

JHU THESIS TEMPLATE TITLE

by

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Doctor of Philosophy**

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Abstract

While next generation sequencing (NGS) has enabled massively parallel DNA sequencing for lower and lower cost, the development of third generation nanopore sequencing offers several key advantages over older sequencing methods. Nanopore sequencers are pocket-sized, making them orders of magnitude cheaper than the next most affordable alternative and the ideal option for wide deployment. They are capable of providing data in real-time, saving valuable hours before data analysis can begin. Additionally, they are able to sequence reads several thousand basepairs long, as opposed to the hundreds of basepairs NGS platforms are capable of, and they embed base modification data without the need for specific treatment beforehand. Given these advantages, in this thesis I examine the application of nanopore sequencing to the study of human pathogens.

First, we use nanopore sequencing to characterize anti-microbial resistance (AMR) in forty clinical isolates. We analyzed real-time data to quickly identify AMR genes, assembled genomes to identify chromosomal mutations, and used short-read sequencing data to correct the errors in the assemblies. With sequencing data, we found that time to effective antibiotic therapy could be shortened by as much as 20 hours compared to standard antimicrobial

susceptibility testing (AST).

Second, we leverage the long reads of nanopore sequencing to assemble the genome of a pathogenic yeast, *Candida nivariensis*. Previous efforts to assemble this yeast genome relied solely on NGS data, resulting in a highly fragmented genome. Using nanopore data, we achieve a much higher contiguity, capture previously missing portions of the genome. Furthermore, we demonstrate that our more contiguous genome can be used to better study long and repetitive genes, such as those involved in pathogenicity to humans.

Third, we use the base modification information embedded in nanopore sequencing data to call methylation in metagenomic assemblies. These calls enable the binning of metagenomic contigs according to methylation signature without the need to collect additional data. We demonstrate the efficacy of this method on a synthetic community sample, a simple two-bacteria system, and a clinical sample with matched proximity ligation binning data.

These applications of nanopore sequencing demonstrate its potential and its utility for all fronts of pathogen genomics research.

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directly or in subtler ways,
to this work.

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Chapter 1

Introduction

Introduce your thesis (Aardvark, 1900)

References

Aardvark, A. A. (1900). "Article title". In: *Journal One* 1.1, pp. 1–8.

Chapter 2

Applying Rapid Whole-Genome Sequencing To Predict Phenotypic Antimicrobial Susceptibility Testing Results among Carbapenem-Resistant *Klebsiella pneumoniae* Clinical Isolates

Portions of this chapter originally appeared in:

Tamma PD, Fan Y, Bergman Y, Pertea G, Kazmi AQ, Lewis S, et al. Applying Rapid Whole-Genome Sequencing To Predict Phenotypic Antimicrobial Susceptibility Testing Results among Carbapenem-Resistant *Klebsiella pneumoniae* Clinical Isolates 2019;63. <https://doi.org/10.1128/AAC.01923-18>

2.1 Abstract

Standard antimicrobial susceptibility testing (AST) approaches lead to delays in the selection of optimal antimicrobial therapy. Here, we sought to determine the accuracy of antimicrobial resistance (AMR) determinants identified by Nanopore whole-genome sequencing in predicting AST results. Using a cohort of 40 clinical isolates (21 carbapenemase-producing carbapenem-resistant *Klebsiella pneumoniae*, 10 non-carbapenemase-producing carbapenem-resistant *K. pneumoniae*, and 9 carbapenem-susceptible *K. pneumoniae* isolates), three separate sequencing and analysis pipelines were performed, as follows: (i) a real-time Nanopore analysis approach identifying acquired AMR genes, (ii) an assembly-based Nanopore approach identifying acquired AMR genes and chromosomal mutations, and (iii) an approach using short-read correction of Nanopore assemblies. The short-read correction of Nanopore assemblies served as the reference standard to determine the accuracy of Nanopore sequencing results. With the real-time analysis approach, full annotation of acquired AMR genes occurred within 8h from subcultured isolates. Assemblies sufficient for full resistance gene and single-nucleotide polymorphism annotation were available within 14h from subcultured isolates. The overall agreement of genotypic results and anticipated AST results for the 40K. *pneumoniae* isolates was 77

2.2 Introduction

Whole-genome sequencing (WGS) has enabled notable advancements to the field of infectious diseases, such as an improved understanding of transmission dynamics and outbreak analysis (1). An exciting possibility from this technology is the ability to predict antimicrobial susceptibility testing (AST) results based on the identification of acquired resistance genes and/or chromosomal mutations (2).

Currently, there are several shortcomings with standard approaches to AST, particularly as they relate to multidrug-resistant Gram-negative (MDRGN) organisms. First, AST results are reported approximately 48 to 72h after the time of culture collection, potentially leading to delays in appropriate empirical antibiotic therapy (3). Second, automated AST panels are limited in the number of antibiotic agents included. For agents that frequently need to be considered for highly drug-resistant pathogens (e.g., colistin, tigecycline, ceftazidime-avibactam, etc.) and newer agents in later phases of development that are unlikely to be routinely included in AST panels for the foreseeable future, there are additional delays in AST determination. As it is generally not evident at the time antibiotics are initiated that a patient will be infected with an MDRGN organism, susceptibility testing for these last-resort agents occurs subsequent to, and not simultaneously with, automated AST testing. Third, standard AST reporting does not include identification of resistance mechanisms (e.g., carbapenemases, extended-spectrum -lactamases [ESBLs], etc.), which can be important for guiding antibiotic treatment decisions, as in vitro activity does not always translate to in vivo activity (4). WGS can

potentially alleviate many of these concerns by offering the potential to predict AST results by identifying the presence or absence of resistance genes, as well as mutations in relevant genes, from which clinicians can infer the activity of antibiotic agents.

Oxford Nanopore Technologies (Oxford, England) has created a Nanopore-based DNA sequencer that sequences DNA by monitoring the electrical current as DNA passes through a protein pore. Unlike second-generation sequencing methods, which require the entire run to be completed before data can be analyzed, Nanopore sequencing streams long-read data in real time (5), allowing for resistance gene identification within as few as 15min of beginning the sequencing run (6–8). As the duration of time needed for DNA extraction and library preparation techniques continues to be reduced, the total time to identification of resistance determinants from organism growth could conceivably be accomplished within a single laboratory shift. To further advance this science, we evaluated the correlation of resistance determinants identified through Nanopore sequencing with AST results in a cohort of 40 clinical *Klebsiella pneumoniae* complex isolates. This also enabled us to quantify the potential decrease in time to effective antibiotic therapy for the patients contributing isolates with the use of WGS using real-time analysis or rapid assembly approaches compared to that with traditional AST methods.

2.3 Results

2.3.1 Percent agreement of WGS in predicting AST results

2.3.2 Time to resistance determination

2.3.3 Drug resistances

2.4 Discussion

2.5 Materials and Methods

2.5.1 Study cohort

2.5.2 Species and antimicrobial susceptibility testing

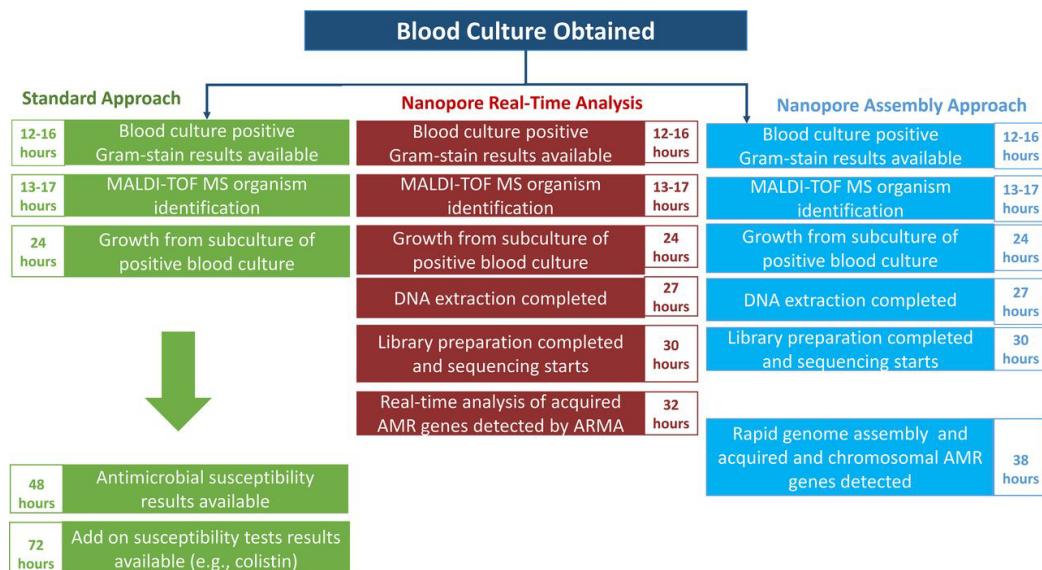


Figure 2.1: Estimated timelines of resistance detection. Schematic of Nanopore sequencing with a real-time analysis and assembly-based approach for identifying resistance genes compared to standard of care testing, using an example of a positive blood culture. MALDI-TOF MS, matrix-assisted laser desorption ionization<U+2013>time of flight mass spectrometry; AMR, antimicrobial resistance; AST, antimicrobial susceptibility testing.

2.5.3 Whole-genome sequencing and antimicrobial resistance gene detection

2.5.4 Predicted correlations between WGS and AST results

2.5.5 Clinical data

2.5.6 Data Availability

Supplemental Dataset: Rapid Nanopore Assembly Statistics				
Isolate Number	# of contigs	n50 (bp)	longest contig (bp)	shortest contig (bp)
1	6	3279607	3279607	39430
2	10	5316522	5316522	14713
3	5	4527157	4527157	16841
4	3	5368538	5368538	17428
5	2	5293767	5293767	129043
6	5	5455776	5455776	14379
7	8	5431055	5431055	17297
8	4	5237837	5237837	27046
9	6	5484734	5484734	34263
10	2	5172417	5172417	209465
11	2	5192109	5192109	198529
12	6	5350919	5350919	26469
13	5	5297830	5297830	33964
14	8	5339196	5339196	21363
15	5	5331989	5331989	9743
16	4	5477999	5477999	36255
17	12	2025764	2123455	2453
18	4	5411221	5411221	51750
19	6	5394690	5394690	16798
20	7	5284029	5284029	23318
21	7	5500581	5500581	29080
22	4	5311389	5311389	24974
23	7	5369624	5369624	30029
25	1	5176691	5176691	5176691
26	3	5322531	5322531	41651
28	5	5442403	5442403	4430
29	8	4425498	4425498	29802
30	5	5440840	5440840	13982
31	5	5192492	5192492	33746
32	4	5162118	5162118	54490
33	4	5236343	5236343	49389
34	2	5210236	5210236	233404
35	3	5325108	5325108	144054
36	4	5380945	5380945	34240
37	5	5373953	5373953	23643
38	3	5068471	5068471	143600
39	2	5239676	5239676	97726
40	3	4418684	4418684	304407

Table 2.1: Rapid Pipeline. Assembly statistics using the rapid pipeline

Chapter 3

Genome assembly of *Candida nivariensis*

Portions of this chapter originally appeared in:

Fan Y, Gale AN, Bailey A, Barnes K, Colotti K, Mass M, et al. Genome and transcriptome of a pathogenic yeast, *Candida nivariensis*. G3 Genes | Genomes | Genetics. 2021;11. doi:10.1093/g3journal/jkab137

3.1 Abstract

We present a highly contiguous genome and transcriptome of the pathogenic yeast, *Candida nivariensis*. We sequenced both the DNA and RNA of this species using both the Oxford Nanopore Technologies and Illumina platforms. We assembled the genome into an 11.8Mb draft composed of 16 contigs with an N50 of 886 Kb, including a circular mitochondrial sequence of 28 Kb. Using direct RNA nanopore sequencing and Illumina cDNA sequencing, we constructed an annotation of our new assembly, supplemented by lifting over genes from *Saccharomyces cerevisiae* and *Candida glabrata*.

3.2 Introduction

For immunocompromised hosts, opportunistic infections caused by drug-resistant fungi of the *Candida* genus are a major source of morbidity and mortality (Borman et al., 2008). In particular, *Candida nivariensis*, a close relative to *Candida glabrata*, has emerged in recent years as especially resistant to antifungal therapies (Borman et al., 2008). However, due to its phenotypic similarities to *C. glabrata*, *C. nivariensis* is generally underidentified and easily misdiagnosed, and currently, only molecular approaches can distinguish the two (Aznar-Marin et al., 2016), spurring whole-genome sequencing studies on the clade (Gabaldón et al., 2013).

Accurate assembly of repetitive genomic regions is crucial for understanding genetic diversity and virulence in pathogenic species. Fungal pathogens have long been known to exhibit a high degree of genome plasticity to enhance fitness in various environments (Croll, Zala, and McDonald, 2013; Ford et al., 2015; López-Fuentes et al., 2018; Carreté et al., 2019; Todd et al., 2019). Repetitive subtelomeric regions in particular play a crucial role in virulence for many pathogenic organisms (Barry et al., 2003; De Las Peñas et al., 2003). Many yeasts' subtelomeric regions contain and regulate the expression of genes crucial for biofilm formation, carbohydrate utilization, and cellular adhesion (Naumov, Naumova, and Louis, 1995; De Las Peñas et al., 2003; Iraqui et al., 2005). These gene families often undergo rapid evolution through changes in copy number and sequence through either SNPs or indels (Carreto et al., 2008; Brown, Murray, and Verstrepen, 2010; Anderson et al., 2015). However, these subtelomeric regions remain one of the most difficult sections of the genome to

accurately assemble due to their repetitive nature and high sequence similarity between genes, making genetic analysis cumbersome (Brown, Murray, and Verstrepen, 2010).

One of the gene families of great interest to the pathogenic yeast field are the GPI-anchored cell wall proteins. This protein family includes many genes that encode for adhesion proteins that are found in various members of the *Candida* genus, and play a key role in pathogenicity, being involved in regulation of biofilm formation, cell-to-cell contact, and host-pathogen interactions (Timmermans et al., 2018; McCall et al., 2019). With the many roles these genes play in infection, the accurate identification and understanding of the genetic variation of these genes is vital to combating fungal pathogens.

Unfortunately, like many eukaryotic pathogens, the current reference genome for *C. nivariensis* (GenBank: GCA_001046915.1) is highly fragmented. Constructed from sequencing of strain CBS9983, the reference genome consists of 123 contigs with an N50 of 248Kb (Gabaldón et al., 2013), meaning that at least half of the total genome length is contained in contigs 248Kb or longer. This is typical of genomes assembled from limited short-read sequencing data; though short reads are highly accurate, assembling them into contiguous genomes is challenging depending on the size and complexity of the genome. Such short read assemblies have limited utility since large scale variants, repetitive regions, and genome structure remain difficult to elucidate, though they are often involved in the genome plasticity of pathogenic yeasts (Carreté et al., 2018). In contrast, long-read sequencing data has been shown to produce much more contiguous assemblies, and have been crucial

in sequencing through large repetitive regions, as well as assessing structural variants. However, read accuracy on the ONT platform in particular ranges from 86% for early basecaller versions (Wick, Judd, and Holt, 2019) to 97% as currently reported by ONT. This is lower than the read accuracy of short-read Illumina sequencing, which achieves 99.9% accuracy (Fox et al., 2014). In consensus sequences, most random errors can be corrected by other reads covering the same genomic loci, resulting in >99% consensus accuracy (Wick, Judd, and Holt, 2019). However, systematic errors occurring in most or all of the reads cannot be corrected this way. For ONT data, indels at homopolymers dominate systematic errors (Wick, Judd, and Holt, 2019). These persistent errors can be problematic for gene prediction and annotation in downstream analysis (Watson and Warr, 2019) and are typically corrected with more accurate short-read data in mappable regions (Garrison and Marth, 2012; Walker et al., 2014; Vaser et al., 2017).

Having a genome alone is not enough; we need to annotate it with genes and other functional elements for the genome to be of greatest use. Knowledge of gene loci is critical to constructing phylogenetic relationships between organisms, and to studying the functional implications of variants, both common uses of reference genomes. While model-based, purely computational gene predictors can be highly accurate in bacteria, gene sparsity and intronic regions make this task more difficult in eukaryotes (Salzberg, 2019). For improved annotations, some RNA-seq information is required (Salzberg, 2019).

Here, as part of our newly developed Methods in Nucleic Acid Sequencing university course, we used a hybrid approach, applying long-read nanopore

sequencing to assemble a highly contiguous genome of *C. nivariensis*, followed by short-read sequencing to polish or correct errors in our assembly. We followed this by a combination of nanopore direct RNA sequencing as well as short-read RNA-seq to annotate our assembly. By combining this data with liftover of annotations from evolutionary “cousins” of *nivariensis*, we have generated a new and annotated reference genome for the community.

3.3 Results

3.3.1 Genome statistics

Using our nanopore and Illumina sequencing data, we generated a new assembly of *Candida nivariensis*, JHU_Cniv_v1 (Methods). Our assembly consists of 11.8 Mb of sequence in 16 contigs with an N50 of 886 Kb (Figure 3.1, Table 3.1). Compared to the reference genome, we have 275kb of additional sequence, 218kb of which is accounted for by gaps in the reference which are newly spanned by JHU_Cniv_v1. Of the 69 newly spanned gap sequences, 54 were identified as repeat regions. Another 13 gap regions were identified to contain a higher than average proportion of multi-mapping short reads (>10% in gap regions vs 7% average across the genome).

	Contigs	N50	Longest Contig	Shortest Contig	Total Length
Reference	123	248 Kb	807 Kb	666 bp	11.56 Mb
JHU_Cniv_v1	16	886 Kb	1.42 Mb	28.5 Kb	11.83 Mb

Table 3.1: Assembly Statistics. Assembly statistics of JHU_Cniv_v1 and the reference genome for *C. nivariensis*.

To determine whether JHU_Cniv_v1 contigs represent full chromosomes,

we looked for telomere repeats in our assembly and attempted to use related yeast reference genomes to scaffold. In our assembly, 11 contigs terminate at both ends in repeats of CTGGGTGCTGTGGGT, the telomere sequence of *Candida glabrata* (McEachern and Blackburn, 1994). The other

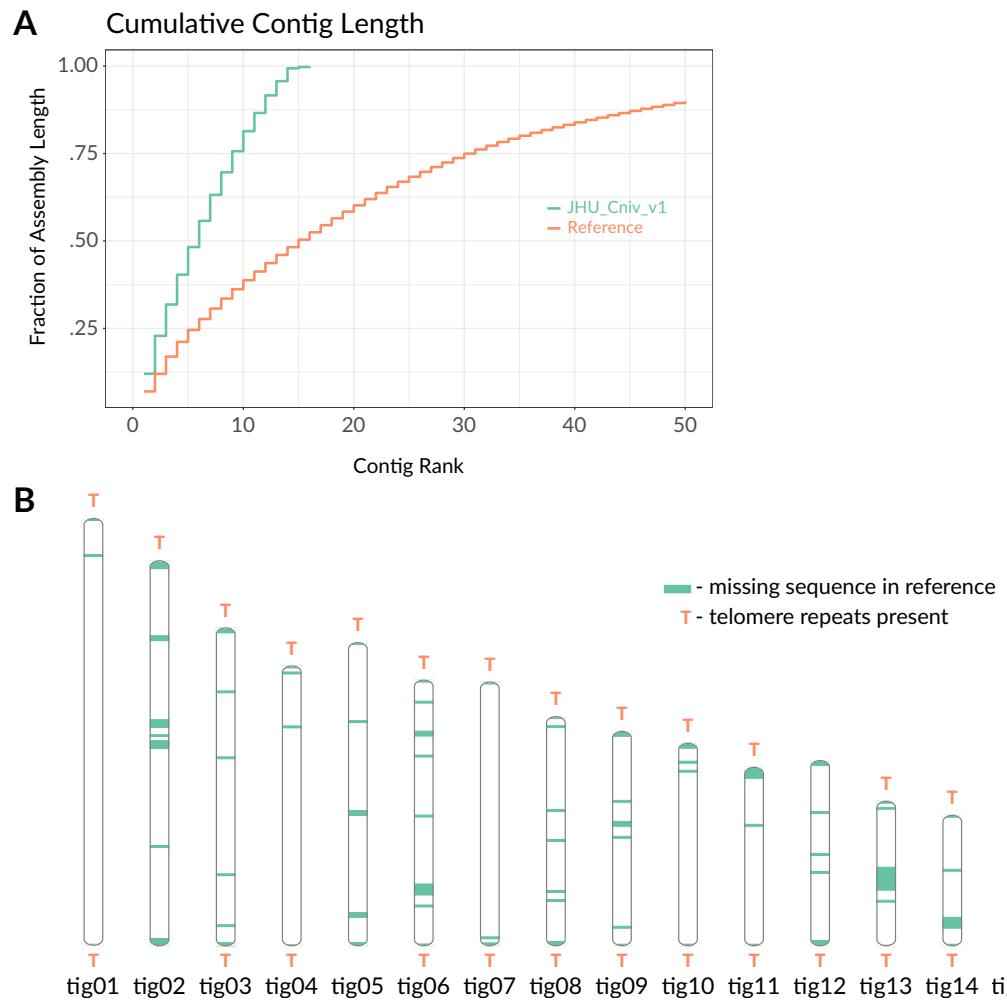


Figure 3.1: Characteristics of the JHU_Cniv_v1 assembly. (A) Cumulative lengths of the 50 longest sequences in our assembly and previous reference genome. (B) Ideogram of assembly. Sequence that is missing in the reference genome is shown along each non-mitochondrial contig, and the positions of telomere repeats are marked.

4 non-mitochondrial sequences terminate only at one end in this telomeric repeat (**Figure 3.1**, **Table 3.2**), suggesting they may scaffold to form two additional chromosomes. This suggests that, like *C. glabrata*, the *C. nivariensis* genome also contains 13 chromosomes.

Contig	Length (bp)	Forward Telomeres	Reverse Telomeres
tig01	1423475	35	38
tig02	1283968	0	39
tig03	1060011	35	39
tig04	933062	36	26
tig05	1010854	0	36
tig06	885783	35	38
tig07	879540	39	35
tig08	763992	34	33
tig09	714796	35	47
tig10	675194	36	36
tig11	594828	32	26
tig12	617546	36	0
tig13	481613	38	41
tig14	434809	33	33
tig24	44616	0	39
JHU_Cniv_v1_mito	28512	0	0

Table 3.2: Contig and telomere lengths. Contig lengths and the number of times the forward and reverse telomere sequence appears in each

We tried to further scaffold our assembly using the more contiguous and highly related *glabrata* genome as a reference, but we found that reference

based scaffolders such as Medusa v1.6 (Bosi et al., 2015) and RagTag v1.0.2 (Alonge et al., 2019) either placed telomeric sequences in the middle of scaffolds or made no improvement (Figure 3.2). Upon aligning the *C. glabrata* genome to JHU_Cniv_v1 using Mummer, we found only sporadic shared segments of negligible length (Figure 3.3), as opposed to a nearly perfect 1:1 alignment between JHU_Cniv_v1 and the current *C. nivariensis* reference genome (Figure 3.4). This indicated that the *C. glabrata* genome is not sufficiently similar to *C. nivariensis* to use as a reference for contig scaffolding. Using the *C. nivariensis* reference genome for scaffolding similarly results in erroneous placement of telomere repeats in the middle of scaffolds, or no change to our assembly. This is unsurprising, as the *C. nivariensis* reference genome is so highly fragmented.

3.3.2 Genome completeness

To assess assembly completeness, fungal single-copy orthologs were checked using BUSCO v5.0.0 (Simão et al., 2015) and its available saccharomycetes_odb10 database. Out of 2137 BUSCOs searched, JHU_Cniv_v1 has only 14 missing, 13 of which are also missing in the current reference (Figure 3.5). This additional missing gene, RNA polymerase archaeal subunit P/eukaryotic subunit RPABC4 (buscoID 41996at4891), though present in the reference, has the second lowest combined match length and match score among all genes searched. From the reference, we extracted the nucleotide sequence of this match using the coordinates reported by BUSCO, and searched for it in JHU_Cniv_v1 using BLAST. We found a full-length match with 99.9% identity, suggesting that this

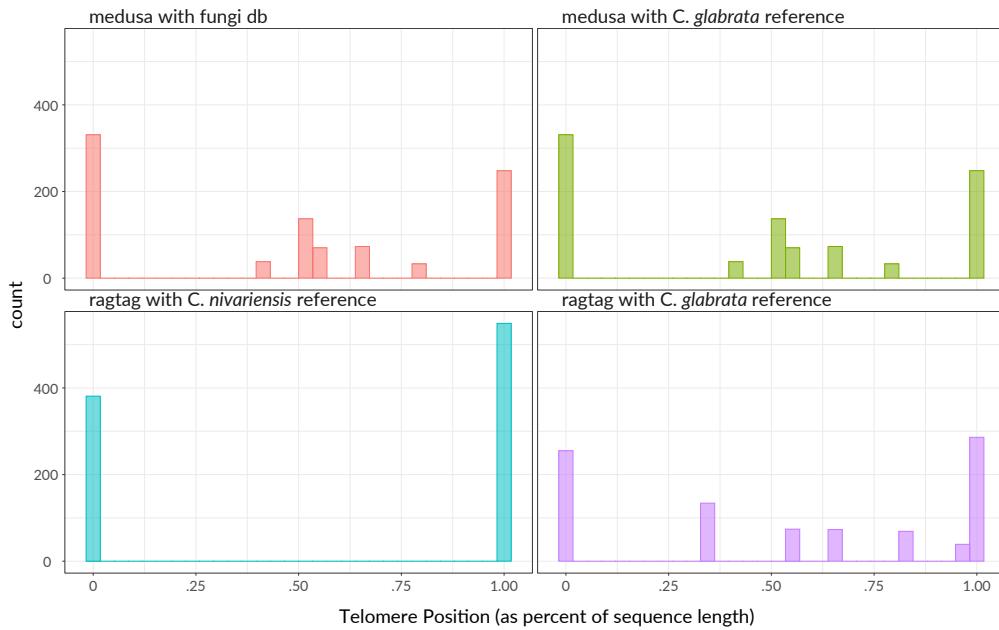


Figure 3.2: Telomere positions reference based scaffolds. Histogram of telomere repeat positions in our assembly, and in scaffolds produced by RagTag and MeDuSa. When MeDuSa is used with a database including the reference genomes of *exititC. nivariensis*, *exititC. glabrata*, *C. bracarensis*, and *N. delphensis*, telomeres are placed in the middle of contigs. The same result is produced when only the *exititC. glabrata* genome is used for scaffolding with MeDuSa, and MeDuSa fails to run when only the *exititC. nivariensis* reference is used. When the *exititC. nivariensis* reference genome is used for scaffolding with RagTag, no changes are made. When the more contiguous *exititC. glabrata* genome is used with RagTag, telomere sequences are again placed in the middle of sequences, suggesting a scaffolding error.

BUSCO is not actually absent in JHU_Cniv_v1. Upon further examination of this alignment, we found that all seven nonmatching nucleotides consist of small deletions associated with poly-A or poly-T homopolymers, known error-prone regions for nanopore sequencing data (Watson and Warr, 2019).

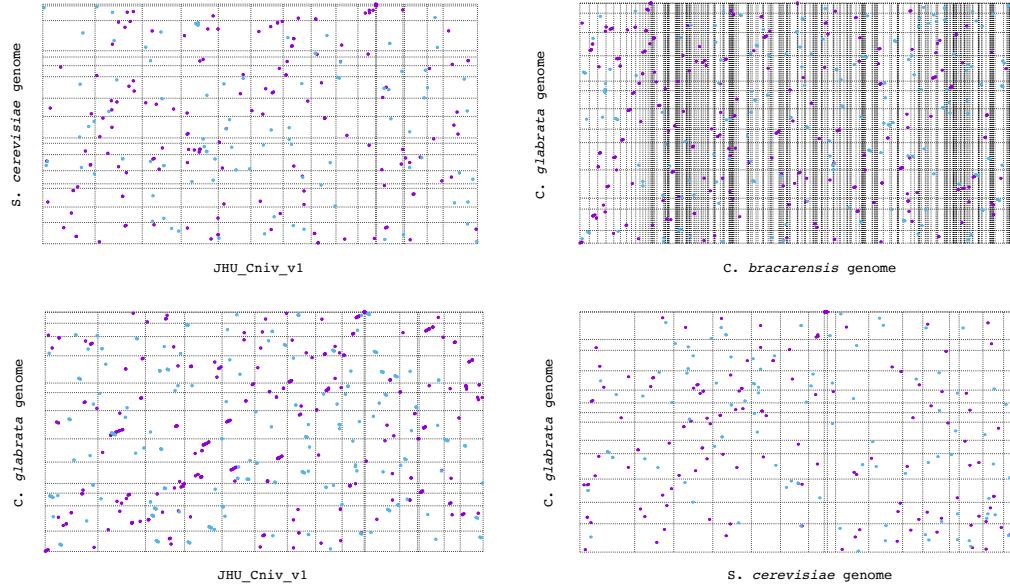


Figure 3.3: Whole genome alignments between related yeasts. Whole genome alignment of our new assembly against the *S. cerevisiae* (top left), and *C. glabrata* (bottom left) reference genomes. For both, there are no long alignments, suggesting that there is little similarity in genome structure between these species and *C. nivariensis*. *C. bracarensis*, a close relative to both *C. glabrata* and *C. nivariensis*, also shares little genome similarity to *C. glabrata* (top right), suggesting that yeast genomes within the *glabrata* clade are not generally similar enough to support inter-species reference based scaffolding. We also compared *C. glabrata* to the highly contiguous and complete *S. cerevisiae* genome (bottom right) to check that genome contiguity alone did not bias the genome similarity detected.

3.3.3 Repetitive genes

As *C. glabrata* subtelomeric regions have been proven to be difficult to correctly assemble using short-read data (Xu et al., 2020), we compare the copy number of *C. glabrata* subtelomere gene homologs between the *C. nivariensis* reference genome and *JHU_Cniv_v1*. Using the assembly and re-annotation of *C. glabrata* from Xu et al. (2020), we extracted the sequences of the *C. glabrata* subtelomere genes and used BLAST (v2.6.0+) to find any matches in the *C.*

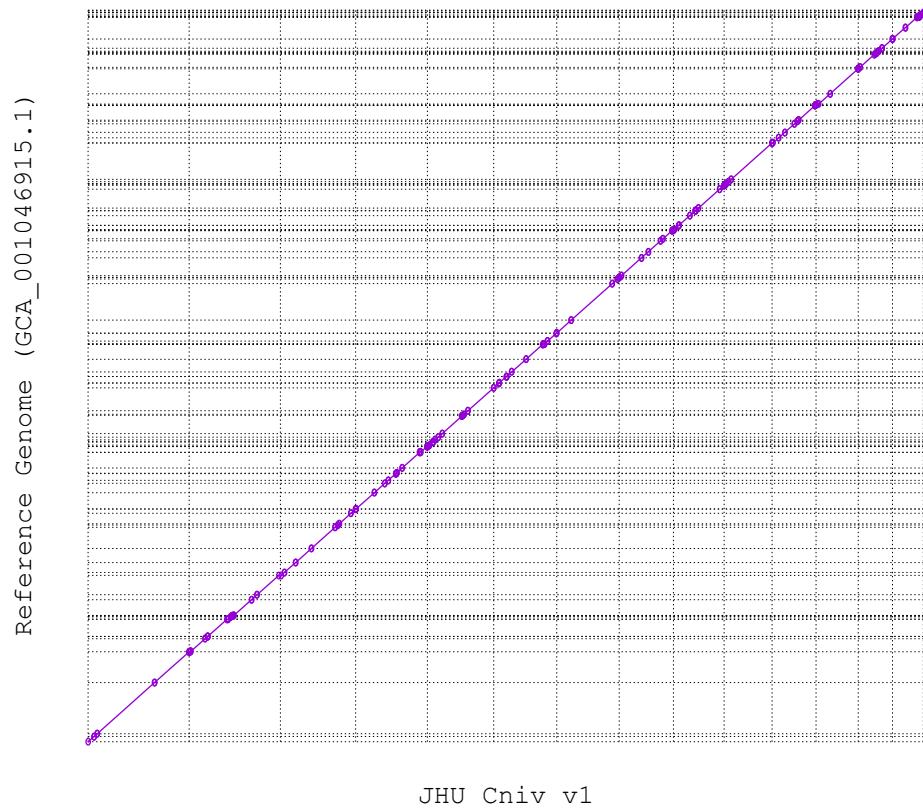


Figure 3.4: Whole genome alignment of JHU_Cniv_v1 and the *C. nivariensis* reference genome. Whole genome alignment of the current reference genome (y axis) compared to our new assembly (x axis). Alignments match with no notable structural variants, and very little missing or duplicated sequence.

nivariensis reference and JHU_Cniv_v1. We observed an identical set of 48 *C. glabrata* subtelomere genes in both *C. nivariensis* genomes but found that the copy number for several genes was greater in JHU_Cniv_v1 (Figure 3.6). To account for genes truncated by short contigs in the reference genome, we calculate copy number by summing the alignment lengths of all the hits of a particular gene and dividing by gene length. Of the 48 *C. glabrata* genes with

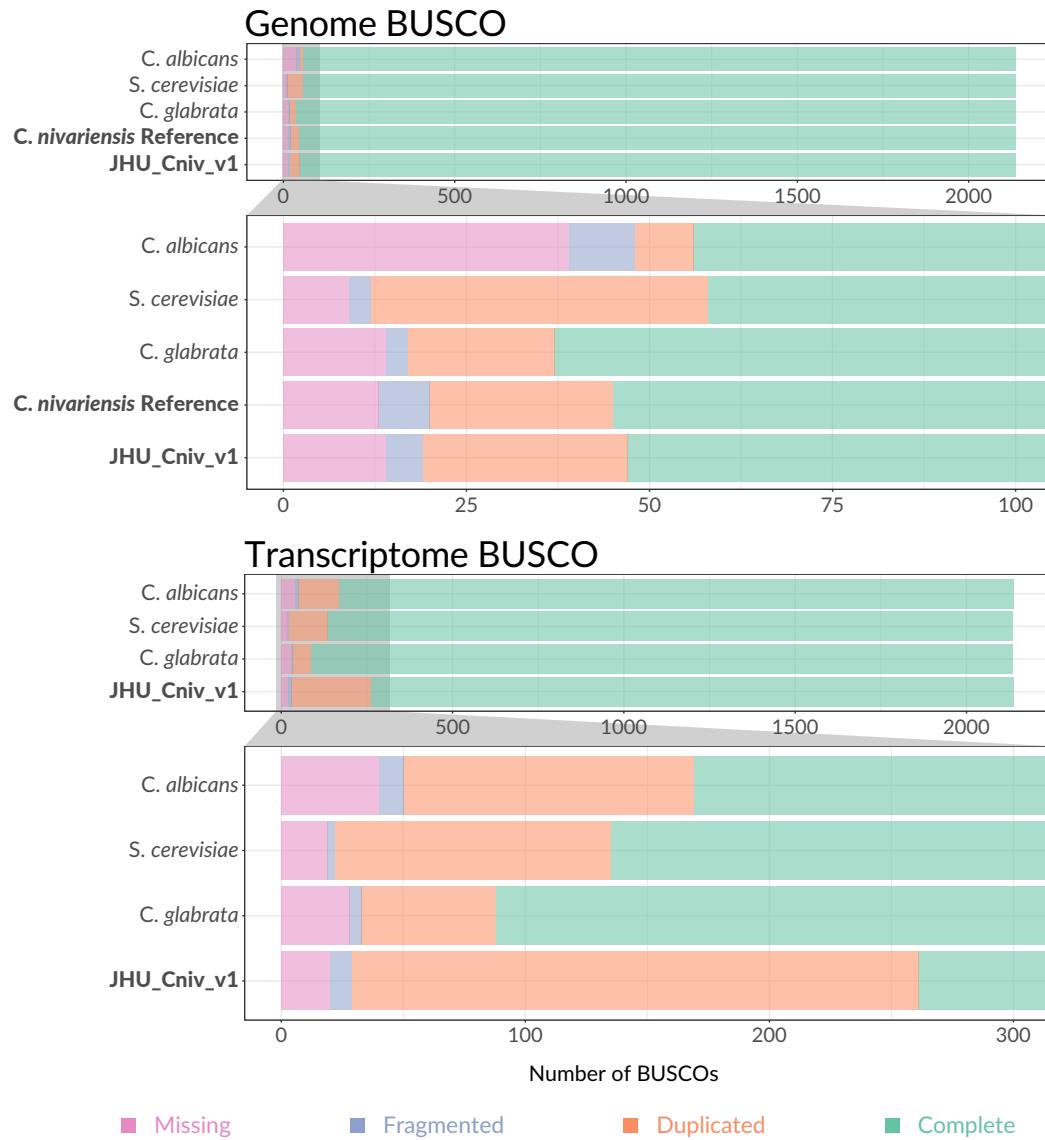


Figure 3.5: Completeness of the JHU_Cniv_v1 assembly. Genome and transcriptome completeness Bar charts comparing BUSCOs detected in JHU_Cniv_v1 and accompanying transcriptome to those of the current *C. albicans*, *S. cerevisiae*, *C. glabrata*, and *C. nivariensis* reference genomes. No reference transcriptome is currently available for *C. nivariensis*.

homology in *C. nivariensis*, 35 are ribosomal. With the exception of just three ribosomal genes, which occur a similar number of times in both *C. nivariensis*

genomes, all homologous ribosomal genes appear once in the reference, and either four or six times in JHU_Cniv_v1 (**Figure 3.6**).

Using JHU_Cniv_v1, we identified GPI-anchored membrane proteins among annotated genes >1000-nt long. Using GffRead (Pertea and Pertea, 2020), we constructed the amino acid sequences for these genes and excluded any with internal stop codons. We then used PredGPI (Pierleoni, Martelli, and Casadio, 2008) to predict which of these encoded GPI proteins, using an FDR cutoff of <0.0005 (Xu et al., 2020) to find 86 total genes. As GPI-anchored fungal adhesins typically contain tandem repeats (Lipke, 2018; Xu et al., 2020), we further filtered for genes overlapping with tandem repeats as classified by Tandem Repeat Finder and identified 53 of the GPI genes as putative adhesins. As with *C. glabrata*, the putative adhesins typically spanned multiple kilobases (**Figure 3.6**), though we do not find very long (>13 kb) genes in contrast to several glabrata GPI-CWPs. To find the corresponding adhesin genes in the *C. nivariensis* reference genome, we again used BLAST, and compared the longest hit of each adhesin gene to the true length of the gene as predicted in JHU_Cniv_v1 (**Figure 3.6**). Notably, no hit in the reference genome exceeded 3.5kb, and 27 of these adhesin genes are not found continuously, suggesting the previous reference either truncated or did not continuously assemble these important pathogenicity genes.

3.4 Discussion

JHU_Cniv_v1 is a high quality, extremely contiguous assembly of *Candida nivariensis* constructed by long reads and polished by short reads. It spans

large, repetitive gaps in the *nivariensis* genome that have fragmented short-read assemblies thus far, and includes a full mitochondrial chromosome, as well as telomere repeats. These telomere repeats are identical to those in *C. glabrata* and have been found to be shared within the entire “*glabrata* group” (Gabaldón et al., 2013). The orientation of the telomeres suggests that *C. nivariensis* has 13 chromosomes, which is in agreement with previous PFGE data (Gabaldón et al., 2013). Furthermore, of the contigs missing telomere

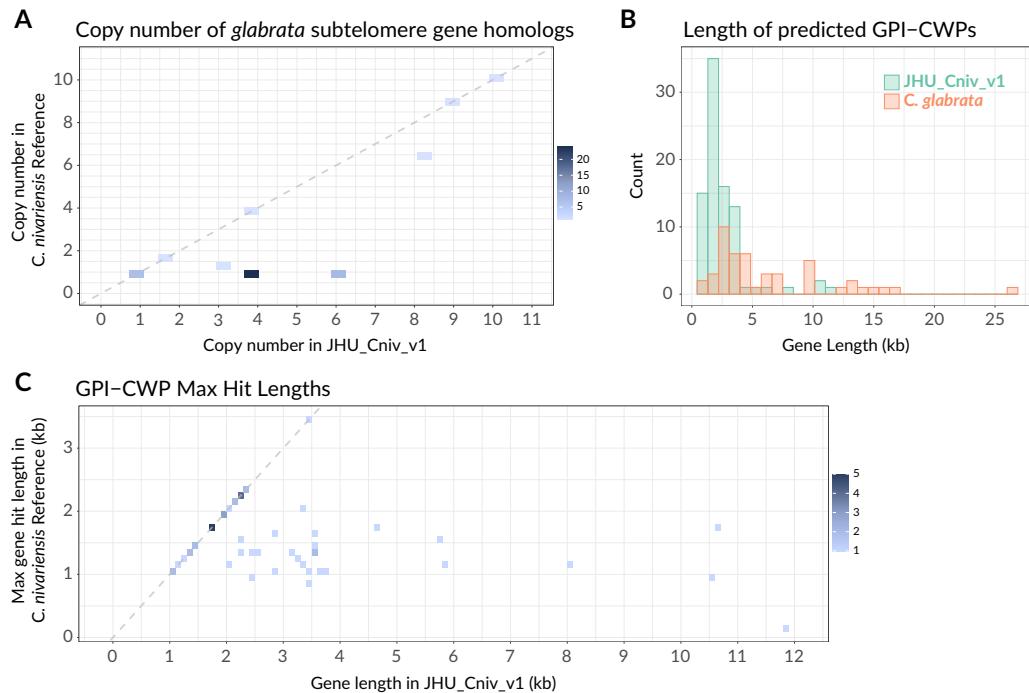


Figure 3.6: GPI genes. (A) Scatterplot showing the number of times each *glabrata* subtelomere gene homolog appears in the *C. nivariensis* reference genome and in JHU_Cniv_v1. Overlapping points are shown on the color scale, and the $y=x$ line is shown in dashed gray. (B) Histogram of adhesion protein lengths in *glabrata* as annotated by Xu et al., and the lengths of predicted adhesion proteins found in JHU_Cniv_v1. (C) Scatterplot showing the maximum BLAST alignment lengths for each predicted *nivariensis* GPI gene in JHU_Cniv_v1 and the *C. nivariensis* reference genome. Overlapping points are shown on the color scale, and the $y=x$ line is shown in dashed gray.

repeats on one end, we note that scaffolding tig05 with tig12 and tig02 with tig24 would result in 13 chromosomes that would all match PFGE length estimates to 8% error or less, which is within the expected range of PFGE error for very large DNA fragments (Cutting et al., 1988).

As assessed by BUSCO, genome completeness of the current *C. nivariensis* reference and JHU_Cniv_v1 are comparable to other related yeasts, with our genome slightly improved over the previous reference. However, while JHU_Cniv_v1 is a much more contiguous assembly than any *C. nivariensis* genome preceding it, the few remaining sequence errors still can pose a problem to downstream analyses, as evidenced by the seemingly absent BUSCO we manually identified.

Our accompanying RNA-seq data enabled us to annotate this genome, achieving a similar level of BUSCO completeness to some of the most highly studied model organisms. Our annotation has comparable or lower levels of missing and fragmented BUSCOs compared to the reference annotations, though more duplicated ones. While our annotation is largely comparable to those of similar yeasts (Table 3.3), it has not been manually curated, and should thus be treated as preliminary. Of course, as these organisms were grown under only one condition before RNA extraction, it remains unlikely that this annotation is fully complete.

To demonstrate the utility of genome and annotation contiguity, we examine genes from a difficult to assemble region in *C. glabrata*. For each subtelomeric *C. glabrata* gene with homology in *C. nivariensis*, more copies were found in JHU_Cniv_v1, as its contiguity allows it to more easily capture repeated

	Total Exons	Total Genes
JHU_Cniv_v1	7,298	5,859
<i>C. glabrata</i>	5,629	5,448
<i>S. cerevisiae</i>	6,760	6,420
<i>C. albicans</i>	6,732	6,263

Table 3.3: Gene and exon counts of JHU_Cniv_v1 and related yeasts. Gene and exon counts of our annotation and currently available reference annotations

genome elements. We note that of subtelomeric *glabrata* genes found, the majority are ribosomal, and of these, only three do not show a four or six times increased copy number in JHU_Cniv_v1. Due to the repetitive nature of rDNA arrays, it can be difficult for short-read genome assemblies to capture them in their full complexity. Conversely, our long-read assembly more easily spans these regions, potentially providing a clearer look at the biology in which they are involved.

In addition to genes arranged in complex and repetitive patterns, our more contiguous assembly enables analysis of large genes with internal repeats, such as GPI adhesins. Since these genes are so large, it can be difficult or impossible to predict them from fragmented assemblies which are unable to capture them in their full length. As adhesins are critical to understanding elements of pathogenicity in these yeasts, fragmented genome assemblies and missing gene annotations can be crippling to this dimension of research in these organisms.

3.5 Methods

3.5.1 Media and growth conditions

For genomic extractions, a single colony of *C. nivariensis* CBS9983, originally isolated from a blood culture of a Spanish woman (Alcoba-Flórez et al., 2005), was inoculated into synthetic complete (SC) medium supplemented with 2% glucose and shaken overnight at 30°C in a glass culture tube. For RNA extractions, *C. nivariensis* CBS9983 was grown to log phase in SC medium supplemented with 2% glucose at 30°C in a glass culture tube.

3.5.2 DNA isolation and sequencing

DNA was extracted from liquid culture using the Zymo Fungal/Bacterial DNA MiniPrep Kit according to manufacturer specifications. Two ONT sequencing libraries were prepared from the extracted DNA using the ONT rapid barcoding sequencing kit (SQK-RBK004), and each was sequenced on a separate MinION flowcell (R9.4). Two Illumina libraries were prepared with the Nextera Flex Library Prep Kit, each using 400ng of extracted DNA. Both Illumina libraries were then sequenced on a single iSeq 100 run.

3.5.3 RNA isolation and sequencing

RNA was extracted from liquid culture using the Zymo Fungal/Bacterial RNA MiniPrep Kit. Using the NEBNext Poly(A) mRNA Magnetic Isolation Module, polyA tailed mRNA was isolated from the total RNA. Two ONT direct RNA sequencing libraries were prepared and sequenced on separate MinION

flowcells, each using 200ng of polyA selected RNA and the SQK-RNA002 sequencing kit. With the NEBNext Ultra II RNA First-Strand Synthesis Module and the NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module, cDNA was prepared from the isolated mRNA. Two individual Illumina libraries were then prepared with the Nextera Flex Library Prep Kit, each using 400ng of cDNA. Both library replicates were then sequenced on a single iSeq 100 run, generating 2×150 paired-end reads.

3.5.4 Genome assembly

Nanopore data were basecalled using Guppy v3.2.4 on default settings. Reads greater than 3kb long with an average basecalling quality score greater than 7 were assembled into 21 contigs using Canu v2.1 (Koren et al., 2017) on default settings with the genome size set to 11m. Illumina DNA reads were trimmed for adapters and quality using Trimmomatic v0.39 (Bolger, Lohse, and Usadel, 2014) using settings LEADING:3 TRAILING:3 SLIDINGWINDOW:4:30 MINLEN:36. The trimmed reads were then used to iteratively correct draft assembly using Freebayes v1.3.4-pre1 (Garrison and Marth, 2012) with alignments made by bwa mem v0.7.17-r1198-dirty (Li, 2013) using default settings. Changes were made at positions where both the alternative allele frequency was greater than 0.5 and the total number of alternate allele observations was greater than 5. We aligned and corrected the assembly iteratively for three rounds, after which further rounds of corrections made no changes.

Of our 21 corrected contigs, 5 were flagged as repeats by Canu and originally constructed from fewer than 180 nanopore reads. The remaining 16

contigs were constructed from over 1800 nanopore reads each. Because the five repetitive contigs were constructed from so few reads and were found to occur elsewhere in the assembly through Mummer v4.0.0rc1 (Marçais et al., 2018) and nanopore read alignment Minimap2 v2.17 (Li, 2018), we excluded them from the final assembly. One 32-Kb contig was suggested to be circular by Canu, and therefore likely to be a mitochondrial sequence. To confirm, we aligned this contig to the complete mitochondrial genome of *C. nivariensis* (NCBI: NC_036379.1) using Mummer, and observed a 3662-bp sequence in the reference mitochondrial genome which appears at both ends of our 32-kb circular contig. Using the Mummer alignments (Figure 3.7), we removed the extraneous 3662bp from the end of our contig, resulting in a 28-kb mitochondrial genome, which we named “JHU_Cniv_v1_mito.” Lastly, we remapped the ONT and Illumina reads back to the assembly, and found no bases with zero coverage, indicating that none of our contigs need to be further broken (Figure 3.8). Henceforth, we refer to this assembly as “JHU_Cniv_v1.”

Repeat regions were identified by Tandem Repeats Finder v4.09 (Benson, 1999) with settings (Xu et al., 2020): `match = 2, mismatch = 7, delta = 7, pm = 80, pi = 10, minscore = 50, maxperiod = 600`. Multimapping short reads were identified using bwa mem (Li, 2013) on default settings.

3.5.5 Annotation

Illumina RNA-seq reads were trimmed using Trimmomatic v0.39 (Bolger, Lohse, and Usadel, 2014) in order to check for any remaining adapter sequences and to filter out reads with low base quality. HISAT2 v2.1.0 was

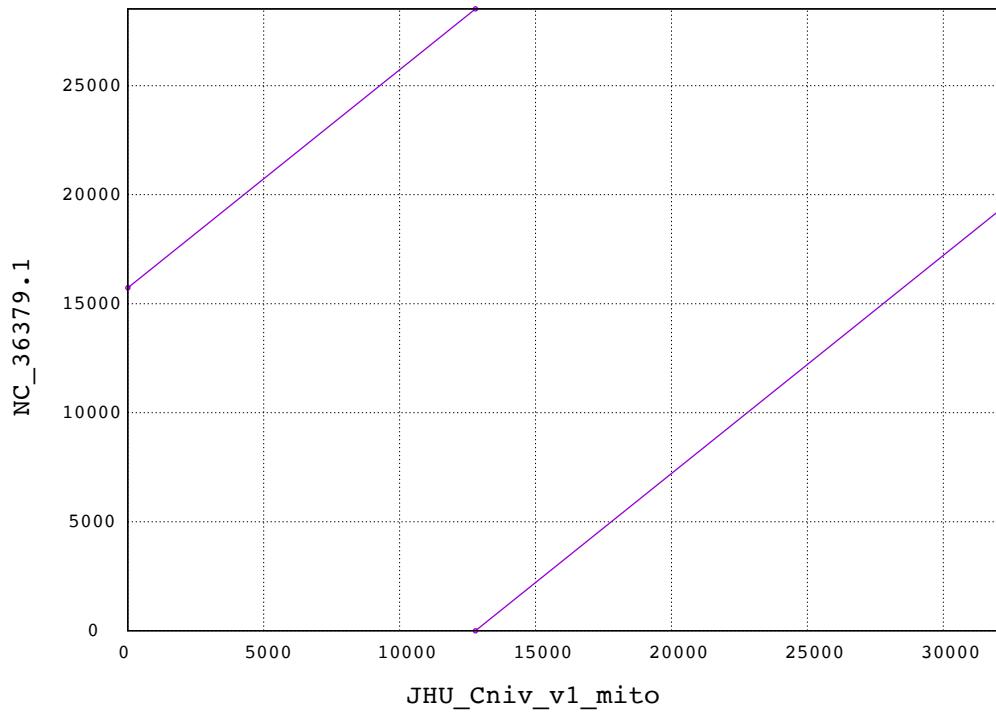


Figure 3.7: Alignment of JHU_Cniv_v1 mitochondrial contig and the exitC. nivariensis mitochondrial genome. Alignment of our 32Kb circular contig (x axis) with the completed mitochondrial genome of the *C. nivariensis* reference genome (y axis). The final 3662bp of this contig appears twice in the reference genome.

used on default settings to align the trimmed cDNA reads to the assembly. The BRAKER v2.1.5 pipeline (Hoff et al., 2019) was then used to make gene predictions using these alignments. Currently, ONT dRNA compatibility with BRAKER is in development, and that data was thus not used for prediction. Instead, ONT dRNA reads were aligned to the genome assembly using Minimap2 on recommended settings for nanopore direct RNA reads (`-ax splice -uf -k14`). Transcripts were then assembled from the dRNA alignments using StringTie2 v2.1.5 (Kovaka et al., 2019) with the long read option (`-L`). Using Liftoff v1.5.0 (Shumate and Salzberg, 2020), we lifted over the annotations

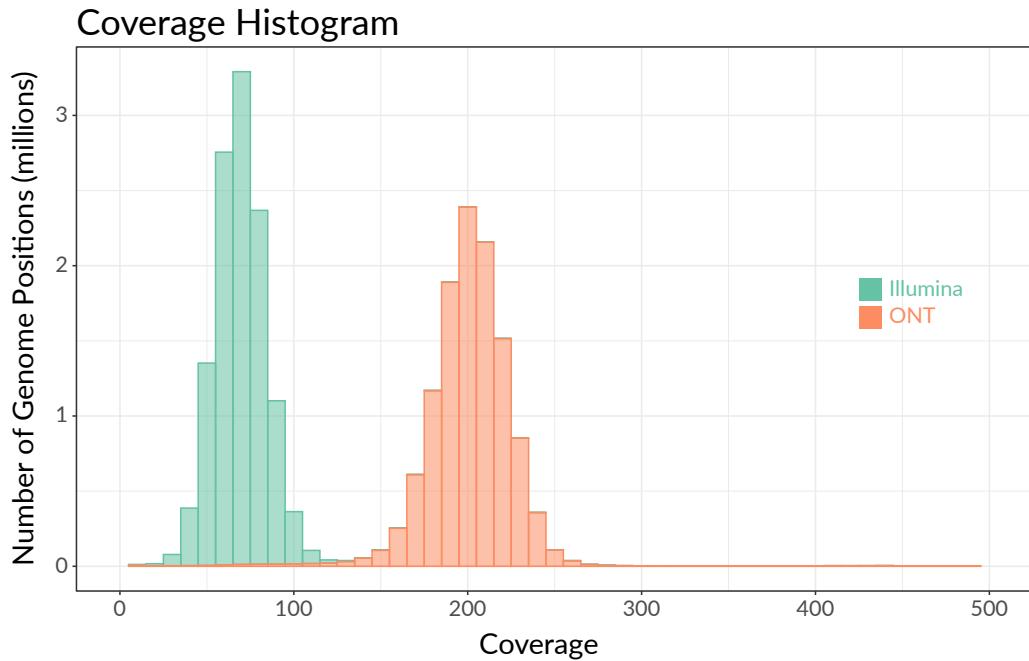


Figure 3.8: Coverage histograms. Histogram of coverage per base in our assembly by filtered (>3kb) ONT reads and trimmed Illumina reads.

from *C. glabrata* (NCBI: GCF_000002545.3), *Saccharomyces cerevisiae* (NCBI: GCF_000146045.2), *Candida albicans* (NCBI: GCF_000182965.3).

Starting with the BRAKER predictions, GffCompare v0.12.1 (Pertea and Pertea, 2020) was used to add nonoverlapping annotations lifted from *C. glabrata*, *S. cerevisiae*, and *C. albicans* in that order. Specifically, we add any annotation with class code “u” in the GffCompare .tmap outputs when comparing our list of genes with a list of potential genes to add, since these refer to intergenic regions devoid of any overlap or proximity to previous annotations. Finally, we compared and added nonredundant transcripts assembled by StringTie2 to the annotation using GffCompare.

	Total	Gene	Exon
Augustus (BRAKER)	23,497	5,028	6,109
Genemark.hmm (BRAKER)	36	6	12
Liftoff glabrata	263	130	2
Liftoff cerevisiae	42	21	0
Liftoff albicans	0	0	0
StringTie	2,141	824	1,175

Table 3.4: Contributions from each annotation software. Number of genes and exons added by each software

3.5.6 Data Availability

All sequence data are available in the Sequence Read Archive, under BioProject PRJNA686979. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAEVGP000000000. The version described in this here is version JAEVGP010000000. Code used for analysis is available at <https://github.com/timplab/nivar>.

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Chapter 4

Discussion and Conclusion

Discuss and conclude your thesis (Abramson, Barbie, and Rider, 1900)

References

Abramson, A. A., B. B. Barbie, and C. C. Rider (1900). "Article title". In: *Journal Three* 1.1, pp. 192–244.



John Doe

Resumé title

Some quote

Education

year–year **Degree**, *Institution, City, Grade.*
Description

year–year **Degree**, *Institution, City, Grade.*
Description

Master thesis

title *Title*
supervisors Supervisors
description Short thesis abstract

Experience

Vocational

year–year **Job title**, *Employer, City.*
General description no longer than 1–2 lines.
Detailed achievements:

- Achievement 1;
- Achievement 2, with sub-achievements:
 - Sub-achievement (a);
 - Sub-achievement (b), with sub-sub-achievements (don't do this!);
 - Sub-sub-achievement i;
 - Sub-sub-achievement ii;
 - Sub-sub-achievement iii;
 - Sub-achievement (c);
- Achievement 3.

year–year **Job title**, *Employer, City.*
Description line 1
Description line 2

Miscellaneous

street and number – postcode city – country

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👤 jdoe • additional information

year–year **Job title**, *Employer*, City.
Description

Languages

Language 1	Skill level	<i>Comment</i>
Language 2	Skill level	<i>Comment</i>
Language 3	Skill level	<i>Comment</i>

Computer skills

category 1	XXX, YYY, ZZZ	category 4	XXX, YYY, ZZZ
category 2	XXX, YYY, ZZZ	category 5	XXX, YYY, ZZZ
category 3	XXX, YYY, ZZZ	category 6	XXX, YYY, ZZZ

Interests

hobby 1	Description
hobby 2	Description
hobby 3	Description

Extra 1

- Item 1
- Item 2
- Item 3. This item is particularly long and therefore normally spans over several lines. Did you notice the indentation when the line wraps?

Extra 2

- Item 1
- Item 2
- Item 3
- Item 4
- Item 5[3]
- Item 6. Like item 3 in the single column list before, this item is particularly long to wrap over several lines.

References

Category 1	Category 2	All the rest & some more
○ Person 1	Amongst others: ○ Person 1, and ○ Person 2 (more upon request)	That person, and those also (all available upon request).

Publications

- [1] John Doe. Title, year.

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- [3] John Doe and Author 1. *Title*. Publisher, edition edition, year.
- [4] John Doe and Author 2. *Title*. Publisher, edition edition, year.
- [5] John Doe and Author 3. Title, year.

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January 01, 1984

Dear Sir or Madam,

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Albert Einstein discovered that $e = mc^2$ in 1905.

$$e = \lim_{n \rightarrow \infty} \left(1 + \frac{1}{n}\right)^n$$

Yours faithfully,

John Doe

Attached: *curriculum vitæ*

John Doe

street and number – postcode city – country

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