together to mitigate low PacBio coverage in this particular sample and obtain a more contiguous assembly. Following the initial assembly, we used Redundans[33] to remove redundant contigs, which were reconstructed as individual alleles due to high heterozygosity. To reduce misassemblies, we further broke up contigs at unexpected drops in coverage based on reads mapped with minimap2[34] and identified with the custom Perl script bam-junctions[21] and bedtools[35]. After exploring different approaches (see Technical Validation) bacterial contamination was identified and removed based on taxonomic assignments generated with Kaiju[36] and the script tax-resolve[21] using the ETE 3 python library[37]. To obtain these assignments, each contig was split into 500 bp fragments, which we classified against the NCBI non-redundant protein database. Contigs with more than 50% of fragments annotated as bacteria were excluded from the assembly. Finally, to remove base-level errors we polished the assemblies in two rounds by mapping back first PacBio, then Illumina reads with minimap2[34], and by generating consensus sequences from the mappings with Racon[38].

Primer name	Forward (5′ to 3′)	Reverse (5′ to 3′)
TBP-PCR#1	CCGCGATGCTTCTGCCTCCA	CGCGCAGTCGAGATTCACAGT
TBP-PCR#2	GCCATCACCAAGCACGGGATCA	CGCGCAGTCGAGATTCACAGT
60sRP-PCR#3	CGCAACCAGACCAAGTTCCACG	GTACGCCAGAGCATGCGGGA
60sRP-PCR#4	CGCACTGAGGAGGTGAACGTC	GCGGGTTGGTGTTCCGCTTC

Table 4. Primers used for the validation of the intron-exon structure in two genes of *C. roenbergensis* strain RCC970-E3.

Gene prediction and functional annotation. Repetitive regions were detected with WindowMasker[39]. Only repetitive regions with a minimum length of 100 bp were retained. tRNA genes were predicted with tRNAscan2.0[40] with a minimum score of 70. Gene prediction was performed with the BRAKER pipeline[41,42], which utilizes BLAST[43,44], Augustus[45,46] and GeneMark-ES[47,48]. Augustus and GeneMark-ES gene models were trained with publicly available transcriptomic data of *C. roenbergensis* E4-10P as extrinsic evidence (http://datacommons.cyverse.org/browse/iplant/home/shared/imicrobe/projects/104/samples/2026)[49]. Prior to gene prediction, splice sites were detected by HISAT[50] and processed with samtools[51,52]. Protein functions were assigned with two different approaches: (1) by blastx best hit against the UniProtKB/Swiss-Prot database v14/01/2019[53] and (2) by eggNOG-mapper[54] best match against the EggNOG v4.5.1 database[55]. Only results with an E-value of 10^{-3} or lower were retained. In addition, blastx hits with bitscores below 250, percentage identities below 30, or raw scores below 70 were ignored.

Mitochondrial genome curation and annotation. To obtain a complete and correctly annotated mitochondrial genome, we first mapped the whole genome assemblies against an existing *C. roenbergensis* mitochondrial reference genome (NCBI accession NC_000946.1) to identify the mitochondrial contig using minimap2[34]. We then extracted the contig, trimmed overlapping ends of the circular sequence with seq-circ-trim and reset the start to the same location as the reference genome - the large subunit ribosomal RNA gene - with seq-circ-restart[21]. Gene annotation was carried out with Prokka[56] and with an adjusted non-standard translation code. Predicted tRNA and coding genes completely overlapping other coding regions were manually removed guided by the reference genome annotation.

PCR and reverse-transcription PCR conditions. Genomic DNA (gDNA) was extracted from 200 µl of suspension culture with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for DNA purification of total DNA from cultured cells, with a single elution step in 100 µl of double-distilled (dd) H2O and storage at −20 °C.

For extraction of total RNA, 500 µl of suspension culture were centrifuged for 5 min at 4,500 g, 4 °C. The supernatants were discarded and the cell pellets were immediately flash-frozen in N2(l) and stored at −80 °C until further use. RNA extraction was performed with the Qiagen RNeasy Mini Kit following the protocol for purification of total RNA from animal cells using spin technology. Cells were disrupted with QIAshredder homogenizer spin columns and an on-column DNase I digest was performed with the Qiagen RNase-Free DNase Set. RNA was eluted in 50 µl of RNase-free molecular biology grade water. The RNA was then treated with 1 µl TURBO DNase (2 U/µl) for 30 min at 37 °C according to the manufacturer's instructions (Ambion via ThermoFisher Scientific, Germany). RNA samples were analyzed for quantity and integrity with a Qubit 4 Fluorometer (Invitrogen via ThermoFisher Scientific, Germany) using the RNA Broad Range and RNA Integrity and Quality kit respectively.

For cDNA synthesis, 6 µl of each RNA sample was reverse transcribed using the Qiagen QuantiTect Reverse Transcription Kit according to the manufacturer's instructions. This protocol included an additional DNase treatment step and the reverse transcription reaction using a mix of random hexamers and oligo(dT) primers. Control reactions to test for gDNA contamination were done for all samples by adding ddH2O instead of reverse transcriptase to the reaction mix. The cDNA was diluted fivefold with RNase-free H2O and analyzed by PCR with gene-specific primers (Table 4).

PCR amplifications were performed using 2 ng of gDNA template or 2 µl of the diluted cDNA in a 20 µl reaction mix containing 10 µl Platinum™ II Hot-Start PCR Master Mix (Invitrogen via ThermoFisher Scientific, Germany), 4 µl Platinum GC Enhancer and 0.2 µM of each primer.

The following cycling conditions were used in a ProFlex PCR System (Applied Biosystems via ThermoFisher Scientific, Germany): 2 min denaturation at 94 °C and 35 cycles of 15 s denaturation at 94 °C, 20 s annealing at 60 °C (for all primers) and 20 s extension at 68 °C. For product analysis, 1 µl of each reaction were mixed with loading dye and pipetted on a 1% (w/v) agarose gel supplemented with GelRed. The marker lanes contained 0.5 µg of GeneRuler 100 bp DNA Ladder (Fermentas, Thermo-Fisher Scientific, USA). The gel was electrophoresed for 1 h at 100 V and visualized on a ChemiDoc MP Imaging System (BioRad, Germany).

Comparative and phylogenetic analysis. Phylogenetic relationships among the *C. roenbergensis* strains and *Halocafeteria seosinensis* (Genbank accession LVLI00000000) were reconstructed from the concatenated alignment of 123 shared single-copy orthologous genes. Orthologs were identified with BUSCO[57,58] with the eukaryotic lineage dataset. Only orthologs present in all five genomes with a BUSCO score of at least 125 and a minimum covering sequence length of 125 bp were taken into account. Orthologous protein sequences were aligned with MAFFT[59] and trimmed for poorly aligned regions with trimAl[60]. The phylogenetic tree was computed with RAxML using the GAMMA model of rate heterogeneity and automatically determined amino acid

substitution models for each partition. The bootstrap confidence values were computed with 100 iterations of rapid bootstrapping. The tree was rooted with *H. seosinensis* as the phylogenetic outgroup using phytools[61] and visualized with ggtree[62].

The rDNA operon was annotated with barrnap (https://github.com/tseemann/barrnap), sequences processed with seqkit[63] and aligned with muscle[64]. Reads were aligned with minimap2[34] for coverage estimation using samtools and SNP calling with bcftools[65]. The haploid copy number of the rDNA operon was estimated by comparing the median coverage across the operon to the median coverage across the 50 longest contigs per assembly.

Average nucleotide identities (ANI) were obtained with fastANI[66].

Data Records

The raw Illumina and PacBio sequencing reads are available from the NCBI Sequence Read Archive[20]. Accession numbers, library size, and coverage statistics can be found in Table 2. The curated and annotated assemblies for CrE4-10P, CrBVI, CrCflag and CrRCC970-E3 have been deposited as Whole Genome Shotgun projects at DDBJ/ENA/GenBank under the accessions VLTO00000000, VLTN00000000, VLTM00000000, VLTL00000000. The versions described in this paper are VLTO01000000[22], VLTN01000000[23], VLTM01000000[24], VLTL01000000[25]. These records also each comprise the curated and annotated mitochondrial genome of the respective strain on a single contig denoted with the suffix "mito". The final assemblies including annotations and the draft assemblies we initially generated to determine the best assembly strategy together with custom code used in the analysis are available from GitHub (http://github.com/thackl/cr-genomes) and Zenodo[21].

Technical Validation

Overall sequencing quality of MiSeq and PacBio read data was assessed with FastQC v0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). To choose the best assemblies for further refinement, we evaluated different assemblers and alternative assembly strategies. In particular, we assembled draft genomes using MiSeq reads and corrected PacBio data with SPAdes[67] in diploid mode, and generated assemblies from raw PacBio reads with Flye[68] and wtdbg2[69]. Using QUAST[70], BUSCO[58], and the misassembly detection procedure described in Material and Methods, we assessed contiguity, completeness, and quality of the different assemblies. We found that the Illumina-based assemblies, in general, were less complete and less contiguous than the PacBio-based assemblies[21]. The PacBio assemblies differed primarily in the number of potential misassembly sites, with Canu being least prone to this potential issue. Therefore, we selected assemblies generated with Canu for further processing and analysis.

Because the read data was obtained from non-axenic cultures, we screened the assemblies carefully for contaminations with a custom R script. Initially, we considered four different criteria: tetra-nucleotide frequencies, coverage, GC-content, and taxonomic assignments (Supplementary Figs. 2–5). Tetra-nucleotide frequencies and GC-content were computed with seq-comp[21]. Medium contig coverage was determined based on MiSeq reads mapped with minimap2 using bam-coverage[21]. Taxonomic assignments were generated with Kaiju[36] (See Materials and Methods for details). We found that all four criteria generated similar and consistent results. All identified contaminations were classified as bacterial. Viral signatures could all be attributed to endogenous viral elements expected to be present in *Cafeteria* genomes. From this analysis, we established a simple rule for decontamination of the assemblies: Contigs with more than 50% of annotated regions classified as bacteria were excluded.

To further assess the completeness and quality of our assemblies, we used BUSCO[57,58] to detect universal eukaryotic orthologous genes (Fig. 1). We found that all our PacBio-based assemblies contain 82–84% of the expected 303 orthologs. Moreover, most missing orthologs are absent from all four assemblies suggesting poor representation in the database or the complete lack of some of these markers from this group as a systemic issue, rather than assembly problems, which would affect different genes in different assemblies. To validate the automated gene predictions, we spot-checked the intron-exon structure for two genes using regular and reverse-transcription PCR (Supplementary Fig. 6). We selected two intron-containing genes, namely genes coding for a TATA-binding protein (locus tag: FNF27_01237) and a 60S ribosomal protein (locus tag: FNF28_01226). Primers were designed to amplify the intronic regions and PCR with these primers resulted in long amplicons when using gDNA as the template and a shorter amplicon when using cDNA as the template. The cDNA was obtained after reverse transcription of total RNA from *C. roenbergensis* strain RCC970-E3.

Code availability

All custom code used to generate and analyze the data presented here is available from https://doi.org/10.5281/zenodo.355113321 and from http://github.com/thackl/cr-genomes.

Software versions and relevant parameters:

Trimmomatic v0.32 (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 SLIDINGWINDOW:10:20 MINLEN:75 LEADING:3 TRAILING:3); proovread v2.12 (config settings: 'seq-filter' => {'–trim-win' => '10,1', '–min-length' => 500}, 'sr-sampling' => {DEF => 0}); Canu v1.8; Flye v2.3.7; WTDBG v2.1; SPAdes v3.6.1 (–diploid); minimap2 v2.13-r858-dirty (PacBio reads: -x map-pb; MiSeq readsL -x sr); bam-junctions SHA: 28dc943 (-a200 -b200 -c5 -d2 -f30 -e30); Redundans v0.14a (–noscaffolding –norearrangements –nogapclosing); Kaiju v1.6.3 (-t kaijudb/nodes.dmp -f kaijudb/kaiju_db_nr_euk.fmi); Prokka v1.13 (–kingdom Mitochondria –gcode 4); DEXTRACTOR rev-844cc20; jellyfish v2.2.4; samtools v1.7; Racon v1.3.1; BUSCO v3.1.0; WindowMasker 1.0.0; tRNAscan v2.0; BRAKER v.2.1.1; BLAST v.2.6.0+; Augustus v3.3.2; GeneMark-ES v.4.38; HISAT v.2.1.0; MAFFT v7.310; trimAl v1.4.rev22 (-strictplus); RAxML v8.2.9 (-p 13178 -f a -x 13178 -N 100 -m PROTGAMMAWAG -q part.txt); phytools v0.6-60; ggtree v1.14.4; barrnap (–kingdom euk); bcftools v1.9 (mpileup; call -mv -Ob);

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Author contributions

T.H. and M.G.F. designed the study and wrote the manuscript with contributions from all other authors. K.B. and M.G.F. maintained the cultures and extracted DNA for sequencing. T.H. generated, curated and analyzed the assemblies and annotation data. R.M. carried out gene annotations. T.H. and R.M. carried out phylogenetic analyses. S.D. conducted the experimental validation of gene models. M.G.F and D.H. supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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