A new method for determining ribosomal DNA copy number shows differences between *Saccharomyces cerevisiae* populations

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Abstract

Ribosomal DNA genes (rDNA) encode the major ribosomal RNAs (rRNA) and in eukaryotic genomes are typically present as one or more arrays of tandem repeats. Species have characteristic rDNA copy numbers, ranging from tens to thousands of copies, with the number thought to be redundant for rRNA production. However, the tandem rDNA repeats are prone to recombination-mediated changes in copy number, resulting in substantial intra-species copy number variation. There is growing evidence that these copy number differences can have phenotypic consequences. However, we lack a comprehensive understanding of what determines rDNA copy number, how it evolves, and what the consequences are, in part because of difficulties in quantifying copy number. Here, we developed a genomic sequence read approach that estimates rDNA copy number from the modal coverage of the rDNA and whole genome to help overcome limitations in quantifying copy number with existing mean coverage-based approaches. We validated our method using strains of the yeast *Saccharomyces cerevisiae* with previously-determined rDNA copy numbers, and then applied our pipeline to investigate rDNA copy number in a global sample of 788 yeast isolates. We found that wild yeast have a mean copy number of 92, consistent with what is reported for other fungi but much lower than in laboratory strains. We show that different populations have different rDNA copy numbers. These differences can partially be explained by phylogeny, but other factors such as environment are also likely to contribute to population differences in copy number. Our results demonstrate the utility of the modal coverage method, and highlight the high level of rDNA copy number variation within and between populations.

43 **Introduction**

44 The ribosomal RNA gene repeats (rDNA) encode the major ribosomal RNA (rRNA) components
45 of the ribosome, and thus are essential for ribosome biogenesis and protein translation. In most
46 eukaryotes the rDNA forms large tandem repeat arrays on one or more chromosomes [1]. Each
47 repeat unit comprises a coding region transcribed by RNA polymerase I (Pol-I) that encodes 18S,
48 5.8S and 28S rRNA [2], and an intergenic spacer region (IGS) that separates adjacent coding
49 regions (**Fig 1**). The number of rDNA repeat copies varies widely between species, typically from
50 tens to hundreds of thousands of copies [1, 3-5]. However, each species appears to have a 'set' or
51 homeostatic (in the sense of [6]) rDNA copy number that is returned to if the number of copies
52 deviates [7-10]. Deviation in rDNA copy number between individuals within a species is well
53 documented and can be substantial [11-16]. This copy number variation is thought to be tolerated
54 because of redundancy in rDNA copies [8, 17]. This redundancy can partly be explained by the
55 striking observation that only a subset of the repeats is transcribed at any one time [2]. Thus, cells
56 can compensate for changes in rDNA copy number by activating or silencing repeats to maintain
57 the same transcriptional output [18]. Variation in rDNA copy number is a consequence of unequal
58 homologous recombination, which results in loss or gain of rDNA copies [8, 19-22]. This copy
59 number variation is, somewhat counter-intuitively, what drives the high levels of sequence
60 homogeneity observed between the rDNA copies within a genome, a pattern known as concerted
61 evolution [23-25]. Recent results in *Saccharomyces cerevisiae* revealed an elegant mechanism
62 through which homeostatic rDNA copy number is maintained in the face of rDNA copy number
63 change via the abundance of the Pol-I transcription factor UAF (upstream activating factor) and
64 the histone deacetylase Sir2 [26]. However, the selective pressure(s) that determines what the
65 homeostatic rDNA copy number is remains unknown. Nevertheless, there is growing evidence that

66 rDNA copy number and the proportion of active/silent rDNA copies impact several aspects of cell
67 biology beyond simply rRNA production [8, 12, 17, 22, 27-35].

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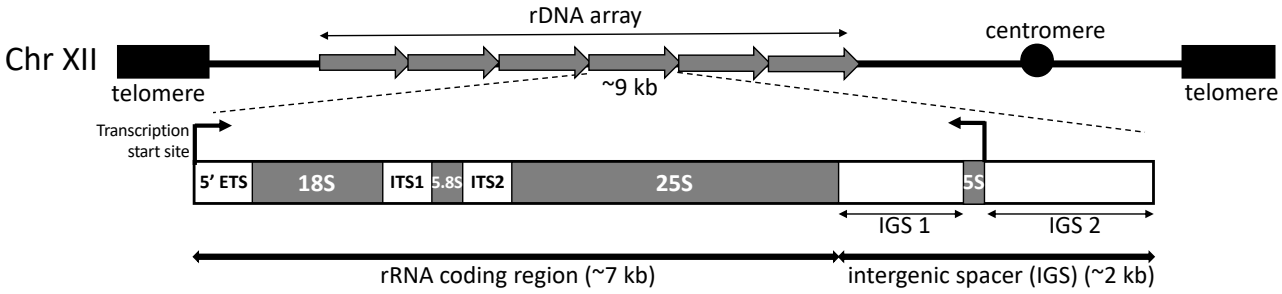


69

70 **Figure 1. Organization of the rDNA repeats in *Saccharomyces cerevisiae*.** Top shows a
71 schematic of tandemly-repeated units in the rDNA array located on chromosome XII. Bottom
72 shows the organization of an individual rDNA repeat including transcription start sites, the 5'
73 external transcribed spacer (5'ETS), the rRNA (18S, 5.8S and 28S) coding genes, the two
74 internal transcribed spacers (ITS1 and 2), and the intergenic spacer (IGS). The IGS is divided
75 into two by a 5S rRNA gene. Schematic is not to scale.

76

77 Interest in the phenotypic consequences of rDNA copy number variation has led to a number of
78 approaches being used to measure it. These include molecular biology approaches such as
79 quantitative DNA hybridization [36-39], pulsed field gel electrophoresis (PFGE) [40, 41],
80 quantitative real-time PCR (qPCR) [15, 42-46] and digital droplet PCR (ddPCR) [47, 48]. A major
81 advance in the measurement of rDNA copy number has been the emergence of bioinformatic
82 approaches that use whole genome (WG) next generation sequencing (NGS) reads to estimate
83 copy number, based on the rationale that sequence coverage of the rDNA correlates with copy
84 number. This correlation is a consequence of concerted evolution, with the high sequence identity

85 between repeats resulting in reads from all rDNA copies mapping to a single reference rDNA unit,
86 thus providing a high coverage signal that is proportional to copy number. Existing bioinformatic
87 approaches calculate the mean rDNA read coverage and normalize to the mean WG coverage to
88 estimate copy number [5, 12, 25, 34, 49], thus assuming that mean coverage represents the "true
89 coverage" for both the rDNA and the WG. However, there are reasons to suspect this mean
90 coverage approach assumption might not always hold. Repetitive elements (e.g. microsatellites
91 and transposons), PCR/sequencing bias (which is particularly evident for the rDNA [50-54];
92 **Supplementary Figure 1**), and large-scale mutations such as aneuploidies and segmental
93 duplications may all cause the measured mean coverage to differ from the real coverage. While
94 efforts have been made to address some of these potential confounders [12, 55, 56], estimated copy
95 number varies depending on which region of the rDNA is used [12, 34], thus the accuracy of this
96 mean read coverage approach has been called into question [5, 46].

97

98 Here we present a bioinformatics pipeline that measures rDNA copy number using modal (most
99 frequent) NGS read coverage as a way to overcome the limitations of the mean coverage
100 bioinformatics approach. We assessed the parameters important for performance and validated the
101 pipeline using *S. cerevisiae* strains with known rDNA copy numbers. We then employed our
102 pipeline to investigate whether *S. cerevisiae* populations maintain different homeostatic rDNA
103 copy numbers.

Materials and Methods

Modified *Saccharomyces cerevisiae* genome

Chromosome sequences for W303 were obtained from the NCBI (accession CM001806.1 - CM001823.1) and concatenated. rDNA copies present within the W303 reference genome were identified using BLAST and removed using Geneious (v. 11.0.3). The *S. cerevisiae* W303 strain rDNA repeat unit from [23] was added as an extrachromosomal rDNA reference, and this modified W303 yeast reference genome (W303-rDNA) was used in subsequent analyses.

Yeast strains/isolates and growth conditions

Yeast strains/isolates that were cultured are listed in **Table 1**. Culturing was performed in liquid or solid (2% agar) YPD (1% w/v yeast extract, 2% w/v peptone and 2 % w/v D+ glucose) medium at 30°C.

119 **Table 1: *S. cerevisiae* strains/isolates cultured in this study**

Strain/isolate	**Details**	**Source**
Wild-type	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3, 112 can1-100 fob1Δ::HIS3*	NOY408-1bf; [17]
20-copy	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3, 112 can1-100 fob1Δ::HIS3*	[17]
40-copy	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3, 112 can1-100 fob1Δ::HIS3*	[17]
80-copy	*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3, 112 can1-100 fob1Δ::HIS3*	[17]
YJM981	Human clinical isolate from Italy; *Mat **a**, ho::HygMX, ura3::KanMX*-Barcode	[57]
DBVPG1373	Netherlands isolate from soil; *Mat **a**, ho::HygMX, ura3::KanMX*-Barcode	[57]
UWOPS03-461-4	Malaysian isolate from nectar	[58]
UWOPS03-461-4 (Mat a)	Derivative of UWOPS03-461-4; *Mat **a**, ho::HygMX, ura3::KanMX*-Barcode	[57]
UWOPS03-461-4 (Mat α)	Derivative of UWOPS03-461-4; *Mat α, ho::HygMX, ura3::KanMX*-Barcode	[57]
YPS128	US isolate from soil beneath *Quercus alba*	[58]
DBVPG1788	Finland isolate from vineyard soil	[58]

120

121 **Genomic DNA extraction**

122 High molecular weight genomic DNA (gDNA) was isolated as follows. Cell pellets from 3–5 mL
123 liquid cultures were washed in 500 µL of 50 mM EDTA pH 8 and resuspended in 200 µL of 50
124 mM EDTA pH 8 supplemented with zymolyase (3 mg/mL). After 1 hr at 37°C, the cell lysate was
125 mixed with 20 µL of 10% sodium dodecyl sulfate then with 150 µL of 3 M potassium acetate
126 (KAc) and incubated on ice for 1 hr. 100 µL of phenol-chloroform-isoamyl alcohol was added to
127 the SDS-KAc suspension, and following vortexing and centrifugation, 600 µL of propanol-2 were
128 added to the aqueous supernatant (≈ 300 µL). The nucleic acid pellet was washed three times in
129 70% EtOH, dried and resuspended in PCR grade water supplemented with RNase A (0.3 mg/mL).
130 After 1 hr at 37°C, samples were stored at -20°C.

131

132 **Whole genome sequence data**

133 gDNA extracted from four isogenic strains with different rDNA copy numbers (WT, 20-copy, 40-
134 copy and 80-copy; **Table 1**) was sequenced using Illumina MiSeq (**Supplementary Table 1**). The
135 raw sequence files are available through the NCBI SRA (accession number SUB7882611).

136

137 **Read preparation**

138 Paired-end reads were combined and quality checked using SolexaQA [59]. Low-quality ends of
139 reads (score cutoff 13) were trimmed using DynamicTrim, and short reads were removed using a
140 length cutoff of 50 bp with LengthSort, both within SolexaQA, as follows:

141 **command:** ~/path/to/solexaQA/SolexaQA++ dynamictrim /fastq/file

142 **command:** ~/path/to/solexaQA/SolexaQA++ lengthsort -l 50 /trimmed/fastq/file

143

144 **Obtaining whole genome and rDNA coverages**

145 The W303-rDNA reference genome was indexed using the bowtie2 (v. 2.3.2) build command as
146 follows:

147 **command:** ~/bowtie2-2.3.2/bowtie2-build <reference_in> <bt2_base>

148 Coverage files for the whole genome and rDNA were obtained using a four step pipeline:

149 **Step-1:** Processed reads were mapped to the indexed W303-rDNA genome using bowtie2 (v.
150 2.3.2)

151 **command:** ~/bowtie2-2.3.2/bowtie2 -x /path/to/indexed/genome/ -U /path/to/trimmed/reads/ -S
152 /output SAM file/

153 **Step-2:** The subsequent SAM format alignment was converted to BAM format using the
154 SAMtools (v. 1.8) view command:

155 **command:** ~/samtools-1.8/samtools view -b -S -o <output_BAM> <input_SAM>

156 **Step-3:** Mapped reads in the BAM file were sorted according to the location they mapped to in
157 W303-rDNA using the SAMtools sort command:

158 **command:** ~/samtools-1.8/samtools sort <input_BAM> -o <output_sorted.bam>

159 **Step-4:** Per-base read coverages across the entire W303-rDNA genome and the rDNA were
160 obtained using BEDtools (v. 2.26.0):

161 **command:** ~/bedtools genomecov -ibam <aligned_sorted.bam> -g <reference_genome.fasta> -d
162 <bedtools_coverage_WG.txt>

command: grep “rDNA_BLAST” <bedtools_coverage_WG.txt> <rDNA_bedtools_coverage.txt>

Calculation of rDNA copy number using modal coverage

Coverage frequency tables for the rDNA and whole genome (excluding mitochondrial DNA and plasmids) were obtained from per-base read coverage files by computing the mean coverage over a given sliding window size with a slide of 1 bp. The mean coverage for each sliding window was then allocated into a coverage bin. The bin that includes read coverage of zero was removed. The three highest frequency coverage bins from both the rDNA and whole genome frequency tables were used to calculate rDNA copy number as follows:

$$\boldsymbol{rDNA\ copy\ number} = \frac{\boldsymbol{Peak\ rDNA\ coverage\ bin\ value}}{\boldsymbol{Peak\ whole\ genome\ coverage\ bin\ value}}$$

The rDNA copy number estimates were taken as the mean of all pairwise combinations of these copy number values (**Supplementary Figure 2**).

Pipeline availability

The pipeline for modal calculation of rDNA copy number from an alignment of sequence reads to a reference genome containing one rDNA copy is available through Github (https://github.com/diksha1621/rDNA-copy-number-pipeline).

Calculation of rDNA copy number using mean and median coverage

183 The per-base read coverage across W303-rDNA from Bedtools was input into custom R-scripts to
184 obtain the mean and median coverage values for the whole genome and rDNA, after removing the
185 rDNA, 2-micron plasmid, and mitochondrial DNA coverage values from the whole genome
186 calculation. The rDNA copy number was then calculated for both mean and median approaches as
187 follows:

188 $$\boldsymbol{rDNA\ copy\ number} = \frac{\boldsymbol{coverage\ across\ rDNA}}{\boldsymbol{coverage\ across\ whole\ genome}}$$

189

190 **Subsampling**

191 To generate different coverage levels for copy number estimation, sequence reads were randomly
192 downsampled using the seqtk tool (https://github.com/lh3/seqtk) as follows:

193 **command:** ~/seqtk/seqtk sample –s$RANDOM <name of fastqfile> <number of reads required>
194 <outputfile>

195

196 **rDNA copy number measurement by ddPCR**

197 At least three independent cultures (biological replicates) were generated for each isolate using
198 one independent colony per culture. To evaluate rDNA copy number variation over generations,
199 cultures were propagated over four days (~60 generations) as follows: individual colonies were
200 initially grown in 3 mL YPD for 24 hr. 30 µL of this was used to inoculate 3 mL YPD and this
201 was grown for another 24 hr. This process was repeated for four days. Cells were harvested after
202 24 hr (~15 generations) and four days, and cell pellets frozen at -80˚C. gDNA was extracted as
203 above, then linearized by *Xba*I in NEB2 buffer following the manufacturer's instructions (NEB)

204 to individualize rDNA repeats. gDNA linearization was verified by separation on agarose gels and
205 DNA concentration measured on a Qubit Fluorometer using the Qubit dsDNA HS assay (Thermo
206 Fisher). Linearized gDNA was brought to 2 pg/µL by serial dilution. EvaGreen master mixes were
207 prepared with an rDNA primer pair (rDNAScSp_F2 5'- ATCTCTTGGTTCTCGCATCG-3',
208 rDNAScSp_R2 5'-GGAAATGACGCTCAAACAGG-3') or a single copy *RPS3* gene primer pair
209 (RPS3ScSp_F2 5'-CACTCCAACCAAGACCGAAG-3', RPS3ScSp_R2 5'-
210 GACAAACCACGGTCTTGAAC-3'). *RPS3* and rDNA ddPCR reactions were performed with 2
211 µL (4 pg) of the same linearized gDNA dilution as template. Droplet generation and endpoint PCR
212 were performed following the manufacturer's instructions, and droplets were read using a QX200
213 droplet reader (BioRad). Quantification was performed using QuantaSoft Analysis Pro (v.
214 1.0.596). rDNA copy number was determined by the (rDNA copy/µL)/(*RPS3* copy/µL) ratio.

215

216 **Pulse field gel electrophoresis (PFGE)**

217 To make chromosome plugs [21], cells from overnight liquid cultures were resuspended in 50 mM
218 EDTA pH 8.0 to 2.10^9 cells/mL, transferred to 45˚C, and mixed with an equal volume of 1.5% low
219 melting point agarose in 50 mM EDTA prewarmed to 45˚C. The mixture was transferred by gentle
220 pipetting to PFGE plug molds (BioRad) to set at 4˚C for 15 min. Plugs were transferred to fresh
221 spheroplasting solution (1 M Sorbitol, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 14 mM 2-
222 mercaptoethanol, 2 mg/mL zymolyase). After 6 hr incubation at 37˚C with occasional inversion,
223 plugs were washed for 15 min in LDS buffer (1% lithium dodecyl sulphate, 100 mM EDTA pH
224 8.0, 10 mM Tris-HCl pH 8.0), before overnight incubation at 37˚C in the same buffer with gentle
225 shaking. Plugs were incubated twice for 30 min each in NDS buffer (500 mM EDTA, 10 mM Tris-
226 HCl, 1% sarkosyl, pH 9.5) and at least three times for 30 min in TE (10 mM Tris-HCl pH 8.0, 1

227 mM EDTA pH 8.0). Plugs were stored at 4˚C in fresh TE. For restriction digestion, half plugs were
228 pre-washed for two hours in TE, three times for 20 min each in TE, and three times for 20 min
229 each in 300 µL restriction buffer supplemented with 100 µg/mL BSA, all at room temp. Restriction
230 digestion was performed overnight at the recommended temperature in 500 µL of restriction buffer
231 containing 100 U of restriction endonuclease. Digested plugs were washed in 50 mM EDTA pH
232 8.0 and stored at 4˚C in 50 mM EDTA pH 8.0 before loading. PFGE was performed using 1%
233 agarose gel in 0.5X TBE (Thermo-Fisher) in a CHEF Master XA 170-3670 system (BioRad) with
234 the following parameters: auto algorithm separation range 5 kb - 2 Mb (angle 120˚C, run 6 V/cm,
235 initial switch time 0.22 s, final switch time 3 min 24 s, run time 916 min) at 14˚C. DNA was
236 visualized by staining in ethidium bromide (5 µg/mL) and imaging (Gel Doc XR+; BioRad).

237

238 **1002 Yeast Genome project rDNA copy number estimation**

239 Illumina reads from the 1002 Yeast Genome project were obtained from the European Nucleotide
240 Archive (www.ebi.ac.uk/) under accession number ERP014555. We omitted clades with few
241 members, mosaic clades, and unclustered isolates, giving a total of 788 isolates. Reads were
242 downsampled to 10-fold-coverage using seqtk() and rDNA copy number for each isolate was
243 calculated using W303-rDNA as the reference. Bin sizes of 1/200th of the mean coverage for rDNA
244 and 1/50th for the whole genome, and a window size of 600 bp for both estimates, were used.
245 Violin plots were plotted using the ggplot() package in R.

246

247 **Phylogenetic analyses**

248 To create a neighbour-joining phylogeny based on rDNA copy number values, rDNA copy number
249 for each isolate (after removing 30 isolates for which SNP data were not available) was normalized

250 on a 0-1 scale. Normalized values were used to calculate pairwise Euclidean distances between
251 each pair of isolates to generate a distance matrix that was applied to construct a phylogeny via
252 neighbour-joining using MEGA X [60].

253

254 Phylocorrelograms of copy number and the SNP phylogeny were generated using phylosignal
255 v.1.3 [61] (https://cran.r-project.org/web/packages/phylosignal/index.html). Phylocorrelograms
256 representing a no phylogenetic signal dataset (a "white noise" random distribution) and a high
257 phylogenetic signal dataset (a character evolving on the SNP tree according to a Brownian motion
258 model) were also generated. For the white noise distribution, data were simulated from a normal
259 distribution with mean and standard deviation matching those of the observed copy number data
260 (mean=92.5, sd=30.8). For the Brownian motion model, we first estimated the ancestral mean
261 (z0=83.2) and the rate parameter (σ2=72557.2) from the observed copy number data using the
262 fitContinuous function from geiger [62] (https://cran.r-project.org/package=geiger). Then, we
263 simulated from these parameters on the SNP tree using fastBM from phytools 0.7 (https://cran.r-
264 project.org/package=phytools). Phylocorrelograms were generated for the observed and the two
265 simulated datasets, estimating correlations at a series of 100 phylogenetic distances using 100
266 bootstrap replicates.

267

268 **Comparing intra-species variation in rDNA copy number**

269 Copy number estimates for twelve isolates from the 1002 Yeast Genome data were randomly
270 drawn 1000 times using a custom bash-script to obtain rDNA copy number ranges.

271

272 **Statistical analyses**

273 All statistical analyses to evaluate differences in rDNA copy number between clades were
274 performed in R. Significance was calculated using the Welch *t*-test (*t*-test), the non-parametric
275 Wilcoxon-Mann-Whitney test (wilcox test) and ANOVA, with *p*-values considered statistically
276 significant at $p < 0.05$.

277 **Results and Discussion**

278 **Establishment of a modal coverage bioinformatics pipeline for estimating rDNA copy**
279 **number**

280 The abundance of data generated from NGS platforms has led a number of studies to use mean
281 read depth to estimate rDNA copy number [5, 12, 25, 34, 49, 55, 56]. However, repeat elements,
282 sequence biases and large-scale changes like aneuploidies can potentially result in non-normal
283 read coverage distributions where the mean coverage does not accurately represent the true
284 coverage. To overcome these limitations, we developed a novel sequence read-based rDNA copy
285 number calculation approach based on the most frequent (modal) coverage. The rationale for this
286 approach is that modal coverage will provide an estimate of the relative coverage representation
287 of a given region in a genome that is more robust to biases away from normality than the mean or
288 median. The approach allocates coverage across a reference genome into coverage bins and the
289 ratio of the most frequently occurring coverage bins for the rDNA and the WG is then used to
290 calculate rDNA copy number (per haploid genome). We implemented this modal coverage
291 approach as a simple pipeline to calculate rDNA copy number from mapped sequence reads (**Fig**
292 **2**). To help smooth across positions that stochastically vary in coverage, an issue that is particularly
293 prevalent with very low coverage datasets, we used a sliding window approach to calculate
294 coverage. Our straightforward pipeline uses a sorted BAM file of reads aligned to a reference
295 genome for which the position of the rDNA is known (either embedded in the genome or as a
296 separate contig) to calculate copy number.

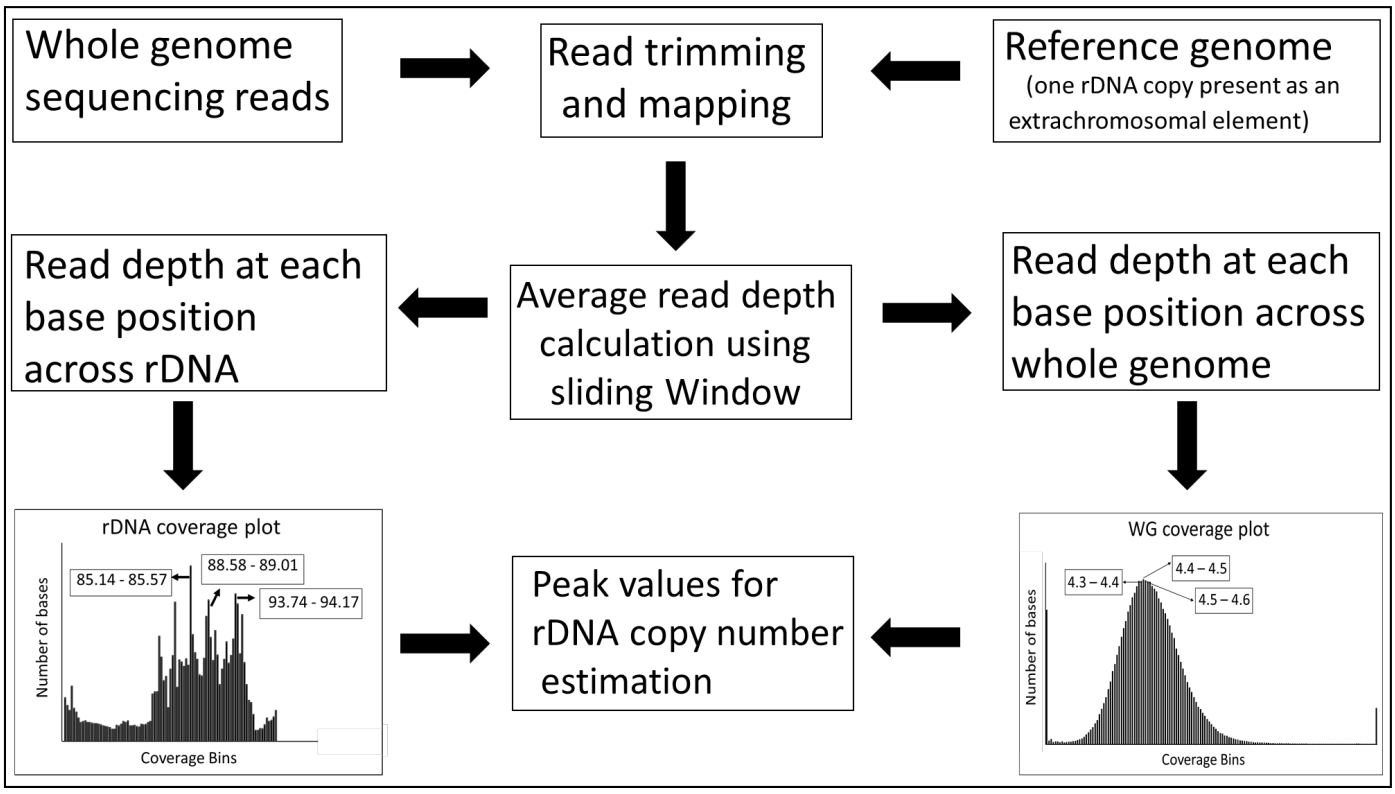


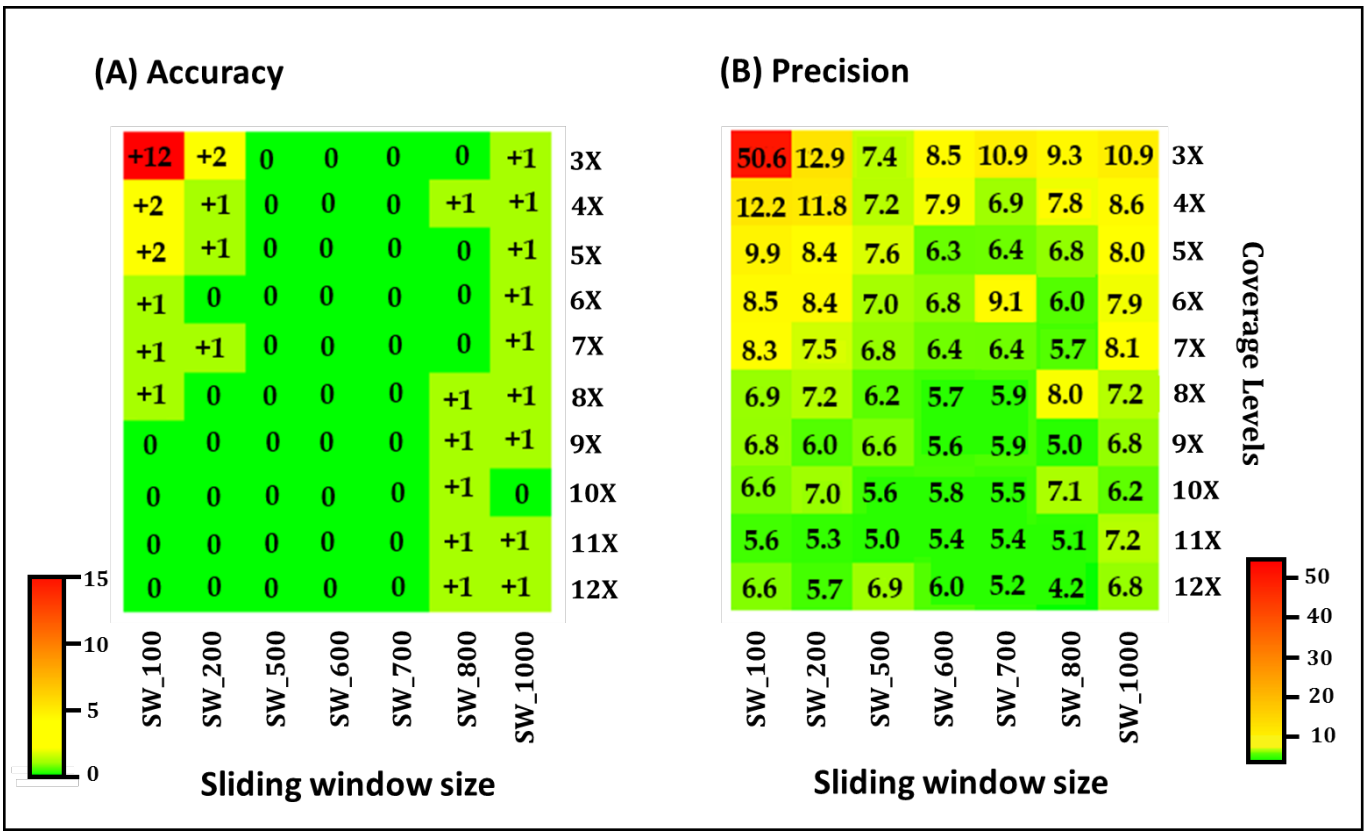
Figure 2. Overview of the modal approach to estimate rDNA copy number from whole genome sequence data. Whole genome (WG) sequence reads are mapped against a reference genome containing a single rDNA copy. Mean read depth for each postion is calculated across the rDNA and the WG using a sliding window, then allocated into coverage bins (shown as histograms). To calculate modal rDNA copy number, the highest frequency coverage bins for both the rDNA and WG are used to compute ratios that represent the rDNA copy number range. The histograms shown were plotted using a 20-copy yeast strain at 5-fold WG coverage with bin sizes of 1/200th of mean coverage for rDNA and 1/50th for WG, and a sliding window of 600 bp for both. The coverage ranges for the three most frequent bins for each are indicated in boxes.

To implement our modal coverage approach, we generated test datasets by performing WG Illumina sequencing of a haploid wild-type laboratory *S. cerevisiae* strain reported to have 150-

311 200 rDNA copies, and three isogenic derivatives where the rDNA has been artificially reduced to
312 20, 40 and 80 copies, and "frozen" in place through disruption of a gene (*FOB1*) that promotes
313 rDNA copy number change [17] (**Table 1**). Initially, we investigated which parameters provide
314 the most accurate results by applying our pipeline to the WG sequence data obtained from a strain
315 with 20 rDNA copies (20-copy strain). We obtained a genome-wide read coverage of 13.1-fold
316 (**Supplementary Table 1**) and mapped these reads to the W303-rDNA yeast reference genome
317 that has a single rDNA copy. The mapping output was used to determine per-base coverage values,
318 which were placed into coverage bins using a sliding window. We investigated a range of sliding
319 window sizes, from 100 bp (previously reported to have an approximately normal distribution of
320 WG sequence read coverage [63]) to 1,000 bp (large sliding window sizes, whilst smoothing
321 stochastic coverage variation, converge on the mean coverage as the window size approaches the
322 rDNA unit length). We also assessed the impact of coverage on copy number estimation by
323 downsampling the sequence reads. We ran analyses with 100 technical replicates and computed
324 the rDNA copy number means and ranges. We found that, as expected, the accuracy and precision
325 (defined here as <u>similarity to known copy number</u> and <u>copy number range</u>, respectively) of the
326 pipeline was poorer at lower coverage levels, while larger sliding window sizes could compensate
327 for a lack of reads to improve both measures (**Fig 3**). Coverage levels above 10-fold with a sliding
328 window size between 500-800 bp produced accurate rDNA estimates. However, our method also
329 demonstrated adequate performance even with a coverage level of 5-fold, when the sliding window
330 was 600-700 bp (**Fig 3**). We found that the method works similarly when just using the rRNA
331 coding region (**Supplementary Figure 3**) rather than the full repeat, which is important as the full
332 rDNA unit sequence is often not available. We also examined the performance of median
333 coverage, but found that while it had greater precision compared to the modal coverage approach,
334 the accuracy was poorer (**Supplementary Figure 4**). Given the rapid rate at which copy number

335 changes even during vegetative growth [21], the lower precision of our method may more
336 accurately represent the range of copy numbers likely to be present in samples that consist of
337 multiple cells.

338



339

340 **Figure 3. Assessing parameters for rDNA copy number estimation accuracy and**
341 **precision.** Cells represent the (**A**) deviation of the calculated modal rDNA copy number from
342 20, and (**B**) maximum variation of rDNA copy number calculated from the 100 technical
343 replicates for each coverage level and sliding window (SW) size combination. The heatmap
344 scales used are indicated. In (**A**), rDNA copy number was rounded to the nearest integer.

345

346 We then assessed the performance of our pipeline with the 40-copy, 80-copy, and WT *S. cerevisiae*
347 strain data. Illumina WG sequence reads (**Supplementary Table 1**) obtained from these strains

348 were downsampled to generate 100 technical replicates at 10-fold coverage for each strain, and
349 rDNA copy numbers were calculated using our modal coverage pipeline with a sliding window of
350 600 bp. The resultant rDNA copy numbers were: 32-40 ($\bar{x}$ = 36 copies) for the 40-copy strain; 57-
351 72 ($\bar{x}$ = 64 copies) for the 80-copy strain; 129-177 ($\bar{x}$ = 157 copies) for the WT strain. These values,
352 while similar to the reported copy numbers for these strains, are not identical. Therefore, to check
353 the actual copy numbers of these strains, and to provide a direct validation of our modal pipeline
354 method, we next experimentally determined the rDNA copy numbers of these strains.

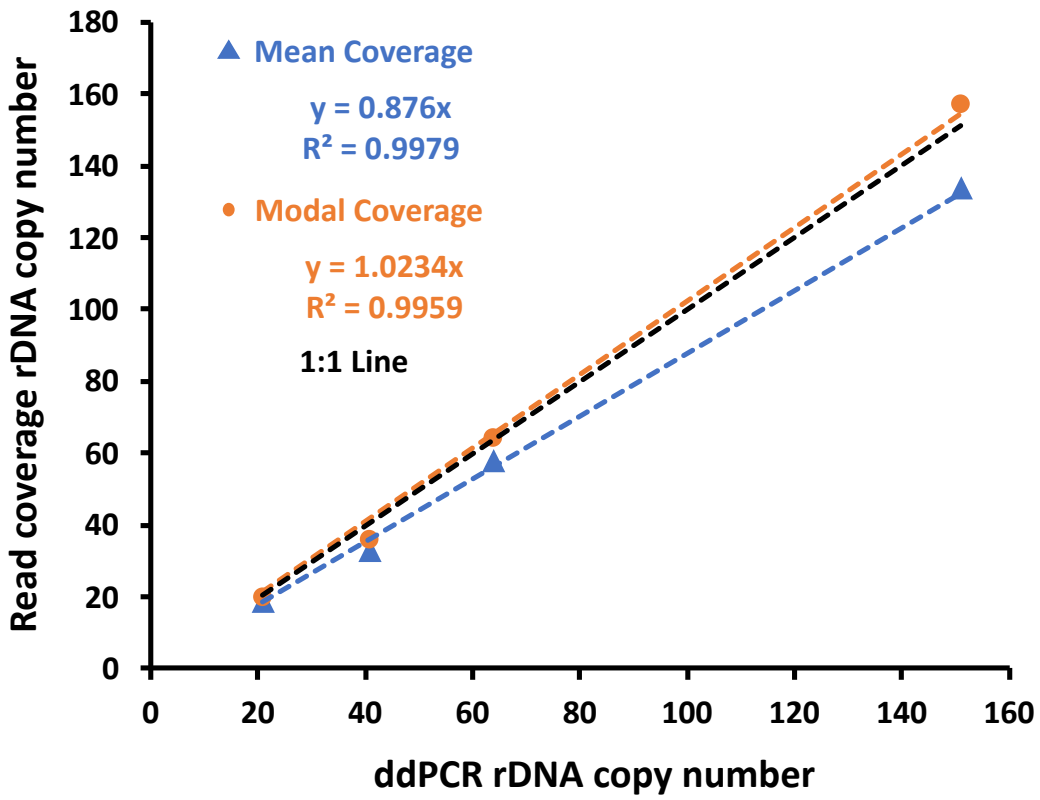
355

356 We chose ddPCR to experimentally determine rDNA copy number because it is less sensitive than
357 qPCR to biases in secondary structure regions that are common in the rDNA coding region [22].
358 The ddPCR data showed that the rDNA copy numbers of our strains are similar to those calculated
359 by our modal coverage method, with both methods suggesting that the "80-copy" strain actually
360 has substantially fewer copies than reported (**Supplementary Table 2**; **Supplementary Figure**
361 **5A**), perhaps due to a stochastic change in copy number that has occurred in our version of this
362 strain. We also compared our modal coverage approach with the mean coverage calculated from
363 the same datasets. We used a simple mean calculation to match the implementation of our modal
364 approach, using the same down-sampled 10-fold WG coverage datasets. The copy number
365 estimates made using the mean coverage approach were uniformly lower than the other estimates
366 (**Supplementary Table 2**), which we suggest is the result of sequencing biases against regions in
367 the rRNA coding region. Importantly, correlating read coverage and ddPCR copy number
368 estimates showed the modal coverage slope was closer to the expected value of 1 than the mean
369 coverage slope (**Fig 4**). We also estimated the copy number using pulsed field gel electrophoresis
370 based on the size of the restriction fragment encompassing the entire rDNA array divided by the
371 rDNA unit size (accounting for the sizes of the flanking regions), again with consistent results

372 (**Supplementary Figure 5B,C**). Together, these results suggest the modal coverage approach is

373 an accurate way to estimate rDNA copy number.

374



375

376 **Figure 4. Comparison of modal and mean coverage copy number estimation methods.**

377 Plot of rDNA copy number for the 20, 40, 80 and WT *S. cerevisiae* strains (10-fold coverage)

378 calculated using modal (orange line) and mean (blue line) coverage methods versus the copy

379 number determined by ddPCR. The expected 1:1 correlation between read coverage and

380 ddPCR methods is shown in black. Note that while the mean coverage method gives a higher

381 R^2, the modal coverage results are a closer fit to the expected 1:1 line.

382

383 Our results suggest that the modal coverage pipeline provides robust estimates of rDNA copy

384 number even when coverage is less than 5-fold. This reliability may partly be a consequence of

385 the larger sliding window size we used compared to that commonly applied for mean coverage
386 methods. It was previously reported that coverage below ~65X results in precision issues when
387 estimating rDNA copy number [5]. However, we did not find this, either for our method or using
388 mean coverage, suggesting that the issues might be specific to the approach or dataset used in that
389 study. The simple implementation of our modal approach coupled with its good performance make
390 it an attractive method for estimating rDNA copy number from sequence read data. Furthermore,
391 a modal approach is expected to be more robust to features that can perturb mean coverage
392 approaches by skewing coverage distributions, such as repeat elements, large duplications and
393 deletions, regions exhibiting sequencing biases, modest sequence divergence from the reference
394 sequence, and aneuploidies [46]. Although we have developed our pipeline for measuring rDNA
395 copy number, in principle it can be used to calculate copy number for any repeated sequence where
396 all reads map to a single repeat copy and the sequence is known, such as mitochondrial and
397 chloroplast genome copy numbers. Given its strong performance, we applied our method to
398 characterize the inter-population distributions of rDNA copy number in *S. cerevisiae*.

399

400 **Within-species evolutionary dynamics of rDNA copy number**

401 Studies in model organisms have provided evidence that each species has a homeostatic copy
402 number which is returned to following copy number perturbations [7-10]. This homeostatic copy
403 number appears to have a genetic basis [5, 26], which suggests it might vary between populations,
404 as well as between species. However, few studies have addressed this question. Given that
405 variation in rDNA copy number has been associated with altered phenotypes [8, 12, 17, 22, 27-
406 35], we decided to undertake a comprehensive assessment of *S. cerevisiae* rDNA copy number at
407 the population level using the global wild yeast dataset from the 1002 Yeast Genome project [64].

408

409 We obtained WG sequence data for 788 isolates from the 1002 Yeast Genome project. Reads for
410 each isolate were downsampled to 10X genome coverage, mapped to our W303-rDNA reference
411 genome, and rDNA copy numbers estimated using our modal coverage pipeline. The rDNA copy
412 numbers ranged between 22-227 ($\bar{x}$ = 92) across the 788 isolates (**Supplementary Table 3**). The
413 copy numbers of 11 wild *S. cerevisiae* isolates included in our dataset had previously been
414 estimated [14, 25], and our results are largely consistent with these (**Supplementary Table 4**).
415 However, the copy number we estimate are, in general, much lower than those (~150-200)
416 measured for most laboratory strains (e.g. [17, 38, 41]). We looked to see whether ploidy affects
417 rDNA copy number, given that laboratory strains are predominantly haploid, while the wild *S.*
418 *cerevisiae* isolates we analyzed are mostly diploid. We observed a small difference in copy number
419 between haploid and diploid isolates (104 vs 91 copies, respectively; **Supplementary Figure 6**
420 and **Supplementary Information**), but overall do not find a strong effect of ploidy on copy
421 number. Thus, the copy number differences between lab and most wild *S. cerevisiae* isolates seem
422 to be a property of these isolates.

423

424 The difference in copy number between lab and wild *S. cerevisiae* isolates suggests that *S.*
425 *cerevisiae* populations may harbor different rDNA copy numbers. To test this, we used the 23
426 phylogenetic clades defined by [64] as proxies for *S. cerevisiae* populations and looked at the
427 distributions of rDNA copy number within and between these populations (**Fig 5)**. ANOVA
428 analysis rejects homogeneity of rDNA copy number between these populations (p = 4.37e^{-15}),
429 suggesting there are population-level differences in copy number within *S. cerevisiae*.

430

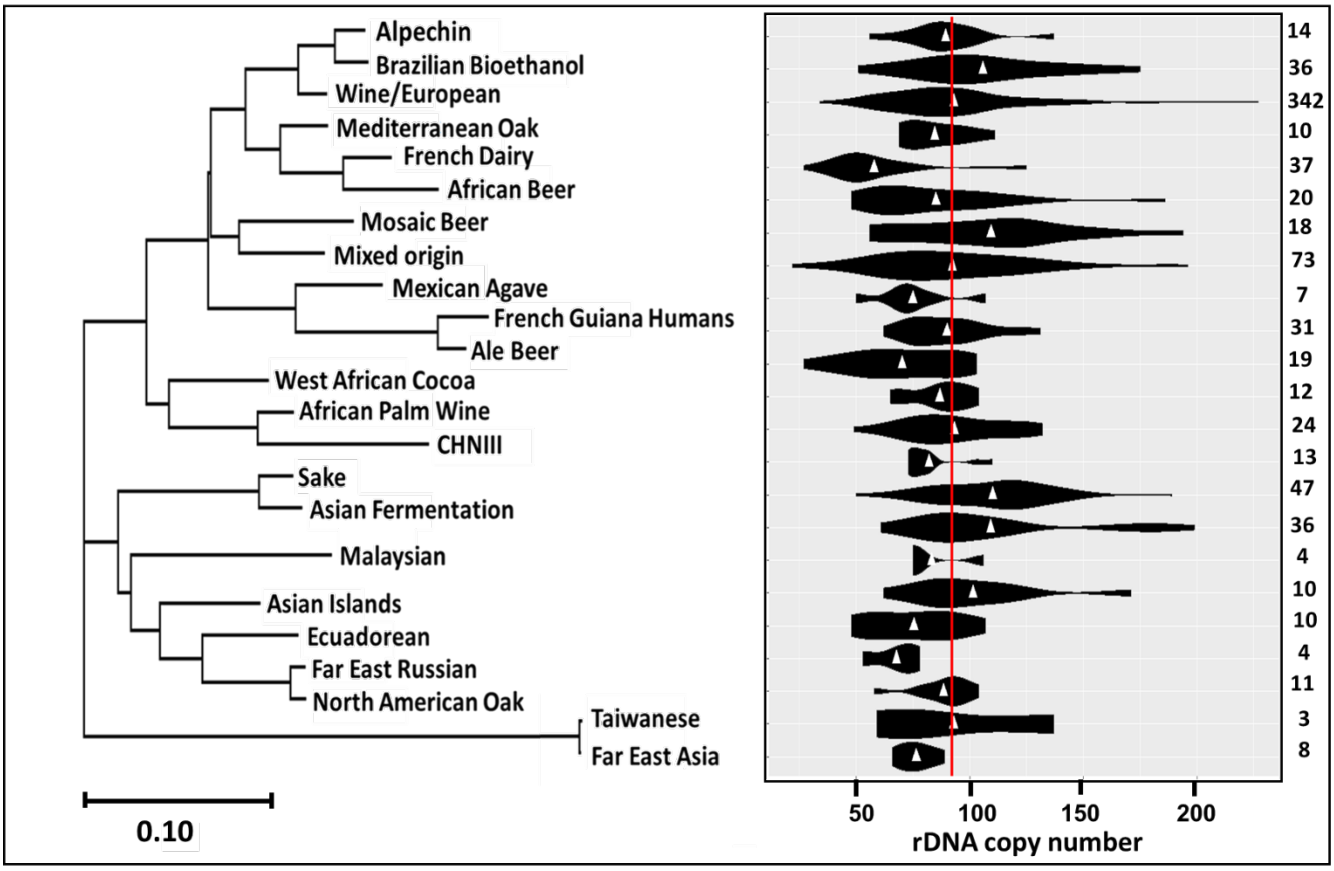


Fig 5. rDNA copy number in *S. cerevisiae* populations. To the left is the phylogeny of the 23 *S. cerevisiae* clades from [64] that encompass the 788 isolates included in this study. The scale represents substitutions per site. On the right, rDNA copy number calculated using the modal coverage method is displayed as a violin plot for each clade with mean population copy numbers indicated by white triangles. Numbers to the right represent the number of isolates in each clade. The red vertical line represents the overall mean rDNA copy number (92 copies). Copy number estimations were determined using 10-fold coverage and a 600 bp sliding window.

We next wanted to look for complementary evidence that *S. cerevisiae* populations have different rDNA copy numbers, as an alternative explanation for our results is different populations happened to have different copy numbers simply due to stochastic variation [21]. If the stochastic variation

444 explanation is correct, we would expect divergent copy numbers to return to the homeostatic value
445 over time. To test this, we used ddPCR to measure the rDNA copy numbers of six of the 1002
446 Yeast Genome project isolates that represent the range of copy numbers observed, including one
447 that we had three different isolates of. We grew three biological replicates of each isolate for ~60
448 generations to allow any fluctuation in rDNA copy number to return to the homeostatic level [7].
449 The rDNA copy numbers, both before and after the ~60 generations, resemble the copy numbers
450 we estimated from the sequence data and show no tendency to converge on the overall *S. cerevisiae*
451 mean copy number (**Table 2; Supplementary Table 5**). These results strongly suggest that our
452 method of estimating rDNA copy number is robust and that the copy numbers of isolates are not
453 recovering towards a common copy number value. From this we conclude that different *S.*
454 *cerevisiae* populations have different homeostatic rDNA copy numbers.

455

456

457 **Table 2. *S. cerevisiae* rDNA copy number does not recover to a common value following ~60**

458 **generations of growth**

Isolates	rDNA CN at start[a]	rDNA CN after ~60 generations[a]		Original modal CN estimation[b]
***S. cerevisiae* wild-type** rep1[c]	213	130	185[d]	157
rep2		217		
rep3		208		
YJM981 rep1	174	120	175	171
rep2		183		
rep3		221		
DBVPG1373 rep1	69	77	85	78
rep2		72		
rep3		107		
UWOPS03-461-4[e] rep1	85	113	95	106
rep2		88		
rep3		83		
UWOPS03-461-4[e] **(Mat**a**)** rep1	244	164	146	
rep2		167		
rep3		106		
UWOPS03-461-4[e] **(Matα)** rep1	ND[f]	108	109	
rep2		115		
rep3		105		
YPS128 rep1	89	87	79	89
rep2		73		
rep3		77		
DBVPG1788 rep1	95	126	108	87
rep2		100		
rep3		97		

459

460 [a] Measured using ddPCR

461 [b] Measured using our modal coverage pipeline

462 [c] rep: biological replicate

463 [d] Mean of the three replicates to the nearest integer

464 [e] UWOPS03-461-4 is the parent isolate of UWOPS03-461-4 Mat**a** and UWOPS03-461-4 Matα
465 derivatives

466 [f] Not determined

467

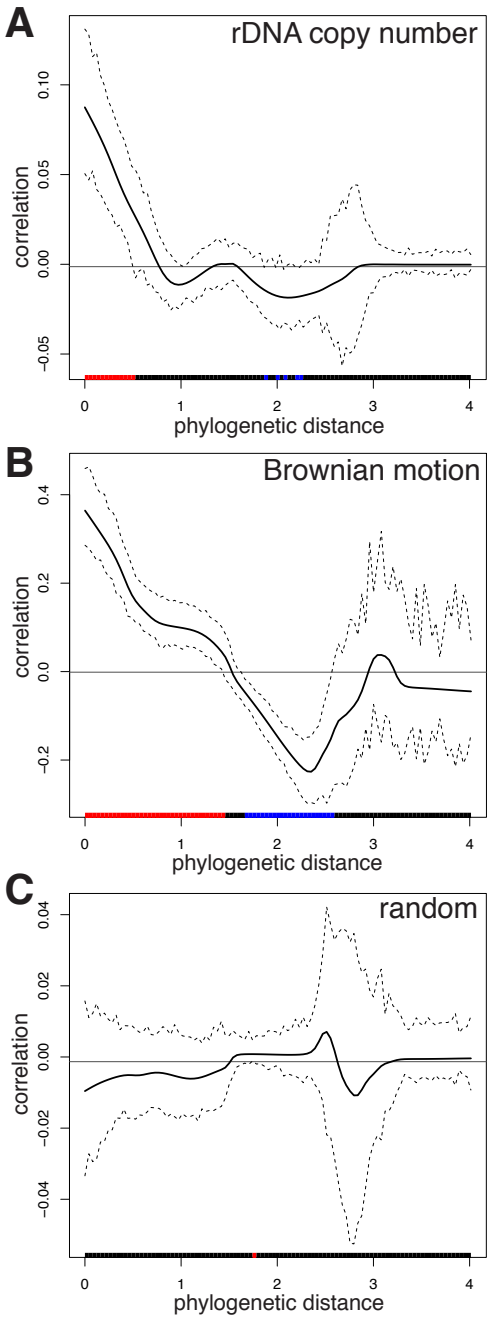
468 Copy number has previously been shown to correlate with phylogeny for species across the fungal

469 kingdom [5]. Given the differences in rDNA copy number we observe, we wondered whether a

470 similar correlation exists between *S. cerevisiae* populations. To test this, we constructed a

471 neighbour-joining phylogeny using rDNA copy number as the phylogenetic character for 758
472 isolates (30 were removed as SNP data were not available) and compared this to the reported *S.*
473 *cerevisiae* phylogeny created from genomic SNP data [64]. To assess how well the two
474 phylogenies correlate, we used Moran's Index of spatial autocorrelation *I*, which quantifies the
475 correlation between two traits. Moran's *I* indicated a modest positive correlation between rDNA
476 copy number and phylogeny at short phylogenetic distances (**Fig 6**), but not a significant negative
477 correlation at greater phylogenetic distances like that previously observed above the species level
478 [5]. These results suggest that phylogeny only partially explains the distribution of rDNA copy
479 numbers amongst *S. cerevisiae* populations.

480



481

482 **Figure 6**. **Phylocorrelograms of autocorrelation based on Moran's *I***. Phylogenetic
483 distance spatial autocorrelations between the SNP-based *S. cerevisiae* phylogeny and the
484 rDNA copy number phylogeny (**A**), a Brownian motion phylogeny (**B**), and random data (**C**)
485 are plotted. Red segments beneath each phylocorrelogram indicate significant positive
486 autocorrelation; black no significant autocorrelation, and blue significant negative

487 autocorrelation. Dotted lines indicate autocorrelation 95% confidence intervals. Significance
488 is based on comparison to zero phylogenetic autocorrelation (horizontal black line at 0).

489

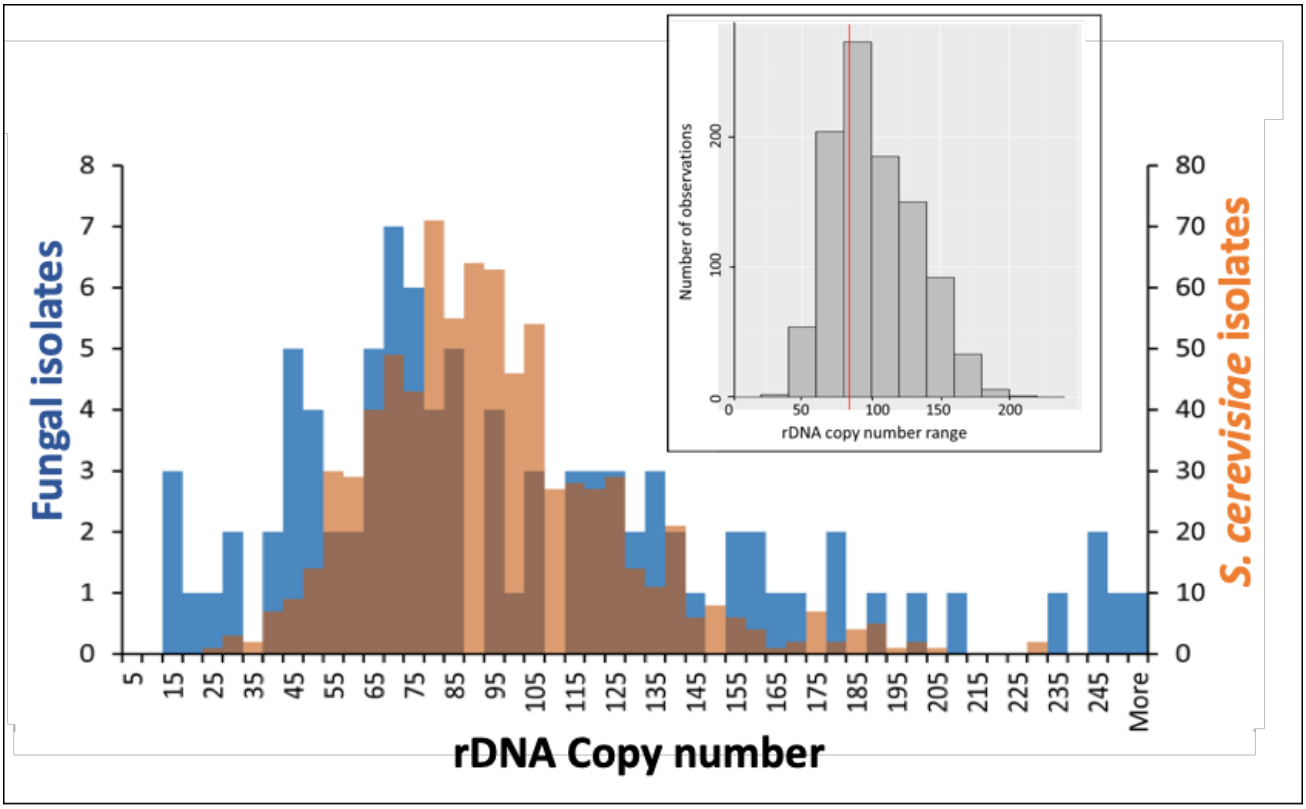
490 Another feature that might explain the distribution of rDNA copy numbers between *S. cerevisiae*
491 populations is the environment, given that nutritional conditions have been proposed to influence
492 copy number [65, 66]. To investigate this, we compared the rDNA copy numbers from two
493 phylogenetically-diverged *S. cerevisiae* populations that are associated with oak trees, which we
494 took as a proxy for similar environments. We found the oak populations did not show significantly
495 different copy numbers (p-value = 0.52), as expected if environment is contributing to copy
496 number. Thus, rDNA copy number might be partially determined by the environmental conditions
497 the population has evolved in. However, we found no consistent pattern of similarities or
498 differences with the copy numbers of the nearest phylogenetic neighbours of these oak clades
499 (**Supplementary Information**), thus we cannot rule out these results simply representing
500 stochastic variation. We suggest that a better understanding of what environmental factors
501 modulate rDNA copy number is necessary before we can properly evaluate the impact of the
502 environment on patterns of rDNA copy number variation.

503

504 Finally, we wondered whether large range in estimated *S. cerevisiae* rDNA copy number (22-227
505 copies) might reflect an unusually large variance in copy number in this species, given this range
506 is almost the same as that reported across 91 different fungal species from three different fungal
507 phyla (11-251 copies, excluding one outlier of 1442 copies) [5]. However, comparing the *S.*
508 *cerevisiae* copy number range generated by drawing twelve *S. cerevisiae* isolates at random from
509 our data 1,000 times to that previously measured for twelve isolates of one fungal species (*Suillus*

510 *brevipes*; [5]) shows that the *S. brevipes* range falls in the middle of the *S. cerevisiae* distribution
511 of copy number ranges (**Fig 7**). These results suggest that *S. cerevisiae* rDNA copy number is no
512 more variable than that of *S. brevipes* at least, and illustrate the tremendous variation in rDNA
513 copy number that is likely to be present in many eukaryotic species.

514



515

516 **Figure 7. Distribution of rDNA copy number for fungal and *S. cerevisiae* isolates.** The
517 main histogram represents rDNA copy number (x-axis) for 91 previously published fungal
518 taxa (blue bars, y-axis on left; [5]) and the 788 *S. cerevisiae* isolates (orange bars; y-axis on
519 right) from this study. Brown represents overlaps. Inset histogram shows the distribution of
520 total rDNA copy number ranges from 1,000 randomly drawn sets of twelve *S. cerevisiae*
521 isolates. The red vertical line represents the total copy number range (84) observed amongst
522 twelve *Suillus brevipes* isolates [5].

523

524 **Conclusions**

525 Our results demonstrate that modal coverage can be used to robustly determine rDNA copy
526 number from NGS data. Using our novel approach, we demonstrate that the mean rDNA copy
527 number across all wild *S. cerevisiae* populations is 92. This is substantially lower than the copy
528 numbers documented for lab *S. cerevisiae* strains, but overlaps the 'typical' rDNA copy numbers
529 reported for fungi [5]. We show that *S. cerevisiae* populations have different homeostatic rDNA
530 copy numbers, consistent with a previous study [14]. We found some correlation between rDNA
531 copy number and phylogeny, but not enough to suggest that homeostatic copy number is simply
532 drifting apart with increasing phylogenetic distance. We provide circumstantial evidence that
533 environmental factors might help drive the homeostatic rDNA copy number differences. This is
534 consistent with demonstrations that nutritional factors can induce physiological rDNA copy
535 number changes [65, 66] and that such differences have phenotypic consequences [8, 12, 17, 22,
536 27-35]. However, it has been shown that rDNA copy number does not correlate with trophic mode
537 in fungi [5]. Therefore, more work is required to determine what really drives copy number
538 dynamics between populations. One caveat to our conclusions is that while studies from a variety
539 of organisms have demonstrated that copy number recovers from perturbation [7-10], presumably
540 as a result of mechanisms maintaining homeostatic copy number [26], some recent studies in *S.*
541 *cerevisiae* and *Drosophila* have reported the persistence of stochastic copy number changes
542 without recovery [65, 67]. It will be important to reconcile these conflicting results and to
543 determine to what extent the population-level differences we observe are the result of copy number
544 homeostasis (as we interpret them) versus copy number inertia.

545

546 Our results showing population-level differences in rDNA copy number suggest that such
547 differences can arise relatively quickly in evolutionary time, although the very high level of copy
548 number variation between individuals obscures this pattern. Therefore, it is important to take the
549 large variances and rapid copy number dynamics of the rDNA into account when interpreting the
550 impact of copy number variation on phenotype. Bioinformatics pipelines, such as the one we have
551 developed here, in conjunction with the increasing availability of appropriate NGS datasets
552 provide a way to establish baseline data on rDNA copy number variation between cells,
553 individuals, populations, and species, as well as to investigate the phenotypic consequences of this
554 variation. Finally, while we report population-level differences in rDNA copy number in *S.*
555 *cerevisiae*, diverse human populations have been reported to not differ in rDNA copy number [12,
556 46]. Whether this reflects a difference in biology (such as differences in the level of genetic
557 divergence between populations) or an incomplete understanding of human population rDNA copy
558 number will require further clarification.

559

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