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

Diksha Sharma[1], Sylvie Hermann-Le Denmat[1,2], Nicholas J. Matzke[1], Katherine Hannan[3,4], Ross D. Hannan[3,4,5,6,7], Justin M. O'Sullivan[8,9,10,11], Austen R. D. Ganley[1]

1. School of Biological Sciences, University of Auckland, Auckland, New Zealand
2. Ecole Normale Supérieure, PSL Research University, F-75005 Paris, France
3. ACRF Department of Cancer Biology and Therapeutics, The John Curtin School of Medical Research, ACT 2601, Australia
4. Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3010, Australia
5. Division of Research, Peter MacCallum Cancer Centre, Melbourne, Victoria, 3000, Australia
6. Sir Peter MacCallum Department of Oncology, University of Melbourne, Parkville, Victoria, 3010, Australia
7. Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia
8. Liggins Institute, University of Auckland, Auckland, New Zealand
9. Maurice Wilkins Center, University of Auckland, New Zealand
10. MRC Lifecourse Unit, University of Southampton, United Kingdom
11. Brain Research New Zealand, The University of Auckland, Auckland, New Zealand

Corresponding Author, email : a.ganley@auckland.ac.nz

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 also 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.

Author summary

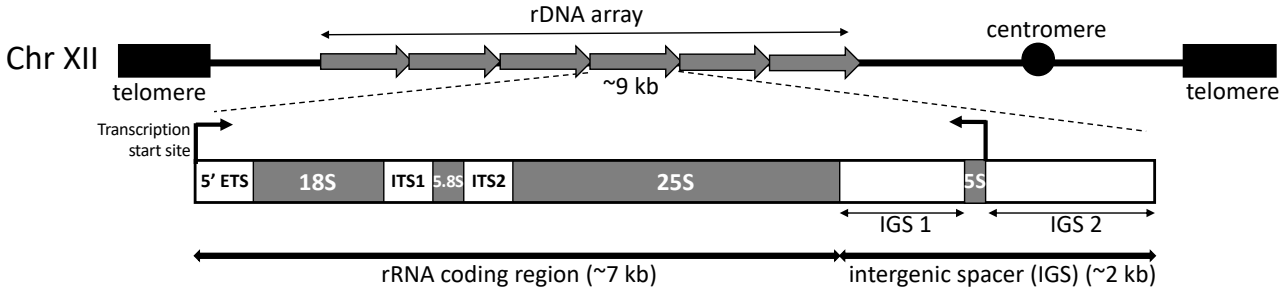
The ribosomal RNA gene repeats (rDNA) form large tandem repeat arrays in most eukaryote genomes. Their tandem arrangement makes the rDNA prone to copy number variation, and there is increasing evidence that this copy number variation has phenotypic consequences. However, difficulties in measuring rDNA copy number hamper investigation into rDNA copy number dynamics and their significance. Here we developed a novel bioinformatics method for measuring rDNA copy number from whole genome sequence data that is based on the modal sequence read coverage. We established parameters for optimal performance of the method and validated it using yeast strains of known rDNA copy numbers. We then applied the method to a dataset of almost 800 global yeast isolates and demonstrate that yeast populations have different rDNA copy numbers that partially correlate with phylogeny. Our work provides a simple and accurate method for determining rDNA copy number that leverages the growing number of whole genome datasets, and highlights the dynamic nature of rDNA copy number.

60 **Introduction**

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

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

85



86

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

93

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

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

114

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

121 **Materials and Methods**

122

123 **Modified *Saccharomyces cerevisiae* genome**

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

129

130 **Yeast strains/isolates and growth conditions**

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

134

135

136 **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]

137

138 **Genomic DNA extraction**

139 High molecular weight genomic DNA (gDNA) was isolated as follows. Cell pellets from 3–5 mL
140 liquid cultures were washed in 500 µL of 50 mM EDTA pH 8 and resuspended in 200 µL of 50
141 mM EDTA pH 8 supplemented with zymolyase (3 mg/mL). After 1 hr at 37°C, the cell lysate was
142 mixed with 20 µL of 10% sodium dodecyl sulfate then with 150 µL of 3 M potassium acetate
143 (KAc) and incubated on ice for 1 hr. 100 µL of phenol-chloroform-isoamyl alcohol was added to
144 the SDS-KAc suspension, and, following vortexing and centrifugation, 600 µL of propanol-2 were
145 added to the aqueous supernatant (≈ 300 µL). The nucleic acid pellet was washed three times in
146 70% EtOH, dried and resuspended in PCR grade water supplemented with RNase A (0.3 mg/mL).
147 After 1 hr at 37°C, samples were stored at -20°C.

148

149 **Whole genome sequence data**

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

153

154 **Read preparation**

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

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

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

160

161 **Obtaining whole genome and rDNA coverages**

162 The W303-rDNA reference genome was indexed using bowtie2 (v. 2.3.2):

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

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

165 **Step-1:** Processed reads were mapped to the indexed W303-rDNA genome using bowtie2:

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

168 **Step-2:** The subsequent SAM format alignment was converted to BAM format using SAMtools
169 (v. 1.8):

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

171 **Step-3:** Mapped reads in the BAM file were sorted according to the location they mapped to in
172 W303-rDNA using SAMtools:

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

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

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

178 **command:** grep "rDNA_BLAST" <bedtools_coverage_WG.txt>
179 <rDNA_bedtools_coverage.txt>

180

181 **Calculation of rDNA copy number using modal coverage**

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

188 $$\boldsymbol{rDNA\ copy\ number} = \frac{\boldsymbol{Peak\ rDNA\ coverage\ bin\ value}}{\boldsymbol{Peak\ whole\ genome\ coverage\ bin\ value}}$$

189 rDNA copy number estimates are the mean of all pairwise combinations of these copy number
190 values (**Supplementary Figure 2**).

191

192 **Pipeline availability**

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

196

197 **Calculation of rDNA copy number using mean and median coverage**

198 Per-base read coverage across W303-rDNA from Bedtools was input into custom R-scripts to
199 obtain mean and median coverage values for the whole genome and rDNA after removing the

200 rDNA, 2-micron plasmid, and mitochondrial DNA coverage values from the whole genome
201 calculation. rDNA copy number was then calculated for the mean and median data as follows:

202 $$\boldsymbol{rDNA\ copy\ number = \frac{coverage\ across\ rDNA}{coverage\ across\ whole\ genome}}$$

203

204 **Subsampling**

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

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

209

210 **rDNA copy number measurement by ddPCR**

211 At least three independent cultures (biological replicates) were generated for each isolate using
212 one independent colony per culture. To evaluate rDNA copy number variation over generations,
213 cultures were propagated over four days (~60 generations) as follows: individual colonies were
214 initially grown in 3 mL YPD for 24 hr. 30 µL of this was used to inoculate 3 mL YPD and this
215 was grown for another 24 hr. This process was repeated for four days. Cells were harvested after
216 24 hr (~15 generations) and four days, and cell pellets frozen at -80˚C. gDNA was extracted as
217 above, then linearized by *Xba*I in NEB2 buffer following the manufacturer's instructions (NEB)
218 to individualize rDNA repeats. gDNA linearization was verified by separation on agarose gels and
219 DNA concentration measured on a Qubit Fluorometer using the Qubit dsDNA HS assay (Thermo
220 Fisher). Linearized gDNA was brought to 2 pg/µL by serial dilution. EvaGreen master mixes were

221 prepared with an rDNA primer pair (rDNAScSp_F2 5'- ATCTCTTGGTTCTCGCATCG-3',
222 rDNAScSp_R2 5'-GGAAATGACGCTCAAACAGG-3') or a single copy *RPS3* gene primer pair
223 (RPS3ScSp_F2 5'-CACTCCAACCAAGACCGAAG-3', RPS3ScSp_R2 5'-
224 GACAAACCACGGTCTTGAAC-3'). *RPS3* and rDNA ddPCR reactions were performed with 2
225 µL (4 pg) of the same linearized gDNA dilution as template. Droplet generation and endpoint PCR
226 were performed following the manufacturer's instructions, and droplets were read using a QX200
227 droplet reader (BioRad). Quantification was performed using QuantaSoft Analysis Pro (v.
228 1.0.596). rDNA copy number was determined by the (rDNA copy/µL)/(*RPS3* copy/µL) ratio.

229

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

231 To make chromosome plugs [21], cells from overnight liquid cultures were resuspended in 50 mM
232 EDTA pH 8.0 to 2.10^9 cells/mL, transferred to 45˚C, and mixed with an equal volume of 1.5% low
233 melting point agarose in 50 mM EDTA prewarmed to 45˚C. The mixture was transferred by gentle
234 pipetting to PFGE plug molds (BioRad) to set at 4˚C for 15 min. Plugs were transferred to fresh
235 spheroplasting solution (1 M Sorbitol, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, 14 mM 2-
236 mercaptoethanol, 2 mg/mL zymolyase). After 6 hr incubation at 37˚C with occasional inversion,
237 plugs were washed for 15 min in LDS buffer (1% lithium dodecyl sulphate, 100 mM EDTA pH
238 8.0, 10 mM Tris-HCl pH 8.0), before overnight incubation at 37˚C in the same buffer with gentle
239 shaking. Plugs were incubated twice for 30 min each in NDS buffer (500 mM EDTA, 10 mM Tris-
240 HCl, 1% sarkosyl, pH 9.5) and at least three times for 30 min in TE (10 mM Tris-HCl pH 8.0, 1
241 mM EDTA pH 8.0). Plugs were stored at 4˚C in fresh TE. For restriction digestion, half plugs were
242 pre-washed for two hours in TE, three times for 20 min each in TE, and three times for 20 min
243 each in 300 µL restriction buffer supplemented with 100 µg/mL BSA, all at room temp. Restriction

244 digestion was performed overnight at the recommended temperature in a total volume of 500 µL
245 containing 100 U of restriction endonuclease. Digested plugs were washed in 50 mM EDTA pH
246 8.0 and stored at 4˚C in 50 mM EDTA pH 8.0 before loading. PFGE was performed using 1%
247 agarose gel in 0.5X TBE (Thermo-Fisher) in a CHEF Master XA 170-3670 system (BioRad) with
248 the following parameters: auto algorithm separation range 5 kb - 2 Mb (angle 120˚C, run 6 V/cm,
249 initial switch time 0.22 s, final switch time 3 min 24 s, run time 916 min) at 14˚C. DNA was
250 visualized by staining in ethidium bromide (5 µg/mL) and imaging (Gel Doc XR+; BioRad).

251

252 **1002 Yeast Genome project rDNA copy number estimation**

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

260

261 **Phylogenetic analyses**

262 To create a neighbour-joining phylogeny based on rDNA copy number values, rDNA copy number
263 for each isolate (after removing 30 isolates for which SNP data were not available) was normalized
264 on a 0-1 scale. Normalized values were used to calculate pairwise Euclidean distances between
265 each pair of isolates to generate a distance matrix that was applied to construct a phylogeny via
266 neighbour-joining using MEGA X [60].

267

268 Phylocorrelograms of copy number and SNP phylogeny were generated using phylosignal [61]
269 (v.1.3; https://cran.r-project.org/web/packages/phylosignal/index.html). Phylocorrelograms
270 representing no phylogenetic signal (a "white noise" random distribution) and high phylogenetic
271 signal (a character evolving on the SNP tree according to a Brownian motion model) were also
272 generated. For the white noise distribution, data were simulated from a normal distribution with
273 mean and standard deviation matching those of the observed copy number data (mean=92.5,
274 sd=30.8). For the Brownian motion model, we first estimated the ancestral mean (z0=83.2) and
275 the rate parameter (σ2=72557.2) from the observed copy number data using the fitContinuous
276 function from geiger [62] (https://cran.r-project.org/package=geiger). Then, we simulated from
277 these parameters on the SNP tree using fastBM from phytools 0.7 (https://cran.r-
278 project.org/package=phytools). Phylocorrelograms were generated for the observed and two
279 simulated datasets, estimating correlations at a series of 100 phylogenetic distances using 100
280 bootstrap replicates.

281

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

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

285

286 **Statistical analyses**

287 Statistical analyses were performed in R. Significance was calculated using the Welch *t*-test (*t*-
288 test), the non-parametric Wilcoxon-Mann-Whitney test (wilcox test) or ANOVA, with *p*-values
289 considered statistically significant at $p < 0.05$.

290 **Results and Discussion**

291 **Establishment of a modal coverage bioinformatics pipeline for estimating rDNA copy**
292 **number**

293 The abundance of data generated from NGS platforms has led a number of studies to use mean
294 read depth to estimate rDNA copy number [5, 12, 25, 34, 49, 55, 56]. However, repeat elements,
295 sequence biases and large-scale changes like aneuploidies can potentially result in non-normal
296 read coverage distributions where the mean coverage does not accurately represent the true
297 coverage. To overcome these limitations, we developed a novel sequence read-based rDNA copy
298 number calculation approach based on the most frequent (modal) coverage. The rationale for this
299 approach is that modal coverage will provide an estimate of the relative coverage representation
300 of a given region in a genome that is more robust to biases away from normality than the mean or
301 median. The approach allocates coverage across a reference genome into coverage bins, and the
302 ratio of the most frequently occurring coverage bins for the rDNA and the WG is then used to
303 calculate rDNA copy number (per haploid genome). We implemented this modal coverage
304 approach as a simple pipeline to calculate rDNA copy number from mapped sequence reads (**Fig**
305 **2**). To help smooth across positions that stochastically vary in coverage, an issue that is particularly
306 prevalent with very low coverage datasets, we used a sliding window approach to calculate
307 coverage. Our straightforward pipeline uses a sorted BAM file of reads aligned to a reference
308 genome for which the position of the rDNA is known (either embedded in the genome or as a
309 separate contig) to calculate copy number.

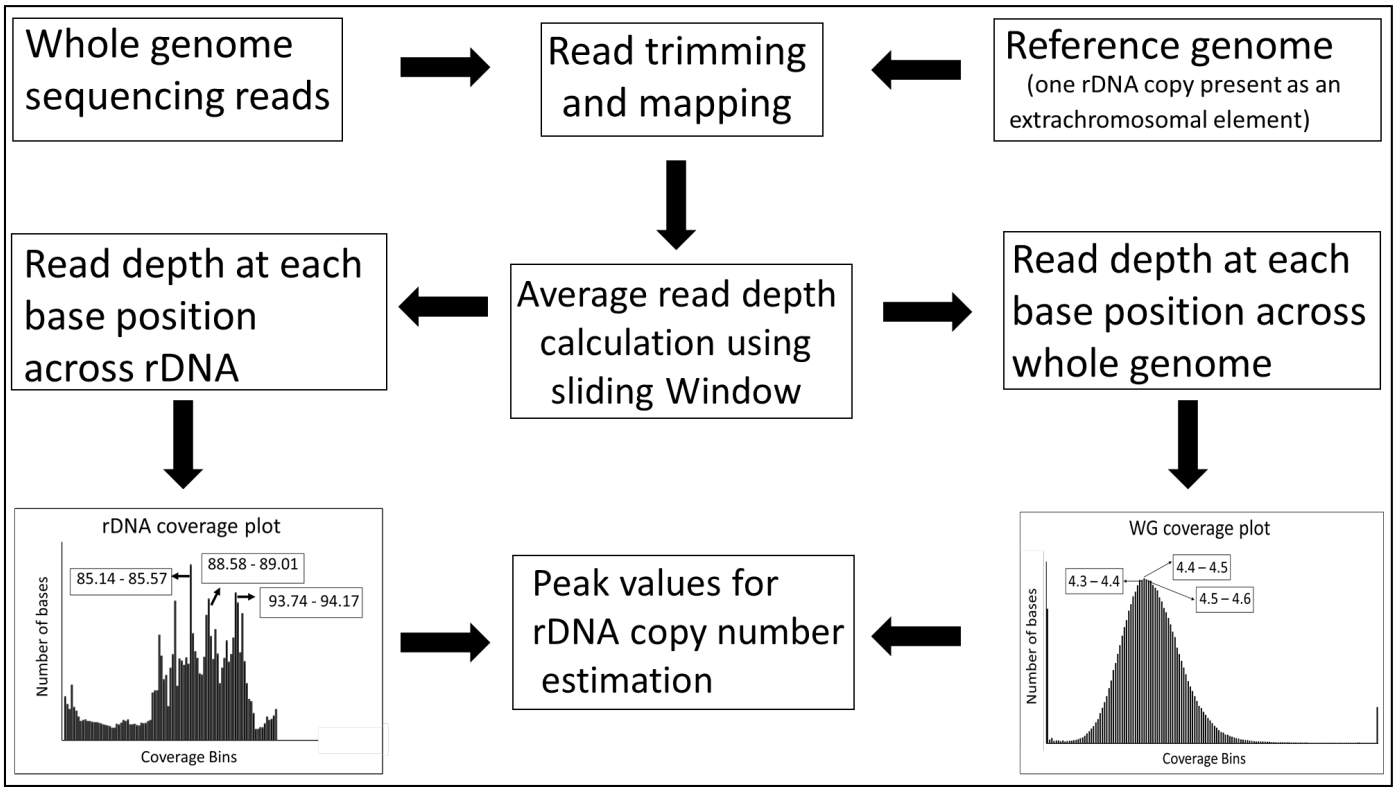


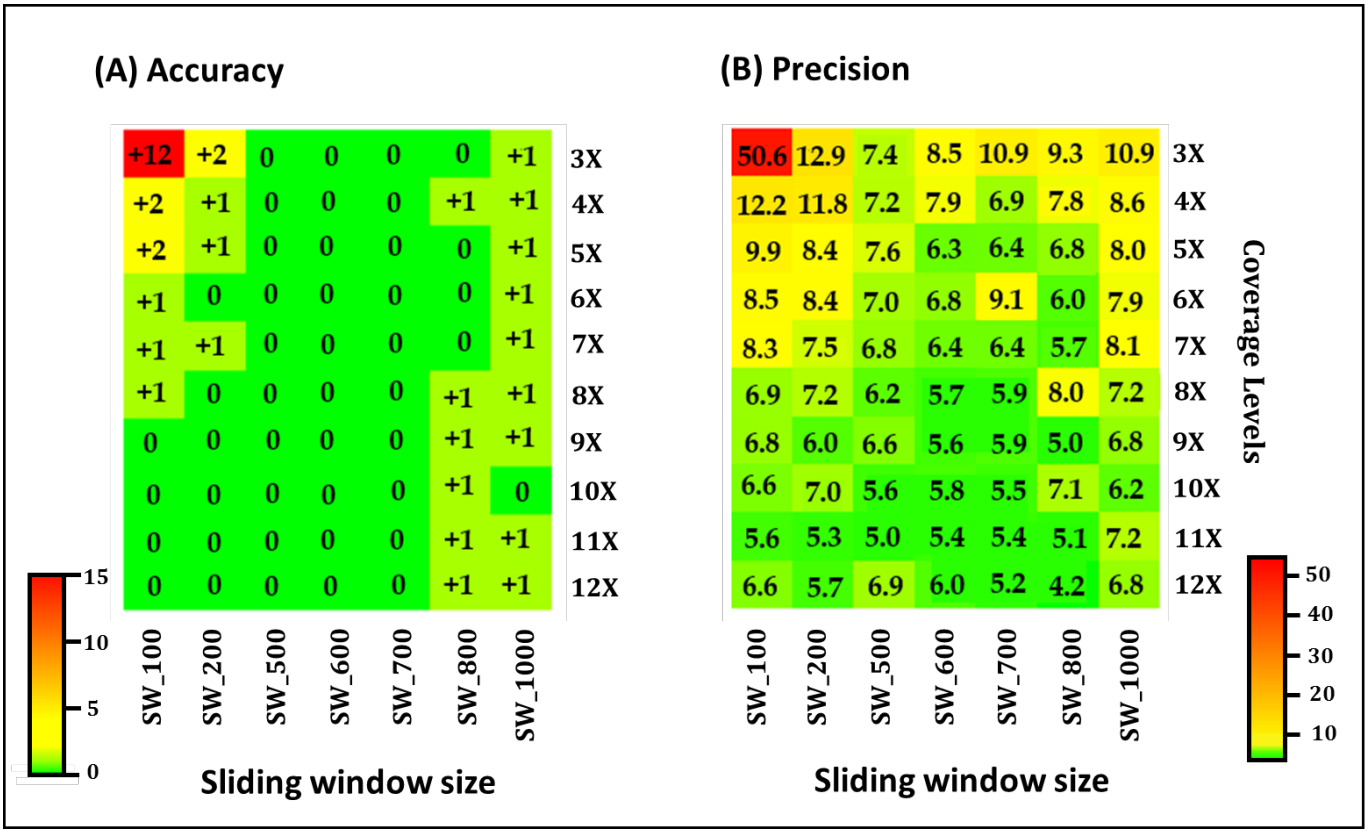
Figure 2. Overview of the modal approach to estimate rDNA copy number from whole genome sequence data. Whole genome (WG) sequence reads are mapped against a reference genome containing a single rDNA copy. Mean read depth for each postion is calculated across the rDNA and the WG using a sliding window, 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-

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

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

351



352

353 **Figure 3. Assessing parameters for rDNA copy number estimation accuracy and**
354 **precision.** Cells represent the (**A**) deviation of the calculated modal rDNA copy number from
355 20, and (**B**) maximum variation of rDNA copy number calculated from the 100 technical
356 replicates for each coverage level and sliding window (SW) size combination. The heatmap
357 scales used are indicated. In (**A**), rDNA copy number was rounded to the nearest integer.

358

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

361 were downsampled to generate 100 technical replicates at 10-fold coverage for each strain, and
362 rDNA copy numbers were calculated using our modal coverage pipeline with a sliding window of
363 600 bp. The resultant rDNA copy numbers were: 32-40 ($\bar{x}$ = 36 copies) for the 40-copy strain; 57-
364 72 ($\bar{x}$ = 64 copies) for the 80-copy strain; 129-177 ($\bar{x}$ = 157 copies) for the WT strain. These values,
365 while similar to the reported copy numbers for these strains, are not identical. Therefore, to check
366 the actual copy numbers of these strains, and to provide a direct validation of our modal pipeline
367 method, we next experimentally determined the rDNA copy numbers of these strains.

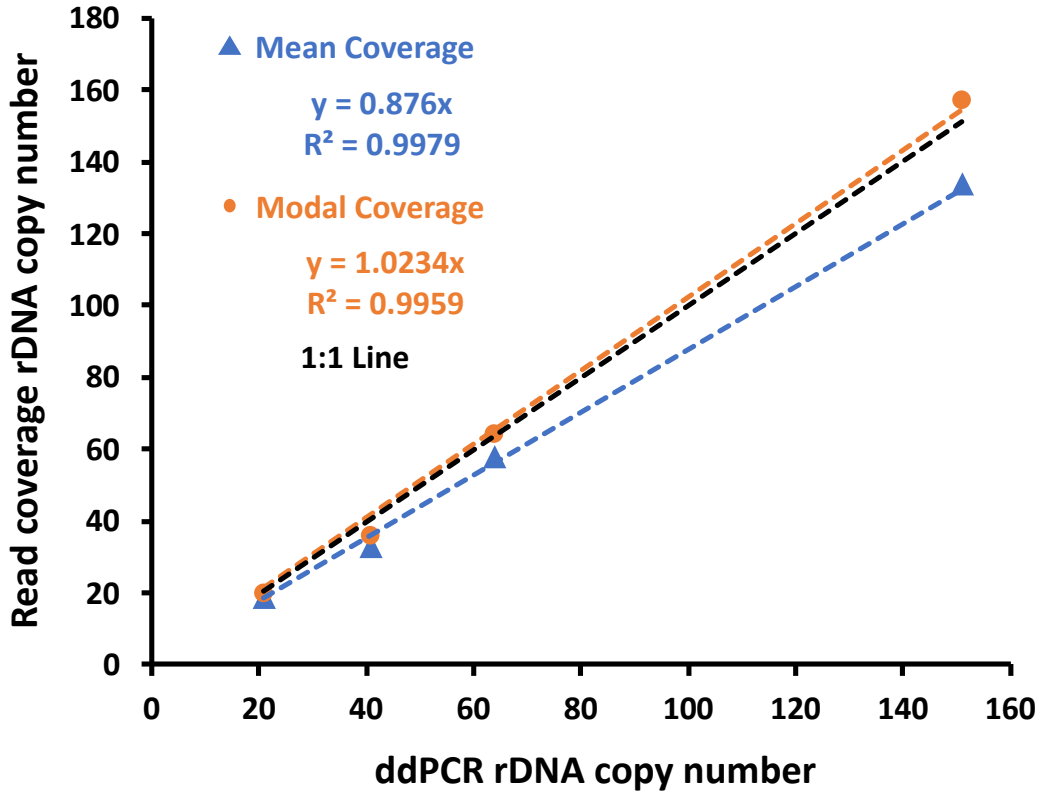
368

369 We chose ddPCR to experimentally determine rDNA copy number because it is less sensitive than
370 qPCR to biases in secondary structure regions that are common in the rDNA coding region [22].
371 The ddPCR data showed that the rDNA copy numbers of our strains are similar to those calculated
372 by our modal coverage method, with both methods suggesting that the "80-copy" strain actually
373 has substantially fewer copies than reported (**Supplementary Table 2**; **Supplementary Figure**
374 **5A**), perhaps due to a stochastic change in copy number that has occurred in our version of this
375 strain. We also compared our modal coverage approach with the mean coverage calculated from
376 the same datasets. We used a simple mean calculation to match the implementation of our modal
377 approach, using the same down-sampled 10-fold WG coverage datasets. The copy number
378 estimates made using the mean coverage approach were uniformly lower than the other estimates
379 (**Supplementary Table 2**), which we suggest is the result of sequencing biases against regions in
380 the rRNA coding region. Importantly, correlating read coverage and ddPCR copy number
381 estimates showed the modal coverage slope was closer to the expected value of 1 than the mean
382 coverage slope (**Fig 4**). We also estimated the copy number using pulsed field gel electrophoresis
383 based on the size of the restriction fragment encompassing the entire rDNA array divided by the
384 rDNA unit size (accounting for the sizes of the flanking regions), again with consistent results

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

386 an accurate way to estimate rDNA copy number.

387



388

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

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

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

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

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

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

395

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

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

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

413

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

415 Studies in model organisms have provided evidence that each species has a homeostatic copy
416 number which is returned to following copy number perturbations [7-10]. This homeostatic copy
417 number appears to have a genetic basis [5, 26], which suggests it might vary between populations,
418 as well as between species. However, few studies have addressed this question. Given that
419 variation in rDNA copy number has been associated with altered phenotypes [8, 12, 17, 22, 27-

420 35], we decided to undertake a comprehensive assessment of *S. cerevisiae* rDNA copy number at
421 the population level using the global wild yeast dataset from the 1002 Yeast Genomes project [64].

422

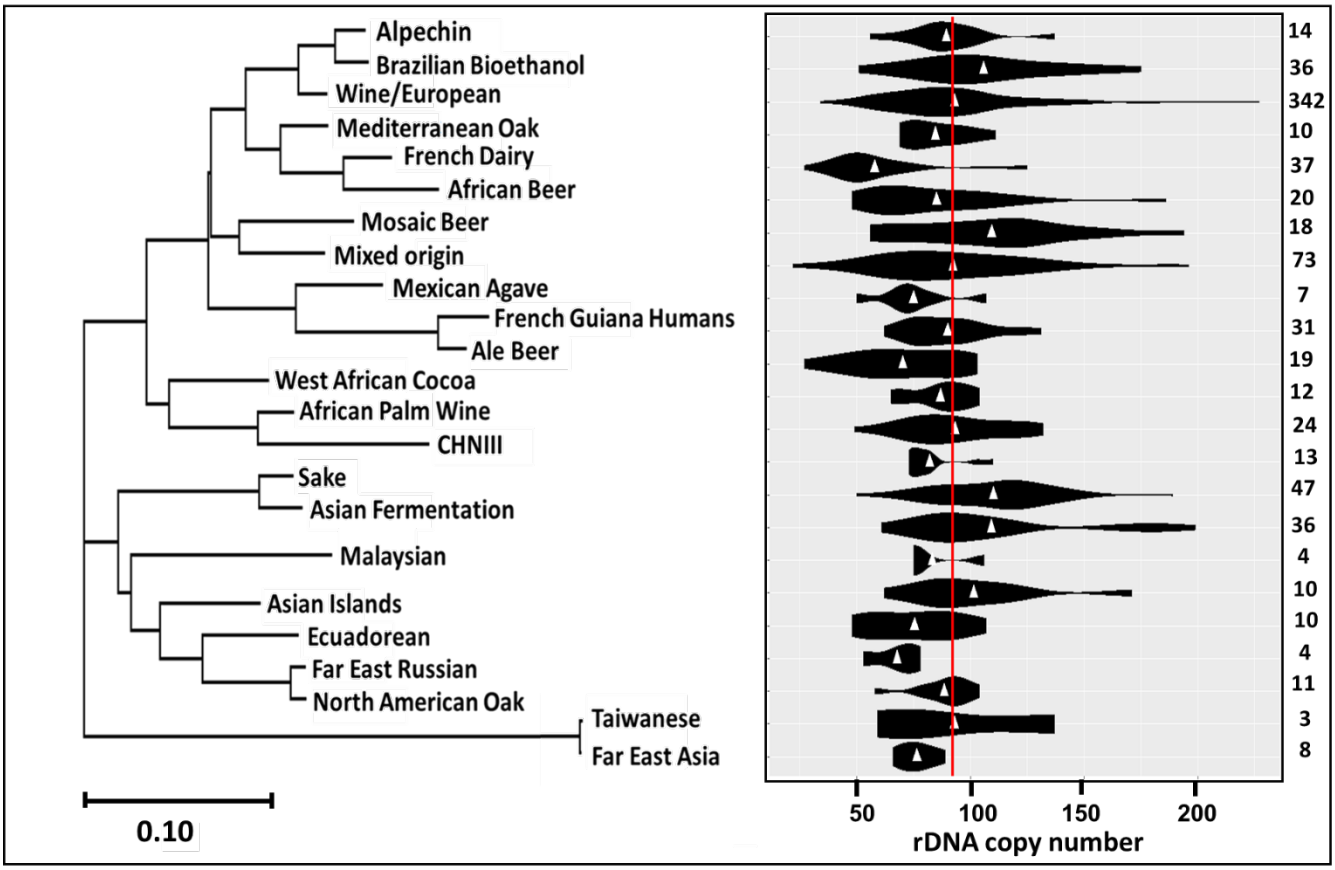
423 We obtained WG sequence data for 788 isolates from the 1002 Yeast Genomes project. Reads for
424 each isolate were downsampled to 10X genome coverage, mapped to our W303-rDNA reference
425 genome, and rDNA copy numbers estimated using our modal coverage pipeline. The rDNA copy
426 numbers ranged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

437

438 The difference in copy number between lab and wild *S. cerevisiae* isolates suggests that *S.*
439 *cerevisiae* populations may harbor different rDNA copy numbers. To test this, we used the 23
440 phylogenetic clades defined by [64] as proxies for *S. cerevisiae* populations and looked at the
441 distributions of rDNA copy number within and between these populations (**Fig 5)**. ANOVA

442 analysis rejects homogeneity of rDNA copy number between these populations (p = 4.37e^{-15}),
443 suggesting there are population-level differences in *S. cerevisiae* copy number.

444



445

446 **Fig 5. rDNA copy number in *S. cerevisiae* populations.** To the left is the phylogeny of the
447 23 *S. cerevisiae* clades from [64] that encompass the 788 isolates included in this study. The
448 scale represents substitutions per site. On the right, rDNA copy number calculated using the
449 modal coverage method is displayed as a violin plot for each clade with mean population
450 copy numbers indicated by white triangles. Numbers to the right represent the number of
451 isolates in each clade. The red vertical line represents the overall mean rDNA copy number
452 (92 copies). Copy number estimations were determined using 10-fold coverage and a 600 bp
453 sliding window.

454

455 We next wanted to look for complementary evidence that *S. cerevisiae* populations have different
456 rDNA copy numbers, as an alternative explanation for our results is different populations happened
457 to have different copy numbers simply due to stochastic variation [21]. If the stochastic variation
458 explanation is correct, we would expect divergent copy numbers to return to a single homeostatic
459 value over time. To test this, we used ddPCR to measure the rDNA copy numbers of six of the
460 1002 Yeast Genomes project isolates that represent the range of copy numbers observed, including
461 one with three different sub-isolates. We grew three biological replicates of each isolate for ~60
462 generations to allow any fluctuation in rDNA copy number to return to the homeostatic level [7].
463 Despite copy number variation between replicates, which is expected given the high level of
464 stochastic copy number variation, the rDNA copy numbers both before and after the ~60
465 generations resemble the copy numbers we estimated from the sequence data and show no
466 tendency to converge on the overall *S. cerevisiae* mean copy number (**Table 2; Supplementary**
467 **Table 5**). These results strongly suggest that our method of estimating rDNA copy number is
468 robust and that the copy numbers of isolates are not recovering towards a common copy number
469 value. From this we conclude that different *S. cerevisiae* populations have different homeostatic
470 rDNA copy numbers.

471

472

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

474 **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		

475

476 [a] Measured using ddPCR

477 [b] Measured using our modal coverage pipeline

478 [c] rep: biological replicate

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

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

482 [f] Not determined

483

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

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

486 similar correlation exists for *S. cerevisiae* populations. To test this, we constructed a neighbour-

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

496

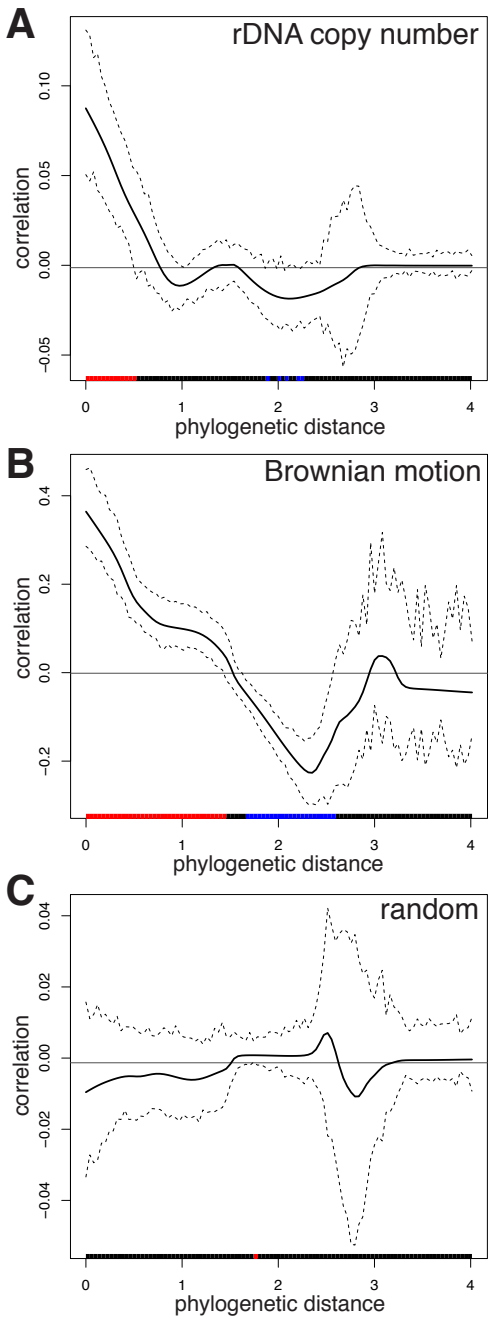


Figure 6. **Phylocorrelograms of autocorrelation based on Moran's *I***. Phylogenetic distance spatial autocorrelations between the SNP-based *S. cerevisiae* phylogeny and the rDNA copy number phylogeny (**A**), a Brownian motion phylogeny (**B**), and random data (**C**) are plotted. Red segments beneath each phylocorrelogram indicate significant positive autocorrelation; black no significant autocorrelation, and blue significant negative autocorrelation. Dotted lines indicate autocorrelation 95% confidence intervals. Significance

504 is based on comparison to zero phylogenetic autocorrelation (horizontal black line at 0). Note
505 the differences in the y-axis scale.

506

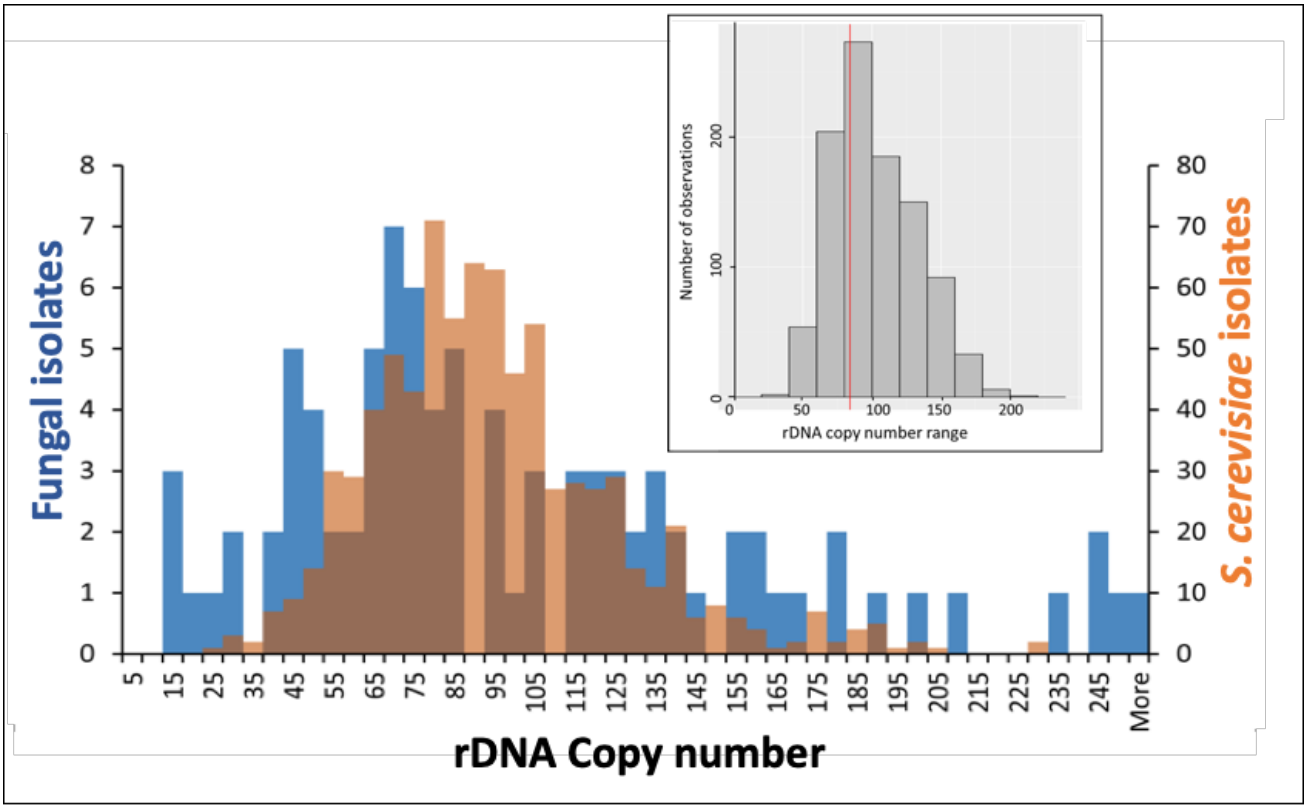
507 Another feature that might explain the distribution of rDNA copy numbers between *S. cerevisiae*
508 populations is the environment, given that nutritional conditions have been proposed to influence
509 copy number [65, 66]. To investigate this, we compared the rDNA copy numbers from two
510 phylogenetically-diverged *S. cerevisiae* populations that are associated with oak trees, which we
511 took as a proxy for similar environments. We found the oak populations did not show significantly
512 different copy numbers (p-value = 0.52), as expected if environment is contributing to copy
513 number. Thus, rDNA copy number might be partially determined by the environmental conditions
514 the population has evolved in. However, we found no consistent pattern of similarities or
515 differences with the copy numbers of the nearest phylogenetic neighbours of these oak clades
516 (**Supplementary Information**), thus these results may simply represent stochastic variation. We
517 suggest that a better understanding of what environmental factors modulate rDNA copy number is
518 necessary before we can properly evaluate the impact of the environment on patterns of rDNA
519 copy number variation.

520

521 Finally, we wondered whether large range in estimated *S. cerevisiae* rDNA copy number (22-227
522 copies) might reflect an unusually large variance in copy number in this species, given this range
523 is almost the same as that reported across 91 different fungal species from three different fungal
524 phyla (11-251 copies, excluding one outlier of 1442 copies; **Fig. 7**) [5]. However, comparing the
525 *S. cerevisiae* copy number range generated by drawing twelve *S. cerevisiae* isolates at random
526 from our data 1,000 times to that previously measured across twelve isolates of one fungal species

527 (*Suillus brevipes*; [5]) shows that the *S. brevipes* range falls in the middle of the *S. cerevisiae*
528 distribution of copy number ranges (**Fig 7**). These results suggest that *S. cerevisiae* rDNA copy
529 number is no more variable than that of *S. brevipes* at least, and illustrate the tremendous inter-
530 individual variation in rDNA copy number that is likely also the case for many other eukaryotic
531 species.

532



533

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

541

542 **Conclusions**

543 Our results demonstrate that modal coverage can be used to robustly determine rDNA copy
544 number from NGS data. Using our novel approach, we demonstrate that the mean rDNA copy
545 number across all wild *S. cerevisiae* populations is 92. This is substantially lower than the copy
546 numbers documented for lab *S. cerevisiae* strains, but overlaps the 'typical' rDNA copy numbers
547 reported for fungi [5]. We show that *S. cerevisiae* populations have different homeostatic rDNA
548 copy numbers, consistent with a previous study using a much smaller sample size [14]. We found
549 some correlation between rDNA copy number and phylogeny, but not enough to suggest that
550 homeostatic copy number is simply drifting apart with increasing phylogenetic distance. We also
551 provide circumstantial evidence that environmental factors might help drive the homeostatic
552 rDNA copy number differences. This is consistent with demonstrations that nutritional factors can
553 induce physiological rDNA copy number changes [65, 66] and that such differences have
554 phenotypic consequences [8, 12, 17, 22, 27-35]. However, it has been shown that rDNA copy
555 number does not correlate with trophic mode in fungi [5] and we cannot exclude stochastic copy
556 number variation explaining our environmental results. Therefore, more work is required to
557 determine what really drives copy number dynamics between populations. One caveat to our
558 conclusions is that while studies from a variety of organisms have demonstrated that copy number
559 recovers from perturbation [7-10], presumably as a result of mechanisms maintaining homeostatic
560 copy number [26], some recent studies in *S. cerevisiae* and *Drosophila* have reported the
561 persistence of stochastic copy number changes without recovery [65, 67]. It will be important to
562 reconcile these conflicting results and to determine to what extent the population-level differences
563 we observe are the result of copy number homeostasis (as we interpret them) versus copy number
564 inertia.

565

566 Our results showing population-level differences in rDNA copy number suggest that such
567 differences can arise relatively quickly in evolutionary time, although the very high level of copy
568 number variation between individuals acts to obscure this pattern. Therefore, it is important to take
569 the large variances and rapid copy number dynamics of the rDNA into account when interpreting
570 the impact of copy number variation on phenotype. Bioinformatics pipelines, such as the one we
571 have developed here, in conjunction with the increasing availability of appropriate NGS datasets
572 provide a way to establish baseline data on rDNA copy number variation between cells,
573 individuals, populations, and species, as well as to investigate the phenotypic consequences of this
574 variation. Finally, while we report population-level differences in rDNA copy number in *S.*
575 *cerevisiae*, diverse human populations have been reported to not differ in rDNA copy number [12,
576 46]. Whether this reflects a difference in biology (such as differences in the level of genetic
577 divergence between populations) or an incomplete understanding of human population rDNA copy
578 number will require further clarification.

579

580 **Acknowledgements**

581 We thank Gianni Liti (IRCAN, CNRS, INSERM, Université Côte d'Azur, University of Nice)
582 for kindly providing strains. We acknowledge the University of Auckland Centre for eResearch
583 (CER) and the New Zealand eScience Infrastructure (NeSI) for providing high performance
584 computing facilities, consulting support, and training services. We thank Auckland Genomics for
585 advice and whole genome sequencing, and Kevin Chang for help with statistical analyses. We
586 thank Alastair Harris for helpful suggestions, and the Ganley Lab for helpful comments and
587 feedback on the manuscript. This work was supported by a grant from the New Zealand Marsden

Fund (14-MAU-053) and a University of Auckland Faculty Research Development Grant (3712288), both to ARDG.

590 **References**

591 1. Long EO, Dawid IB. Repeated genes in eukaryotes. Annu Rev Biochem. 1980;49:727-64.

592 2. McStay B, Grummt I. The epigenetics of rRNA genes: From molecular to chromosome
593 biology. Annu Rev Cell Dev Biol. 2008;24:131-57.

594 3. Prokopowich CD, Gregory TR, Crease TJ. The correlation between rDNA copy number
595 and genome size in eukaryotes. Genome. 2003;46:48-50.

596 4. Torres-Machorro AL, Hernández R, Cevallos AM, López-Villasenor I. Ribosomal RNA
597 genes in eukaryotic microorganisms: witnesses of phylogeny? FEMS Microbiol Rev.
598 2010;34:59–86.

599 5. Lofgren LA, Uehling JK, Branco S, Bruns TD, Martin F, Kennedy PG. Genome-based
600 estimates of fungal rDNA copy number variation across phylogenetic scales and ecological
601 lifestyles. Mol Ecol. 2019;28(4):721-30. doi: 10.1111/mec.14995.

602 6. Iida T, Kobayashi T. How do cells count multi-copy genes?: "Musical Chair" model for
603 preserving the number of rDNA copies. Curr Genet. 2019;65(4):883-5. doi: 10.1007/s00294-
604 019-00956-0.

605 7. Kobayashi T, Heck DJ, Nomura M, Horiuchi T. Expansion and contraction of ribosomal
606 DNA repeats in *Saccharomyces cerevisiae*: requirement of replication fork blocking (Fob1)
607 protein and the role of RNA polymerase I. Genes and Development. 1998;12:3821-30.

608 8. Hawley RS, Marcus CH. Recombinational controls of rDNA redundancy in *Drosophila*.
609 Annu Rev Genet. 1989;23:87-120.

610 9. Russell PJ, Rodland KD. Magnification of rRNA gene number in a *Neurospora crassa*
611 strain with a partial deletion of the nucleolus organizer. Chromosoma. 1986;93:337-40.

612 10. Rodland KD, Russell PJ. Regulation of ribosomal RNA cistron number in a strain of
613 *Neurospora crassa* with a duplication of the nucleolus organizer region. Biochimica et
614 Biophysica Acta. 1982;697:162-9.

615 11. Lyckegaard EM, Clark AG. Ribosomal DNA and Stellate gene copy number variation on
616 the Y chromosome of *Drosophila melanogaster*. PNAS. 1989;86(6):1944-8. doi:
617 10.1073/pnas.86.6.1944.

618 12. Gibbons JG, Branco AT, Yu S, Lemos B. Ribosomal DNA copy number is coupled with
619 gene expression variation and mitochondrial abundance in humans. Nature Communications.
620 2014;5:4850. doi: 10.1038/ncomms5850.

621 13. Cowen LE, Sanglard D, Calabrese D, Sirjusingh C, Anderson JB, Kohn LM. Evolution of
622 drug resistance in experimental populations of *Candida albicans*. J Bacteriol. 2000;182:1515-22.

623 14. West C, James SA, Davey RP, Dicks J, Roberts IN. Ribosomal DNA sequence
624 heterogeneity reflects intraspecies phylogenies and predicts genome structure in two contrasting
625 yeast species. Syst Biol. 2014;63(4):543-54. doi: 10.1093/sysbio/syu019.

626 15. Herrera ML, Vallor AC, Gelfond JA, Patterson TF, Wickes BL. Strain-dependent
627 variation in 18S ribosomal DNA Copy numbers in *Aspergillus fumigatus*. J Clin Microbiol.
628 2009;47(5):1325-32. doi: 10.1128/JCM.02073-08.

629 16. Stults DM, Killen MW, Pierce HH, Pierce AJ. Genomic architecture and inheritance of
630 human ribosomal RNA gene clusters. Genome Res. 2008;18:13-8.

631 17. Ide S, Miyazaki T, Maki H, Kobayashi T. Abundance of ribosomal RNA gene copies
632 maintains genome integrity. Science. 2010;327:693-6.

633 18. French SL, Osheim YN, Cioci F, Nomura M, Beyer AL. In exponentially growing
634 *Saccharomyces cerevisiae* cells, rRNA synthesis is determined by the summed RNA polymerase
635 I loading rate rather than the number of active genes. Mol Cell Biol. 2003;23:1558-68.

636 19. Kobayashi T, Ganley ARD. Recombination regulation by transcription-induced cohesin
637 dissociation in rDNA repeats. Science. 2005;309:1581-4.

638 20. Szostak JW, Wu R. Unequal crossing over in the ribosomal DNA of *Saccharomyces*
639 *cerevisiae*. Nature. 1980;284:426-30.

640 21. Ganley ARD, Kobayashi T. Monitoring the rate and dynamics of concerted evolution in
641 the ribosomal DNA repeats of *Saccharomyces cerevisiae* using experimental evolution. Mol Biol
642 Evol. 2011;28:2883-91.

643 22. Salim D, Gerton JL. Ribosomal DNA instability and genome adaptability. Chromosome
644 Research. 2019;27(1-2):73-87. doi: 10.1007/s10577-018-9599-7.

645 23. Ganley ARD, Kobayashi T. Highly efficient concerted evolution in the ribosomal DNA
646 repeats: total rDNA repeat variation revealed by whole-genome shotgun sequence data. Genome
647 Res. 2007;17:184-91.

648 24. Eickbush TH, Eickbush DG. Finely orchestrated movements: evolution of the ribosomal
649 RNA genes. Genetics. 2007;175:477-85.

650 25. James SA, O'Kelly MJT, Carter DM, Davey RP, van Oudenaarden A, Roberts IN.
651 Repetitive sequence variation and dynamics in the ribosomal DNA array of *Saccharomyces*
652 *cerevisiae* as revealed by whole-genome resequencing. Genome Res. 2009;19:626-35.

653 26. Iida T, Kobayashi T. RNA polymerase I activators count and adjust ribosomal RNA gene
654 copy number. Mol Cell. 2019;73(4):645-54. doi: 10.1016/j.molcel.2018.11.029.

655 27. Delany ME, Muscarella DE, Bloom SE. Effects of rRNA gene copy number and
656 nucleolar variation on early development: inhibition of gastrulation in rDNA-deficient chick
657 embryos. J Hered. 1994;85(3):211-7. doi: 10.1093/oxfordjournals.jhered.a111437.

658 28. Kobayashi T. Regulation of ribosomal RNA gene copy number and its role in modulating
659 genome integrity and evolutionary adaptibility in yeast. Cell Mol Life Sci. 2011;68:1395-403.

660 29. Paredes S, Maggert KA. Ribosomal DNA contributes to global chromatin regulation.
661 PNAS. 2009;106:17829-34.

662 30. Paredes S, Branco AT, Hartl DL, Maggert KA, Lemos B. Ribosomal DNA deletions
663 modulate genome-wide gene expression: "rDNA-sensitive" genes and natural variation. PLoS
664 Genet. 2011;7:e1001376.

665 31. Michel AH, Kornmann B, Dubrana K, Shore D. Spontaneous rDNA copy number
666 variation modulates Sir2 levels and epigenetic gene silencing. Genes and Development.
667 2005;19:1199-210.

668 32. Bughio F, Maggert KA. The peculiar genetics of the ribosomal DNA blurs the boundaries
669 of transgenerational epigenetic inheritance. Chromosome Research. 2019;27(1-2):19-30. doi:
670 10.1007/s10577-018-9591-2.

671 33. Cullis CA. Quantitative variation of ribosomal RNA genes in flax genotrophs. Heredity.
672 1979;42:237-46.

673 34. Xu B, Li H, Perry JM, Singh VP, Unruh J, Yu Z, et al. Ribosomal DNA copy number loss
674 and sequence variation in cancer. PLoS Genet. 2017;13(6):e1006771. doi:
675 10.1371/journal.pgen.1006771.

676 35. Zhou J, Sackton TB, Martinsen L, Lemos B, Eickbush TH, Hartl DL. Y chromosome
677 mediates ribosomal DNA silencing and modulates the chromatin state in *Drosophila*. PNAS.
678 2012;109(25):9941-6. doi: 10.1073/pnas.1207367109.

679 36. Ritossa FM, Spiegelman S. Localization of DNA complementary to ribosomal RNA in
680 the nucleolus organizer region of *Drosophila melanogaster*. PNAS. 1965;53:737-45. doi:
681 10.1073/pnas.53.4.737.

682 37. Wallace H, Birnstiel ML. Ribosomal cistrons and the nucleolar organizer. Biochimica et
683 Biophysica Acta. 1966;114(2):296-310. doi: 10.1016/0005-2787(66)90311-x.

684 38. Schweizer E, MacKechnie C, Halvorson HO. The redundancy of ribosomal and transfer
685 RNA genes in *Saccharomyces cerevisiae*. J Mol Biol. 1969;40:261-77.

686 39. Matsuda K, Siegel A. Hybridization of plant ribosomal RNA to DNA: the isolation of a
687 DNA component rich in ribosomal RNA cistrons. PNAS. 1967;58(2):673-80. doi:
688 10.1073/pnas.58.2.673.

689 40. Maleszka R, Clark-Walker GD. Yeasts have a four-fold variation in ribosomal DNA copy
690 number. Yeast. 1993;9:53-8.

691 41. Saka K, Takahashi A, Sasaki M, Kobayashi T. More than 10% of yeast genes are related
692 to genome stability and influence cellular senescence via rDNA maintenance. Nucleic Acids
693 Res. 2016;44(9):4211-21. doi: 10.1093/nar/gkw110.

694 42. Paredes S, Maggert KA. Expression of *I-Cre*I endonuclease generates deletions within the
695 rDNA of Drosophila. Genetics. 2009;181:1661-71.

696 43. Chestkov IV, Jestkova EM, Ershova ES, Golimbet VE, Lezheiko TV, Kolesina NY, et al.
697 Abundance of ribosomal RNA gene copies in the genomes of schizophrenia patients.
698 Schizophrenia Research. 2018;197:305-14. doi: 10.1016/j.schres.2018.01.001.

699 44. LeRiche K, Eagle SH, Crease TJ. Copy number of the transposon, *Pokey*, in rDNA is
700 positively correlated with rDNA copy number in *Daphnia obtuse*. PLoS One.
701 2014;9(12):e114773. doi: 10.1371/journal.pone.0114773.

702 45. Son J, Hannan KM, Poortinga G, Hein N, Cameron DP, Ganley ARD, et al. rDNA
703 chromatin activity status as a biomarker of sensitivity to the RNA polymerase I transcription
704 inhibitor CX-5461. Frontiers in Cell and Developmental Biology. 2020;8:568.

705 46. Valori V, Tus K, Laukaitis C, Harris DT, LeBeau L, Maggert KA. Human rDNA copy
706 number is unstable in metastatic breast cancers. Epigenetics. 2020;15(1-2):85-106. doi:
707 10.1080/15592294.2019.1649930.

708 47. Alanio A, Sturny-Leclere A, Benabou M, Guigue N, Bretagne S. Variation in copy
709 number of the 28S rDNA of *Aspergillus fumigatus* measured by droplet digital PCR and analog
710 quantitative real-time PCR. J Microbiol Methods. 2016;127:160-3. doi:
711 10.1016/j.mimet.2016.06.015.

712 48. Salim D, Bradford WD, Freeland A, Cady G, Wang J, Pruitt SC, et al. DNA replication
713 stress restricts ribosomal DNA copy number. PLoS Genet. 2017;13(9):e1007006. doi:
714 10.1371/journal.pgen.1007006.

715 49. Rosato M, Kovarik A, Garilleti R, Rossello JA. Conserved organisation of 45S rDNA
716 sites and rDNA gene copy number among major clades of early land plants. PLoS One.
717 2016;11(9):e0162544. doi: 10.1371/journal.pone.0162544.

718 50. Xu J, Xu Y, Yonezawa T, Li L, Hasegawa M, Lu F, et al. Polymorphism and evolution of
719 ribosomal DNA in tea (*Camellia sinensis*, Theaceae). Mol Phylogen Evol. 2015;89:63-72. doi:
720 10.1016/j.ympev.2015.03.020.

721 51. Xu J, Zhang Q, Xu X, Wang Z, Qi J. Intragenomic variability and pseudogenes of
722 ribosomal DNA in stone flounder *Kareius bicoloratus*. Mol Phylogen Evol. 2009;52(1):157-66.
723 doi: 10.1016/j.ympev.2009.03.031.

724 52. Agrawal S, Ganley ARD. Complete sequence construction of the highly repetitive
725 ribosomal RNA gene repeats in eukaryotes using whole genome sequence data. Methods in
726 Molecular Biology. 2016;1455:161-81. doi: 10.1007/978-1-4939-3792-9_13

727 53. Buckler ES, Ippolito A, Holtsford TP. The evolution of ribosomal DNA: Divergent
728 paralogues and phylogenetic implications. Genetics. 1997;145:821-32.

729 54. Mayol M, Rosselló JA. Why nuclear ribosomal DNA spacers (ITS) tell different stories in
730 *Quercus*. Mol Phylogen Evol. 2001;19:167-76.

731 55. Wang M, Lemos B. Ribosomal DNA copy number amplification and loss in human
732 cancers is linked to tumor genetic context, nucleolus activity, and proliferation. PLoS Genet.
733 2017;13(9):e1006994. doi: 10.1371/journal.pgen.1006994.

734 56. Gong W, Marchetti A. Estimation of 18S gene copy number in marine eukaryotic
735 plankton using a next-generation sequencing approach. Frontiers in Marine Science. 2019;6:219.

736 57. Cubillos FA, Louis EJ, Liti G. Generation of a large set of genetically tractable haploid
737 and diploid *Saccharomyces* strains. FEMS Yeast Res. 2009;9(8):1217-25. doi: 10.1111/j.1567-
738 1364.2009.00583.x.

739 58. Liti G, Carter DM, Moses AM, Warringer J, Parts L, James SA, et al. Population
740 genomics of domestic and wild yeasts. Nature. 2009;458(7236):337-41. doi:
741 10.1038/nature07743.

742 59. Cox MP, Peterson DA, Biggs PJ. SolexaQA: At-a-glance quality assessment of Illumina
743 second-generation sequencing data. BMC Bioinformatics. 2010;11:485. doi: 10.1186/1471-
744 2105-11-485.

745 60. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary
746 genetics analysis across computing platforms. Mol Biol Evol. 2018;35(6):1547-9. doi:
747 10.1093/molbev/msy096.

748 61. Keck F, Rimet F, Bouchez A, Franc A. phylosignal: an R package to measure, test, and
749 explore the phylogenetic signal. Ecology and Evolution. 2016;6(9):2774-80. doi:
750 10.1002/ece3.2051.

751 62. Pennell MW, Eastman JM, Slater GJ, Brown JW, Uyeda JC, FitzJohn RG, et al. geiger
752 v2.0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees.
753 Bioinformatics. 2014;30(15):2216-8. doi: 10.1093/bioinformatics/btu181.

754 63. Yoon S, Xuan Z, Makarov V, Ye K, Sebat J. Sensitive and accurate detection of copy
755 number variants using read depth of coverage. Genome Res. 2009;19(9):1586-92. doi:
756 10.1101/gr.092981.109.

757 64. Peter J, De Chiara M, Friedrich A, Yue JX, Pflieger D, Bergstrom A, et al. Genome
758 evolution across 1,011 *Saccharomyces cerevisiae* isolates. Nature. 2018;556(7701):339-44. Epub
759 2018/04/13. doi: 10.1038/s41586-018-0030-5.

760 65. Aldrich JC, Maggert KA. Transgenerational inheritance of diet-induced genome
761 rearrangements in Drosophila. PLoS Genet. 2015;11(4):e1005148. Epub 2015/04/18. doi:
762 10.1371/journal.pgen.1005148.

763 66. Jack CV, Cruz C, Hull RM, Keller MA, Ralser M, Houseley J. Regulation of ribosomal
764 DNA amplification by the TOR pathway. PNAS. 2015;112(31):9674-9. Epub 2015/07/22. doi:
765 10.1073/pnas.1505015112.

766 67. Mansisidor A, Molinar T, Srivastava P, Dartis DD, Pino Delgado A, Blitzblau HG, et al.
767 Genomic copy-number loss is rescued by self-limiting production of DNA circles. Mol Cell.
768 2018;72(3):583-93. Epub 2018/10/09. doi: 10.1016/j.molcel.2018.08.036.

769