Title: GIP: An open-source computational pipeline for mapping genomic instability from protists to cancer cells

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Abstract:

Genome instability has been recognized as a key driver for microbial and cancer adaptation and thus plays a central role in many human pathologies. Even though genome instability encompasses different types of genomic alterations, most available genome analysis software are limited to just one kind mutation or analytical step. To overcome this limitation and better understand the role of genetic changes in enhancing pathogenicity we established GIP, a novel, powerful bioinformatic pipeline for comparative genome analysis. Here we show its application to whole genome sequencing datasets of *Leishmania*, *Plasmodium, Candida*, and cancer. Applying GIP on available data sets validated our pipeline and demonstrated the power of our analysis tool to drive biological discovery. Applied to *Plasmodium vivax* genomes, our pipeline allowed us to uncover the convergent amplification of erythrocyte binding proteins and to identify a nullisomic strain. Re-analyzing genomes of drug adapted *Candida albicans* strains revealed correlated copy number variations of functionally related genes, strongly supporting a

mechanism of epistatic adaptation through interacting gene-dosage changes. Our results illustrate how GIP can be used for the identification of aneuploidy, gene copy number variations, changes in nucleic acid sequences, and chromosomal rearrangements. Altogether, GIP can shed light on the genetic bases of cell adaptation and drive disease biomarker discovery.

One Sentence Summary:

GIP - a novel pipeline for detecting, comparing and visualizing genome instability.

Keywords:

Genome instability, aneuploidy, copy number variations, single nucleotide variation, structural variation, genetic adaptation.

Main Text:

In recent years, the field of genomics has rapidly expanded with a fast increase in the number of newly sequenced genomes (1). This surge is a direct consequence of the development of new and ever more efficient, high-throughput capable sequencing technologies (2). On the one hand, the improvement in long reads technology allowed the generation of high-quality genome assemblies (3,4). On the other hand, the decreasing costs for short-reads sequencing and the parallel increase in sequencing throughput propelled the exponential increase of available whole genome sequencing (WGS) data (5). Thanks to these advances one can reasonably expect WGS to rapidly become a key component of personalized medicine and clinical applications. In this context several consortia-based projects have been established with the goal to produce WGS for the study of different biological systems (5), and a number of publicly available databases have been compiled or updated (6). Parallel to data availability, many bioinformatics

49 tools have been developed to perform specific genome analysis tasks (7,8). For instance, tools
50 such as Freebayes (9), CNVnator (10) and DELLY (11) have been respectively used for the
51 detection or characterization of DNA single nucleotide variants (SNVs), copy number
52 variations (CNVs), and structural variations (SVs), but their scope is limited to the analysis of
53 one genomic feature at the time. A number of integrative WGS pipelines and workflows have
54 been established combining the execution of multiple bioinformatics algorithms serving
55 different analysis steps (12). Even though continuous progress has been made, there is no
56 standardized or unified approach for genomic investigation. For the development of improved
57 WGS data analysis pipelines, several important requirements need be considered, including
58 portability, reproducibility, scalability and compatibility with high-performance computing
59 (HPC) clusters and remote cloud computing. Here we introduce a novel genome instability
60 pipeline (GIP) that fulfills all these requirements. GIP facilitates the genome-wide detection,
61 quantification, comparison and visualization of chromosome aneuploidies, gene CNVs, SNVs
62 and SVs. GIP is implemented in Nextflow (13), a workflow language that allows to execute
63 GIP seamlessly in local workstation, on an HPC or remotely in the cloud. All required
64 environment and software dependencies of GIP are fulfilled and provided with a Singularity
65 container, thus making GIP reproducible, easy-to-install and easy-to-use. GIP allows the use of
66 giptools, a tool-suit of R-based modules for genome data exploration, enabling the comparison
67 of sample sub-sets. GIP and giptools generate a summary report with publication-quality figures
68 and spreadsheet tables. GIP and giptools constitute a single framework for WGS analysis
69 suitable both for large scale batch analysis of individual genomes and comparison of samples
70 from different experimental conditions or origins. Lastly, a key strength of GIP and giptools is
71 the general applicability to different eukaryotic species. We already successfully applied GIP
72 on the analysis of *Leishmania* genomes (14-16). In this study, we validate the use of GIP and
73 giptools using WGS data from published datasets of the three major human pathogens

74 *Leishmania infantum*, *Plasmodium vivax* and *Candida albicans* and as well as three human
75 cancer cell lines. Furthermore, we demonstrate how the extensive and powerful analytical
76 approach operated by GIP and giptools can be used to find new biological signal that escaped
77 previous analyses.
78

79 ## Results:

80 **The GIP workflow.** GIP is a tool for scientific investigation compatible with Linux systems,
81 requiring minimal configuration and distributed as a self-contained package. GIP consists of
82 three files: the Nextflow pipeline code, the configuration and the Singularity container files.
83 The minimum required input is a paired-end WGS data set and a reference genome assembly
84 in the standard fastq and FASTA format, respectively. GIP analyses include (i) extracting
85 genomic features such as assembly gaps or repetitive elements, (ii) mapping the reads, (iii)
86 evaluating chromosome, gene and genomic bin copy numbers, (iv) identifying and visualizing
87 copy number variation with respect to the reference genome, (v) identifying and quantifying
88 gene clusters, (vi) detecting and annotating SNVs, (vii) measuring non-synonymous (N) and
89 synonymous (S) mutations for all genes, (viii) detecting SVs including tandem duplications,
90 deletions, inversions and break-ends translocations using split-read and read-pair orientation
91 information, and (ix) producing a report file providing summary statistics, tables and
92 visualizations (**Fig. 1**). GIP allows to customize filtering and visualization options via the
93 configuration file (see methods). The output of GIP can be used as input for giptools, a tool-
94 suite to compare sample sub-sets and highlight chromosome copy number, gene copy number
95 and SNV differences.
96

97 **Applying giptools on a *Leishmania infantum* case study.** GIP permits the batch analysis of a
98 set of individual samples, where each sample is considered separately and compared only with
99 respect to the provided reference genome assembly. As a consequence, all variants and copy
100 number alterations detected in a sample merely reflect the differences between the sequenced
101 and the reference genomes. While this application may be sufficient in some circumstances,
102 research projects often involve downstream comparison between samples. Examples include
103 the comparison of gene or chromosome copy variation number between for example drug
104 resistant and drug susceptible samples, or the juxtaposition of SNVs detected in isolates from
105 different geographic areas. For this purpose, we developed giptools, a suit of thirteen modules
106 that allows to compare samples processed by GIP (**Table 1**). All modules in giptools are fully
107 embedded in the Singularity container and they are provided with their own documentation.

108 **Table 1: giptools modules**

Module name	Purpose
karyotype	Compare the chromosome sequencing coverage distributions
binCNV	Compare bin sequencing coverage in 2 samples
geCNV	Compare gene sequencing coverage in 2 samples
ternary	Compare gene sequencing coverage in 3 samples
ternaryBin	Compare bin sequencing coverage in 3 samples
SNV	Compare SNVs in multiple samples
binDensity	Density plot of bin sequencing coverage of many samples
geInteraction	Detect CNV genes in many samples and produce correlation-based networks
genomeDistance	Compare samples genomic distance
phylogeny	Extract the SNVs union and infer the phylogenetic tree
convergentCNV	Detect convergent CNV gene amplifications
overview	Overview of the sequencing coverage of chromosomes, genomic bins and genes
panel	Extract genomic information of a gene panel

109

110 To illustrate the type of exploratory data analyses and the biological questions that can be
111 addressed, we tested giptools on a previously analyzed dataset of seven clinical *Leishmania*
112 *infantum* isolates from Tunisia (14). *Leishmania* is the etiological agent of leishmaniasis, a life-
113 threatening human and veterinary disease affecting 12 million people worldwide (17). The

114 parasites were derived from patients affected with visceral leishmaniasis, expanded in cell
115 culture and their genomes were sequenced. The dataset includes four Glucantime drug
116 susceptible isolates and three isolates from relapsed patients, and thus comparison may inform
117 on genetic factors resulting in treatment failure. giptools allowed the detection and visualization
118 of pervasive intra-chromosomal CNVs across the thirty-six *Leishmania* chromosomes (**Fig.**
119 **2A**). Additionally, giptools enables targeted comparison of normalized genomic bin sequencing
120 coverage of sample pairs. We used giptools' `binCNV` module to compute the ratio between
121 corresponding genomic bins of the strains LIPA83 over ZK43, which correspond to a first-
122 episode and a relapse leishmaniasis isolate, respectively. giptools represents different
123 chromosomes as separate panels (**Fig. 2B**), as part of single genome-wide overview (**Fig. S1A**)
124 or as distinct plots (**Fig. S1B**). This analysis allowed the identification of 2,905 and 2,208 bins
125 that were respectively amplified or depleted in LIPA83 with respect to ZK43. The results are
126 returned by giptools as a Microsoft Excel table (.xlsx format) providing ratio scores at each
127 genomic position (**Table S1**). Likewise, giptools permits three-way comparisons of normalized
128 genomic bin sequencing coverage with ternary plots (**Fig. 2C**). We used this representation to
129 display the genomic bin relative abundance in samples ZK43, LIPA83 and ZK28. The analysis
130 shows important strain-specific differences in bin copy number that are visualized by shifts of
131 the signals out of the center. Similar to genomic bin analysis, giptools makes it possible to
132 compare the sequencing depth of annotated genes and thus the copy number in two or three
133 samples (**Fig. 2D** and **Fig. S2**). However, the determination of gene copy number might be
134 impeded by (i) short read length or fragment insert size, (ii) the complexity of the target genome,
135 or (iii) the presence of repetitive elements. The read map quality (MAPQ) score is a measure
136 that reflects how much each gene is supported by unambiguously mapped reads (high MAPQ)
137 in contrast to multimapping reads (low MAPQ). Together with the coverage, GIP also computes
138 the mean read MAPQ score for each gene and allows a different strategy to determine the copy

139 number of low MAPQ genes (see methods). The evaluation of LIPA83 and ZK43 gene
140 coverage ratio scores revealed 13 gene CNVs with a stringent MAPQ cutoff of 50 (**Table S2**).
141 The maximum normalized coverage value of 6.5 was observed for a putative amastin surface
142 glycoprotein (LINF_310009800). Other examples of gene CNVs in this set include the putative
143 surface antigen protein 2 (LINF_120013500) and the heat shock protein HSP33
144 (LINF_300021600) (**Table S2**). Genes falling below a user defined MAPQ score and sharing
145 high level of sequence similarity are assigned to the same gene cluster, and their measured
146 coverage scores are averaged across all members of the group. Low MAPQ scores can also be
147 associated with single genes, e.g. in the case of internal repetitive elements that cause multiple
148 ambiguous alignments inside the gene itself, or if mapping occurs in possibly misannotated
149 intergenic regions. The LIPA83/ZK43 comparison showed 27 CNV gene clusters, including
150 cluster clstr303 (3 genes annotated as 'amastin-like') and cluster clstr16 (2 tb-292 membrane-
151 associated protein-like proteins) (**Table S2**). These results demonstrate the power of GIP and
152 giptools to detect and compare intra-chromosomal CNVs in *Leishmania* at genomic bin level.
153 Conveniently, analogous two- or three-ways comparisons can be applied to reveal copy number
154 variations at individual gene or gene cluster levels.

155

156 **Comparative genomics of a *Plasmodium vivax* WGS dataset.** We next applied GIP and
157 giptools on other biological systems to demonstrate its broad applicability outside the
158 *Leishmania* field, including the human apicomplexan parasite *Plasmodium vivax*. *Plasmodium*
159 *vivax* is a protist parasite and a human pathogen causing malaria. *Plasmodium vivax* gives rise
160 every year to 130 million clinical cases (18), and it is estimated that 2.5 billion people are at
161 risk of infection worldwide (19-21). We applied GIP and giptools to investigate genomic
162 variations across a sizeable dataset of 222 *Plasmodium vivax* genomes isolated from clinical
163 samples of 14 countries worldwide (22,23) (**Table S3**). The phylogenetic tree reconstruction

164 and PCA analyses (**Fig. 3A** and **B**) showed a high correlation between genotypes and the
165 geographic origin of the samples. However, we detected substantial genomic variability
166 between isolates collected at smaller geographical scale, with 14,555 SNVs (~42% of the total)
167 uniquely characterizing representative samples from five Ethiopian study sites (23) (**Fig. 3C**
168 and **D**). This result may reflect diverging evolutionary trajectories radiating from few founder
169 strains. At gene level we profiled the copy number variations of two gene panels. The first panel
170 accounts for 43 previously described genes encoding for potential erythrocyte binding proteins
171 suggested to operate at the interface of the parasite-host invasion process (23). The second panel
172 includes two drug resistance markers comprising the chloroquine resistance transporter
173 PVP01_0109300 and the multidrug resistance protein 1 PVP01_1010900, and four proteins
174 implicated in red blood cell invasion, such as the merozoite surface protein gene MSP7
175 PVP01_1219700, the reticulocyte binding protein gene 2c PVP01_0534300, the serine-repeat
176 antigen 3 PVP01_0417000, and the reticulocyte binding protein 2b PVP01_0800700) (24-33).
177 Read depth analysis indicated that four genes in the panel (PVP01_0623800, PVP01_1031400,
178 PVP01_1031200, PVP01_1031300) show a high degree of variability, with amplifications
179 observed in samples from distinct geographic (**Fig. 3E**). This convergence is sign of strong
180 natural selection, which further sustains the functional importance of these genes in the
181 infection process. Furthermore, 6 genes positioned on chromosome 14 are absent in the Thai
182 strain PD0689_C as a result of the loss of this chromosome (nullisomy) (**Fig. 3E** and **F**). Finally,
183 the comparison of synonymous and non-synonymous SNVs in the panel of genes revealed
184 important differences between sample groups. Our analysis indicates an overall higher number
185 of non-synonymous mutations in Ethiopian compared to Cambodian isolates, therefore
186 suggesting a stronger evolutionary pressure acting on the African strains (**Fig. 3G**). Taken
187 together these analyses well illustrate how GIP and giptools can be readily applied for bulk

188 analysis of *Plasmodium vivax* genomes to assess genome diversity, extract evolutionary
189 information and identify potential disease biomarkers.

190

191 **Gene CNV analysis of *Candida albicans* evolutionary adapted strains.** We next applied GIP
192 and giptools to the human fungal pathogen *Candida albicans*, an opportunistic yeast exhibiting
193 major genome plasticity (34-43) and causing hundreds of thousands of severe infections each
194 year (44). Candidemia, a bloodstream infection with *Candida*, are often associated with high
195 rates of morbidity and mortality (15–50%) notwithstanding existing antifungal treatments
196 (45,46). We applied GIP and giptools to a *Candida albicans* WGS dataset described in a recent
197 study that covers five different progenitor strains (P75063, P75016, P78042, SC5314,
198 AMS3050) and investigates CNVs driving tolerance and resistance to anti-fungal azole drugs
199 (47). We analyzed nineteen samples, including (i) four clinical isolates (P75063, P75016,
200 P78042, SC5314), (ii) seven strains selected *in vitro* against the anti-fungal drug fluconazole
201 (FLC) (AMS4104, AMS4105, AMS4106, AMS4107, AMS4397, AMS4444, AMS4702), (iii)
202 four isogenic colonies adapted to the drug miconazole (AMS3051, AMS3052, AMS3053 and
203 AMS3054) together with their progenitor (AMS3050), and (iv) three colonies derived from a
204 miconazole-adapted population and isolated on a rich medium (AMS3092, AMS3093 and
205 AMS3094) (47-49). GIP and giptools were able to reproduce previous observations of the
206 amplification of the genes for the drug efflux pumps TAC1 (orf19.3188) and ERG11
207 (orf19.922), for the stress response proteins HSP70 (orf19.4980), CGR1 (orf19.2722), ERO1
208 (orf19.4871), TPK1 (orf19.4892), ASR1 (orf19.2344), PBS2 (orf19.7388) and CRZ1
209 (orf19.7359), and for proteins involved in membrane and cell wall integrity, including CDR3
210 (orf19.1313), NCP1 (orf19.2672), ECM21 (orf19.4887), MNN23 (orf19.4874), RHB1
211 (orf19.5994) and KRE6 (orf19.7363) (**Table S4**). Furthermore, the powerful comparative
212 approach of our pipeline permitted the discovery of 1,505 genes showing correlating or anti-

213 correlating copy number variations (**Fig. 4A** and **B**, **Table S4**), which could be assigned to nine
214 distinct correlation clusters (CC) (**Fig. S3A**, **Table S5**) that escaped previous analyses. We
215 verified the sequencing coverage of genomic regions encompassing gene CNVs, including
216 three regions amplified in fluconazole resistant strains (**Fig. 4B**, **Fig. S3B**) (47) and a region
217 whose amplification correlates with the level of miconazole resistance (47) (**Fig. S3C**), as well
218 as the loss of heterozygosity associated to the depletion of chromosome 3 left arm in sample
219 AMS3051 (**Fig. S3D**) (47). Eventually, by representing genes and absolute correlation
220 respectively as nodes and edges of a network, we identified 9 highly interconnected network
221 clusters (NC) (**Fig. 4C**, **Table S6**). NC7, NC8 and NC9 embody genes from individual
222 chromosomes, respectively chromosomes 1, 3 and 4. The most parsimonious explanation for
223 the high levels of correlation observed in these NCs (**Fig. 4C**) is the occurrence of sub-
224 chromosomal amplifications affecting several adjacent genes. A different scenario is pictured
225 for each of the remaining NCs (NC1-6) where the genes are located on different chromosomes
226 thus suggesting genetic interactions that causes coordinated changes in gene copy number. The
227 gene ontology (GO) and metabolic pathway analyses revealed a significant functional
228 enrichment of genes expressed on the cell surface and involved in the interaction with the host
229 (NC2), gibberellin biosynthesis (NC3), transmembrane nucleobase transporters (NC4) and
230 gluconeogenesis (NC5) (**Table S7**). Altogether, GIP and giptools are validated by reproducing
231 previously published results, and beyond that can drive new biological findings as documented
232 by the discovery of a network of epistatic CNV interactions supporting genomic adaptation in
233 *Candida albicans* populations under drug selection.
234
235 **Exploring instability of larger genomes using cancer cell lines as a benchmark.** The larger
236 genome size, and the higher number of genes and WGS reads can represent a challenge when
237 working with higher eukaryotes. For the purpose of comparison, the human genome is ~216
238 times larger than the one of *Candida albicans* we analyse in this study. Therefore, we sought

239 to evaluate the applicability of the GIP and giptools framework to human data and utilized a
240 panel of genomes from cancer cell lines as a test set. In our analyses we considered publicly
241 available WGS data of the cell lines T47D, NCI_H460 and K562 (50), which respectively
242 derive from human breast, lung and blood cancers. The karyotype analysis revealed aneuploidy
243 for all chromosomes except chromosome 4 (**Fig. 5A**). The observed heterogeneity in read depth
244 across chromosomes, illustrated by large interquartile range in the boxplot, suggests sub-
245 chromosomal or episomal copy number variations, or the co-existence of karyotypically
246 different sub-populations. Indeed, the coverage analysis confirmed the pervasive presence of
247 CNVs both at chromosomal and sub-chromosomal levels (**Fig. S4**) with remarkable instability
248 observed for specific chromosomes, e.g. chromosomes 6, 9, 10 and 16 (**Fig. 5B**). Overall, we
249 detected 1,647,016 SNVs (**Supplementary Data 1**) and allele frequency shifts with respect to
250 the reference genome, suggesting haplotype selection and the preferential expression of distinct
251 alleles in different cell lines (**Fig. 5C** and **Fig. S5**). Furthermore, we identified repeated loss of
252 heterozygosity events and uneven distribution of SNVs that form "patches" of high frequency
253 correlating with chromosomal and sub-chromosomal CNVs (**Fig. 5D**, **Fig. 5E** and **Fig. S6**).
254 These results identify GIP and giptools as a powerful new platform to reveal loci, genes or
255 alleles that are under natural selection in cancer cells, thus allowing important new insight into
256 the genetic basis of tumor development, cancer cell evolution and drug resistance.

257

258 **Discussion:**

259 Genome instability is a key driver of evolution for microbial pathogens and cancer cells (51)
260 and a major source of human morbidity. Here we introduce GIP and giptools, an integrated
261 framework for the genotype profiling of biological systems exploiting genome instability for
262 adaptation. We document the power and versatility of GIP and giptools by performing genomic
263 screenings of three major pathogenic eukaryotes and human cancer cell lines. While originally

264 deployed for *Leishmania* genome analysis, in this study we validate the use of our pipeline on
265 other organisms reproducing expected results. For example, in *Candida albicans* we confirmed
266 the CNVs correlating to drug resistance as well as a loss of heterozygosity event (**Fig. S3B-C**
267 and **D**). Parallel to this we also show how GIP and giptools can be used for data mining and
268 scientific discovery. New findings include (i) the discovery of the convergent amplification of
269 erythrocyte binding proteins in *Plasmodium vivax* strains sampled from distinct geographic
270 areas (**Fig. 3E**), (ii) the detection of a nullisomic strain (**Fig. 3F**), (iii) the identification of
271 correlated copy number variations between genes positioned on separate chromosomes of
272 *Candida albicans* adapting strains, and (iv) the functional association of such genes, strongly
273 supporting a mechanism of epistatic interactions exerted through gene-dosage changes, and
274 corroborating previous reports on adapting *Leishmania* populations (52).

275 Importantly, GIP and giptools overcome key limitations of current analysis tools, such
276 as the breadth of analysis that is often limited to individual types of mutations, and the lack of
277 genome-wide, comprehensive reports. To ease genome instability investigations our pipeline
278 offers a single solution to karyotype, gene CNV, SNV and SV batch analyses, providing
279 summary reports and high-quality, genome-wide visualizations. Furthermore, many current
280 tools identify variations with respects to a reference assembly only, which leaves the between
281 samples comparisons to external tools that need installing, may be incompatible in terms of file
282 format, and may rely on different analytical assumptions. To address this limitation giptools
283 enables custom sample comparisons, to explore differences and common features between
284 genomes, and provides a vast choice of analytical tools with compatible features. Likewise,
285 current tools are often restricted to the analysis of data from one or few species only (53-57),
286 but are not generally applicable to different biological systems, which interferes with the
287 investigation of genome variations across multiple species and the exploration potentially
288 conserved genomic adaptation mechanisms. By contrast, our pipeline limits as much as possible

289 the use of hardcoded parametrization, which could limit its use to a specific organism.
290 Therefore, GIP's flexible design makes it adapted for the genome analysis of both model and
291 non-model organisms, including *Leishmania* or human.

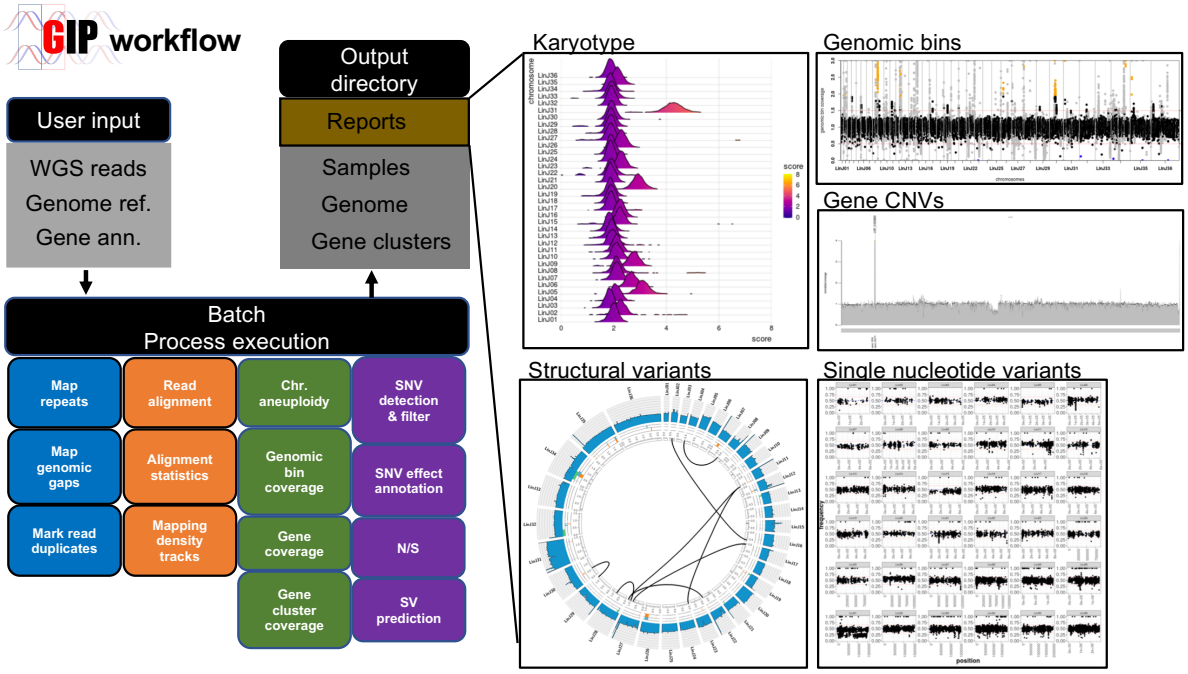
292 Many current tools are further limited in software portability and reproducibility across
293 different computer environments, which can produce faulty results calling their clinical
294 application into question. Conversely, thanks to the Singularity implementation all required
295 software are embedded and provided within the software container. As a consequence, users
296 can easily recreate the same work environment just by downloading the pipeline container, and
297 reproduce exactly the same publication-quality plots and tables presented in this study. Lastly,
298 one more common limitation is posed by software scalability. In the WGS domain, with the
299 rapid increase new samples made available and the enormous amount of data generated in each
300 sequencing run, the CPU and memory resources of local workstation risk to quickly become
301 inadequate for data analysis. Therefore, it is paramount that WGS tools are implemented to run
302 on high-performance computing (HPC) clusters and feature remote cloud computing solutions.
303 Because of its Nextflow implementation GIP can be executed on a local machine, on cluster
304 resource manager or the cloud. GIP can be applied on individual samples and without additional
305 effort on large WGS data sets for batch computation as shown for the 222 *Plasmodium vivax*
306 genomes.

307 These results well illustrate how GIP and giptools can be applied to perform extended
308 genomic analyses in different biological systems and drive biomedical discovery. To conclude,
309 we believe that GIP and giptools represent a step forward toward reproducible research in
310 genomics, and provide a robust computational framework to study how microbes and tumor
311 cells harness genome instability for environmental adaptation and fitness gain.

312

313 **Figures**

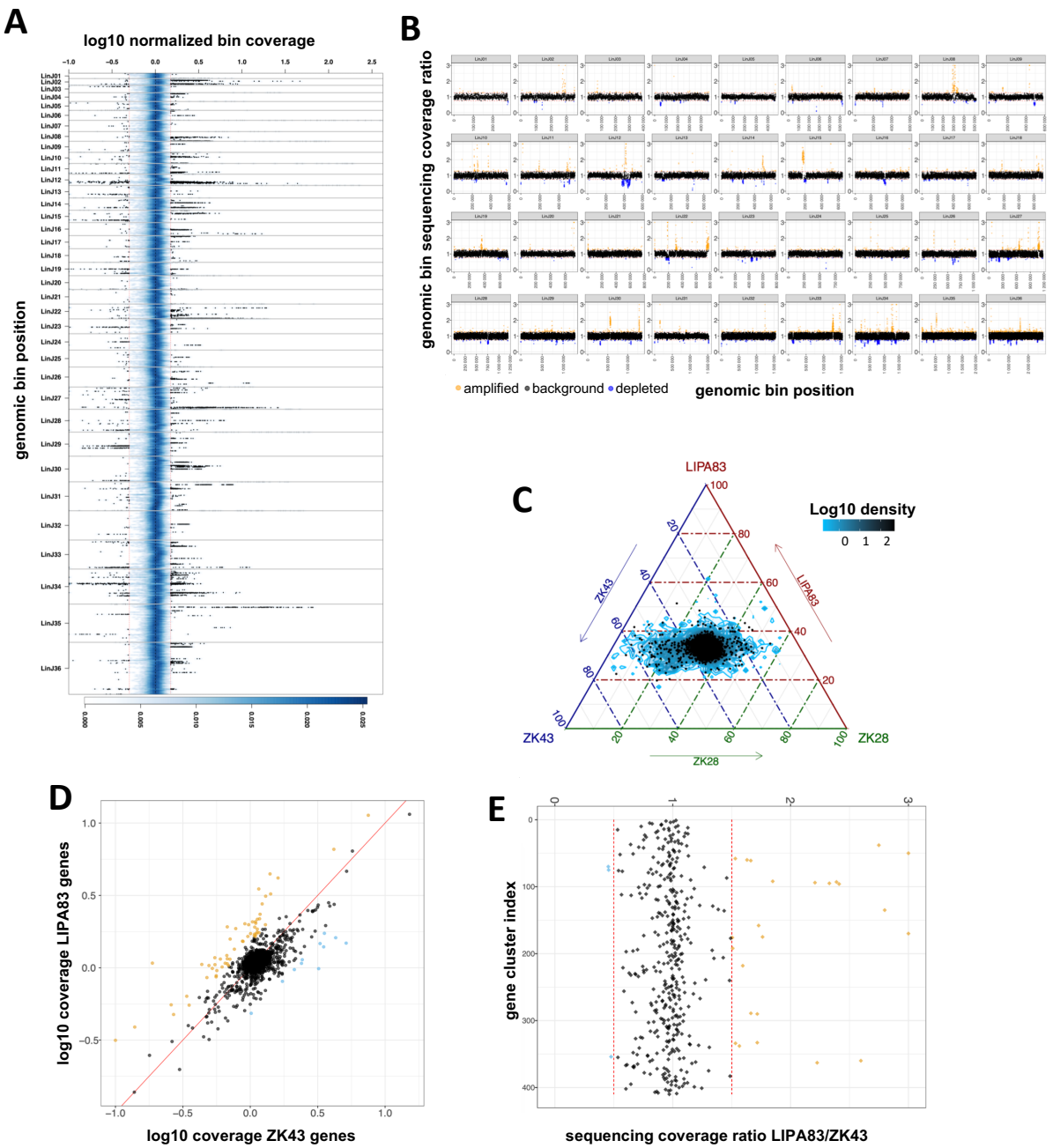
314 **Fig. 1**



315

316 GIP workflow. The schema on the left recapitulates the GIP inputs, processes and outputs (see
317 methods). Blue, orange, green and purple boxes indicate genome reference, read mapping,
318 quantification and variants computation steps, respectively. The panels on the right demonstrate
319 example plots included in the GIP report computed for individual samples. The "Karyotype"
320 plot shows the coverage distributions for each chromosome (y-axis). The "Genomic bins" plot
321 shows the genomic position (x-axis) and the normalized genomic bin sequencing coverage (y-
322 axis). The "Gene CNVs" plot shows the normalized gene sequencing coverage. The "Structural
323 variants" panel shows a Circos plot representing break-end translocations (black links in the
324 inmost part of the plot), and other possible structural variations in the outer tracks, including
325 insertions, duplications, deletions and inversions. The outmost track shows the normalized
326 sequencing coverage. The "Single nucleotide variants" plot shows on the x and y axes
327 respectively the genomic position and variant allele frequency of detected SNVs.

328 **Fig. 2:**

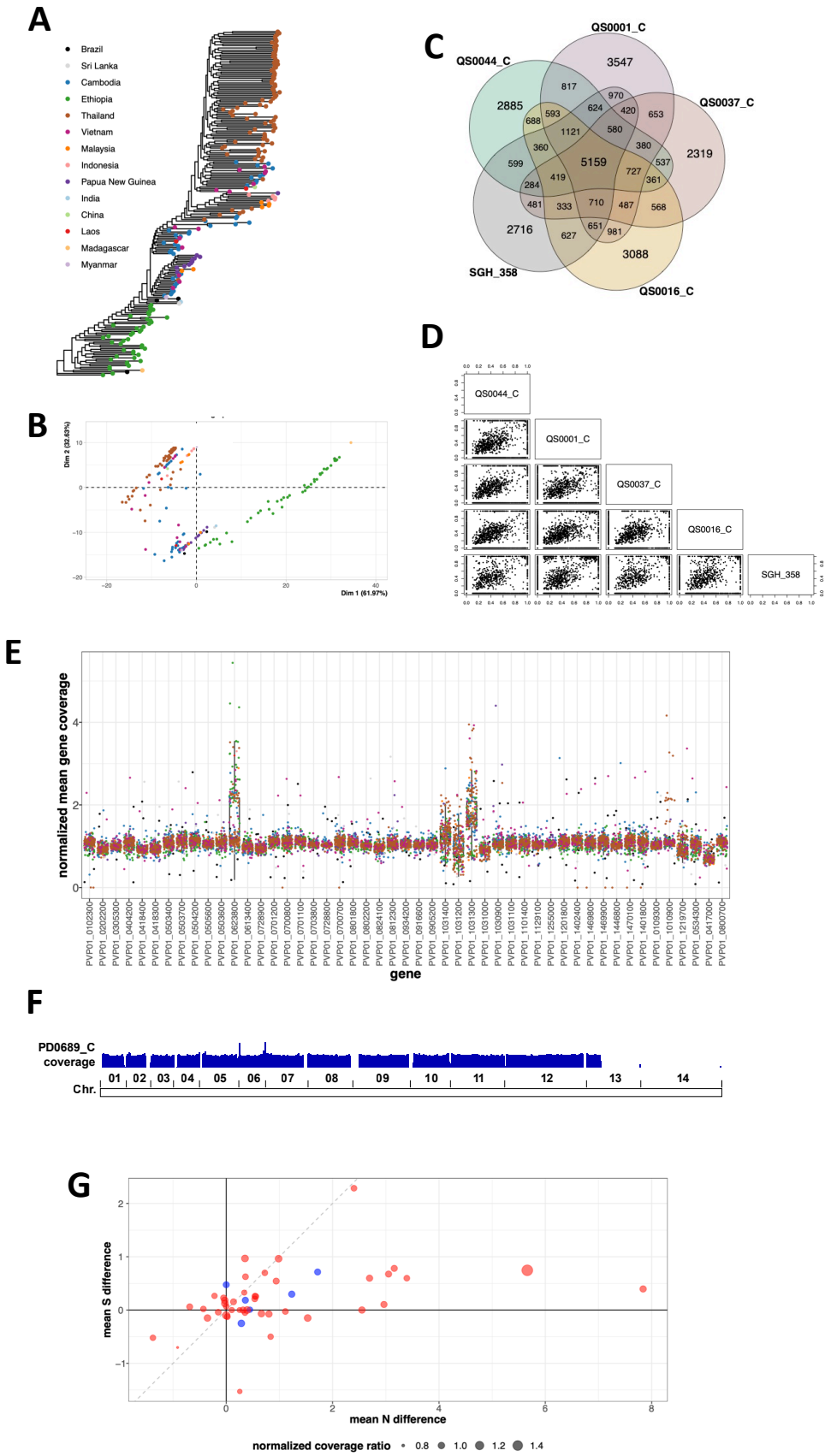


329

330 Comparing *Leishmania infantum* genomes with giptools. (**A**) Density plot representing the
331 genomic coverage of the seven *Leishmania infantum* isolates. The x-axis shows the log 10
332 normalized coverage of genomic bins. The y-axis reflects the genomic position. The thirty-six
333 different chromosomes are materialized as separate panels. The blue shading indicates the (2D)
334 kernel density estimates of genomic bins. The two red vertical lines mark the 1.5 and 0.5

335 coverage values. A selection of 50 000 bins with coverage > 1.5 or < 0.5 is shown as black dots.
336 (**B**) Scatterplot of the genomic bin normalized sequencing coverage ratio of samples LIPA83
337 over ZK43. The x any y axes show the ratio score and the genomic position respectively. Ratio
338 scores > 1.25 are labelled in orange and indicate genomic bin amplification. Ratio scores < 0.75
339 are labelled in blue and indicate genomic bin depletion. (**C**) Ternary comparison showing the
340 relative abundance of the genomic in samples LIPA83, ZK43 and ZK28. The axes report the
341 fraction of the bins normalized sequencing coverage in the three strains. The blue contour
342 indicates the log10 bin density. A subset of 5,000 bins is shown as black dots. Each given point
343 in the plot is adding up to 100. The density area at the center of the plot indicates bins with
344 equal copy number and thus a ~33 distribution across the three axes. (**D**) Scatterplot showing
345 the log10 normalized sequencing coverage of annotated genes in ZK43 (x-axis) and LIPA83
346 (y-axis). The red line indicates the bisector. Dots represent individual genes. (**E**) Sequencing
347 coverage ratio of gene clusters in samples LIPA83 and ZK43. Dots represent gene clusters. For
348 plots **D** and **E,** the ratio scores > 1.5 or < 0.5 are labelled in orange and blue respectively.

Fig. 3:


A
Brazil
Sri Lanka
Cambodia
Ethiopia
Thailand
Vietnam
Malaysia
Indonesia
Papua New Guinea
India
China
Laos
Madagascar
Myanmar
B
Dim 2 (22.63%)
Dim 1 (61.97%)
C
QS0001_C
QS0044_C
QS0037_C
SGH_358
QS0016_C
3547
2885
2319
2716
3088
5159
D
QS0044_C
QS0001_C
QS0037_C
QS0016_C
SGH_358
E
normalized mean gene coverage
gene
F
PD0689_C
coverage
Chr.
01
02
03
04
05
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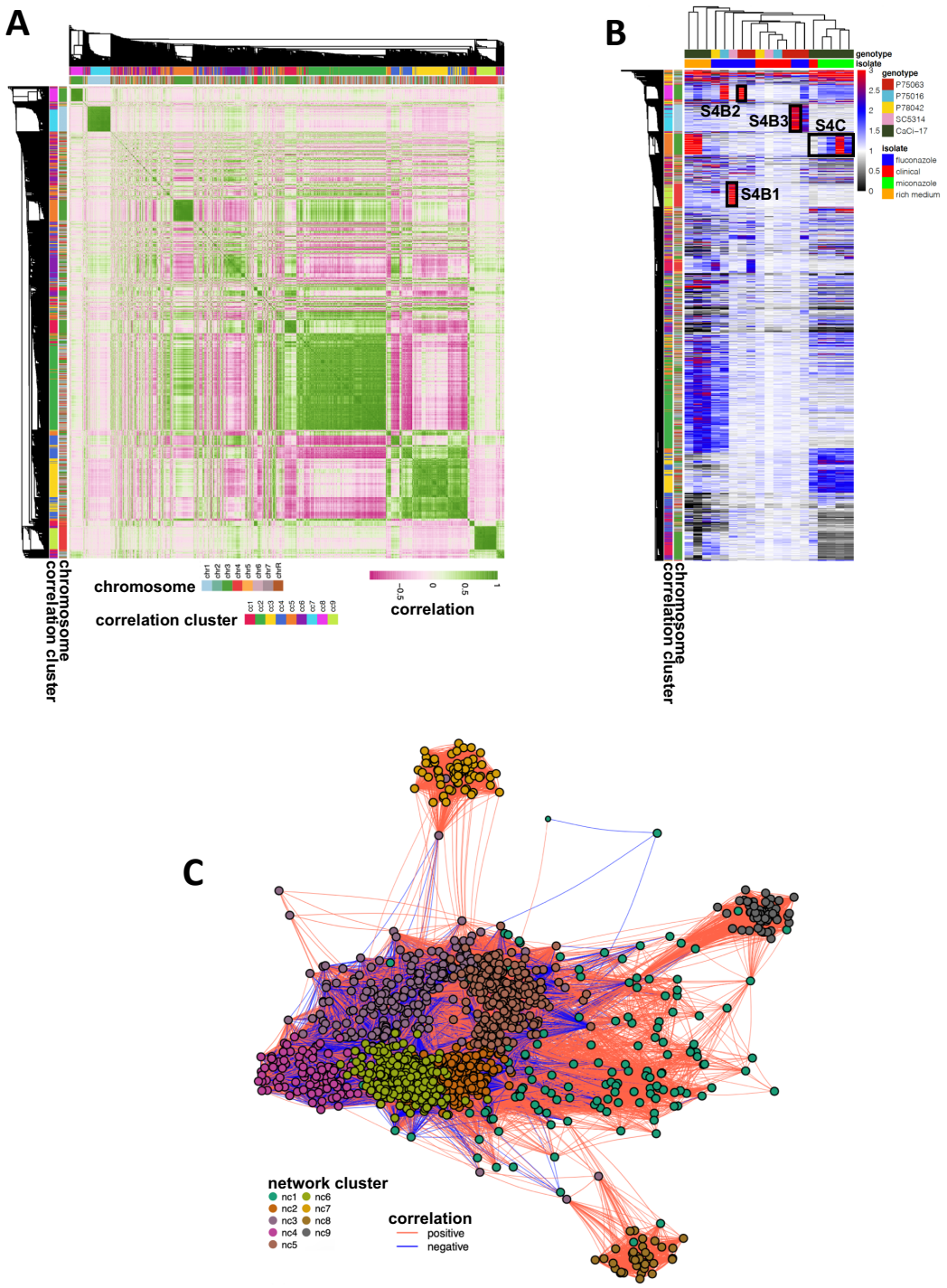
09
10
11
12
13
14
G
mean S difference
mean N difference
normalized coverage ratio
0.8
1.0
1.2
1.4

351 *Plasmodium vivax* genomic diversity. (**A**) Predicted maximum likelihood phylogenetic tree
352 reconstruction. (**B**) PCA analysis of the phylogenetic distances estimated from the tree in (**A**).
353 Each dot indicates a sample. The colour code reflects the geographic origin of the samples and
354 matches with the colours of the legend in (**A**). (**C**) Venn diagram comparing the SNVs of five
355 representative Ethiopian strains. (**D**) Pairwise scatterplot comparing the variant allele frequency
356 of all detected SNVs in the five Ethiopian strains. (**E**) Gene panel analysis. The x-axis reports
357 a set of genes of interest. The y-axis indicates the normalized mean gene coverage. The boxplots
358 demonstrate the coverage values distributions for each gene across all samples. Each dot
359 represents the coverage of the indicated gene in a given sample. Dot colours reflect the sample
360 geographic origin as in (**A**). (**F**) Reads per kilo base per million mapped reads (RPKM)
361 normalized sequencing coverage density track of sample PD0689_C. The boundaries of the 14
362 chromosomes are shown on the bottom. (**G**) Comparison of non-synonymous (N) and
363 synonymous (S) mutations between Ethiopia and Cambodia sample groups. Dots represent
364 genes. The x-axis represents the difference between the mean non-synonymous mutation count
365 in the two sample groups. The y-axis represents the difference between the mean synonymous
366 mutation count in the two sample groups. The dot size demonstrates the ratio of the mean
367 normalized sequencing coverage between the two sample groups for each gene. Red and blue
368 dot colors indicate genes belonging to the 43 genes panel (23) and the custom 6 genes panel
369 respectively.

370 **Fig. 4:**

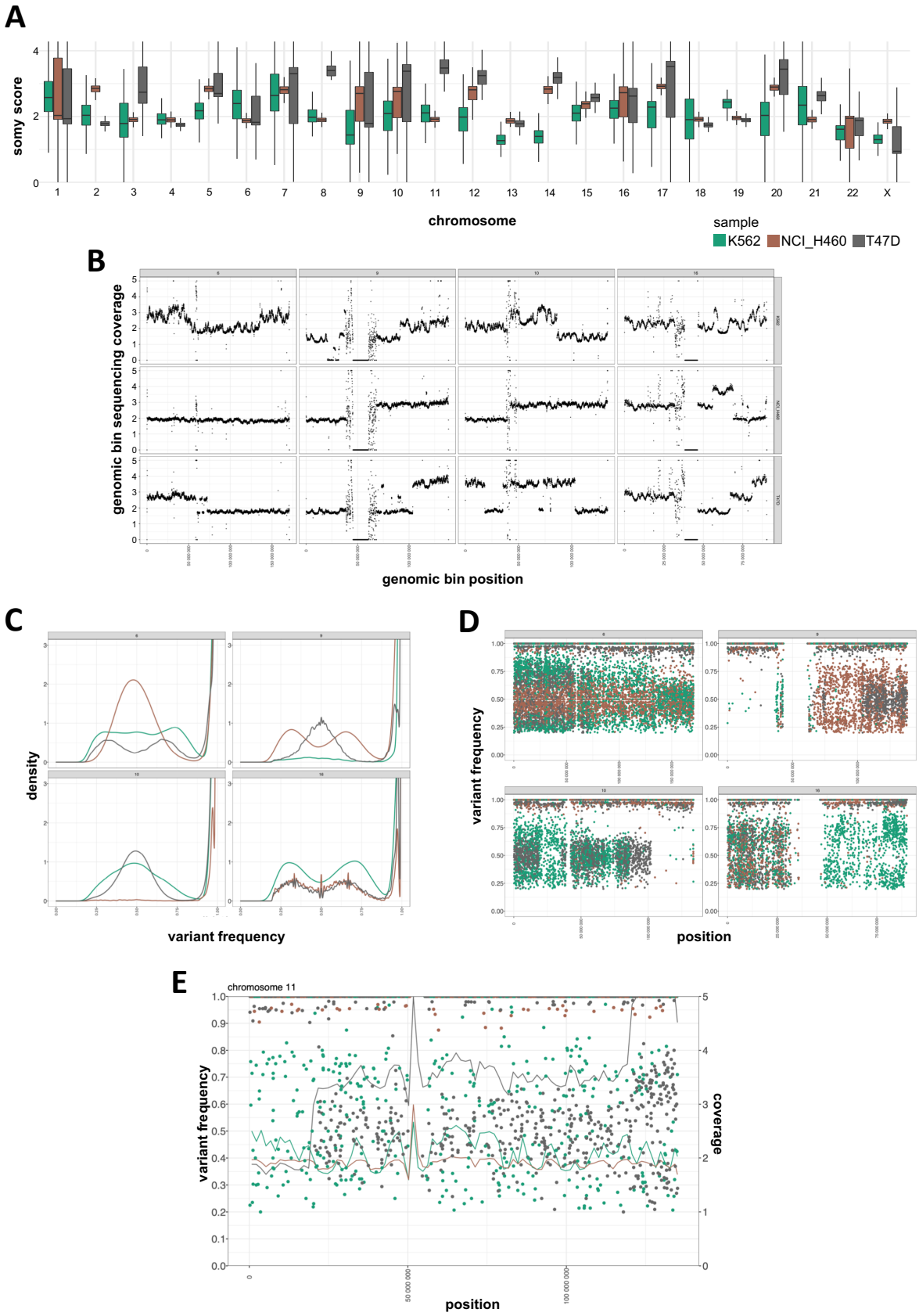


371

372 Gene CNVs interactions. (**A**) All-vs-all normalized sequencing coverage correlation heatmap.
373 The heatmap is symmetrical along its diagonal and reports both on the rows and the columns
374 the detected gene CNVs. The colour scale indicates with green and pink high levels of positive
375 and negative Pearson correlation, respectively. The side ribbons demonstrate in different

376 colours the chromosome and the correlation cluster of each gene. (**B**) Gene CNV heatmap. The
377 columns and the rows report respectively the samples and the detected gene CNVs. The colour
378 scale indicates the normalized sequencing coverage of the genes. To ease visualization,
379 coverage values greater than 3 are reported as 3 (red). Black boxes highlight the genomic
380 regions shown in **Fig. S3B** (panels 1, 2 and 3) and **Fig. S3C**. The ribbons on the left indicate
381 the chromosome and the correlation cluster of each gene. Top ribbons indicate the genotype
382 and the strains resulting from the different evolutionary experiments. (**C**) Gene interaction
383 network. Nodes indicate gene CNVs. Edges reflect the absolute Pearson correlation value. The
384 closer the nodes are, the higher is the correlation. Only significant interactions (Benjamini-
385 Hochberg adjusted p-value < 0.01) are shown. The colour of the edges indicates in red and blue
386 respectively positive and negative correlations. The colour of the nodes denotes the predicted
387 network cluster for each gene.

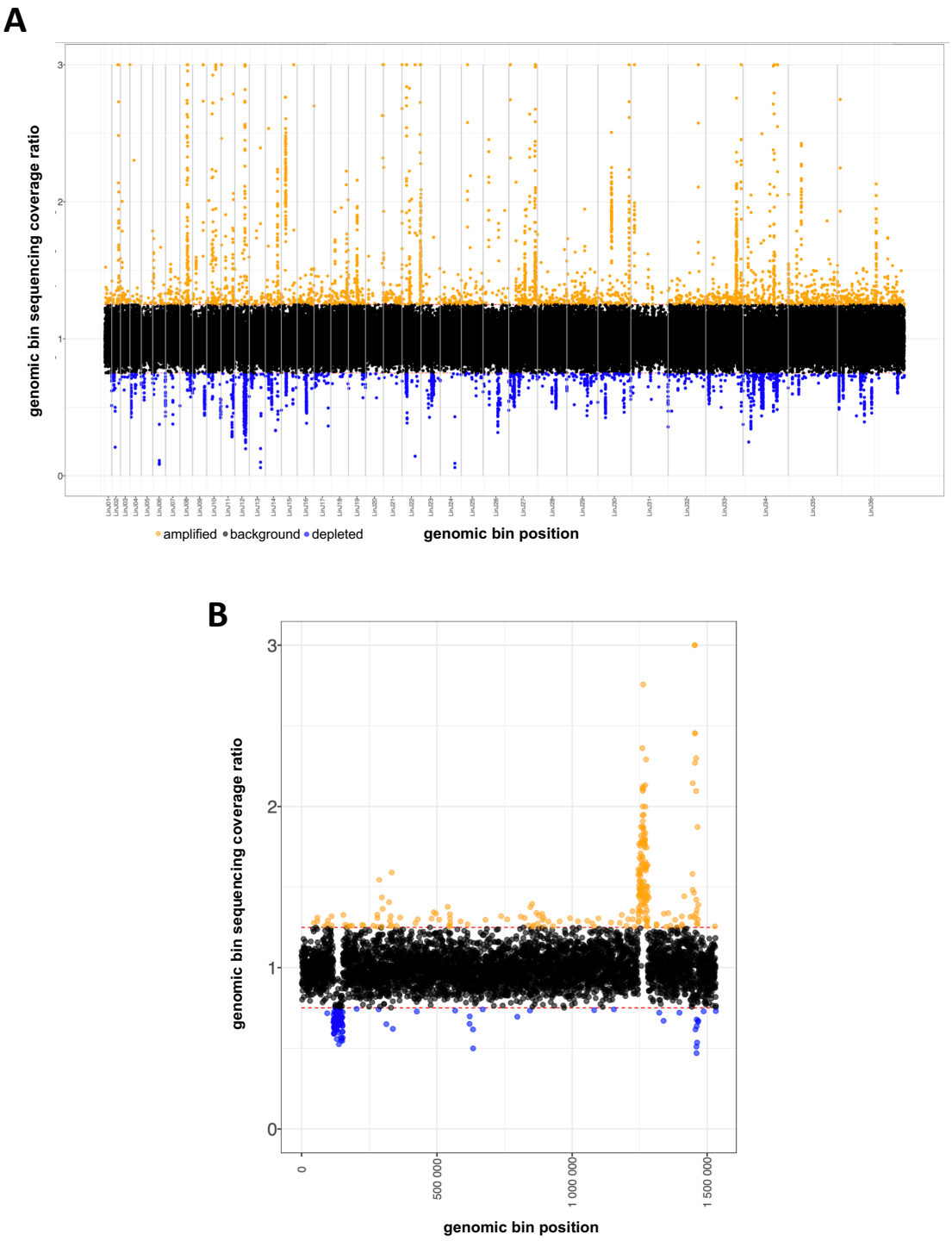
Fig. 5:


A
somy score
chromosome
sample
K562
NCI_H460
T47D
B
genomic bin sequencing coverage
genomic bin position
C
density
variant frequency
D
variant frequency
position
E
chromosome 11
variant frequency
coverage
position

390 Cancer cell lines genome instability. Green, brown, and grey colours indicate respectively
391 samples K562, NCI_H460 and T47D. (**A**) Chromosome coverage analysis. The x-axis reports
392 the chromosomes. The y-axis reports the estimated somy score. The boxes show the somy score
393 distributions. (**B**) Sub-chromosomal copy number variation. Dots indicate genomic bins.
394 Different panels indicate different chromosomes. The panel columns indicate from left to right
395 four selected chromosomes: 6, 9, 10 and 16. The panel rows show top to bottom the samples
396 K562, NCI_H460 and T47D. The x-axis indicates the genomic position. The y-axis indicates
397 the normalized genomic bin sequencing coverage values. Coverage values greater than 5 are
398 reported as 5. (**C**) SNV frequency density plots. The four different panels represent different
399 selected chromosomes: 6, 9, 10 and 16. The x-axis reports the variant allele frequency. The y-
400 axis the estimated kernel density between 0 and 3. (**D**) SNV frequency scatter plots. The four
401 different panels represent different selected chromosomes: 6, 9, 10 and 16. The x-axis indicates
402 the genomic position. The y-axis indicates the variant allele frequency. (**E**) Chromosome 11
403 combined SNV and bin coverage plot. To ease visualization, giptools allows the simultaneous
404 displaying of variant allele frequencies (y-axis, left) and sequencing coverage (y-axis, right).
405 Dots represent SNVs. The lines represent the normalized bin sequencing coverage. The x-axis
406 indicates the genomic position. Coverage values greater than 5 are shown as 5.
407

408 **Supplementary figures**

409 **Fig. S1**:



410

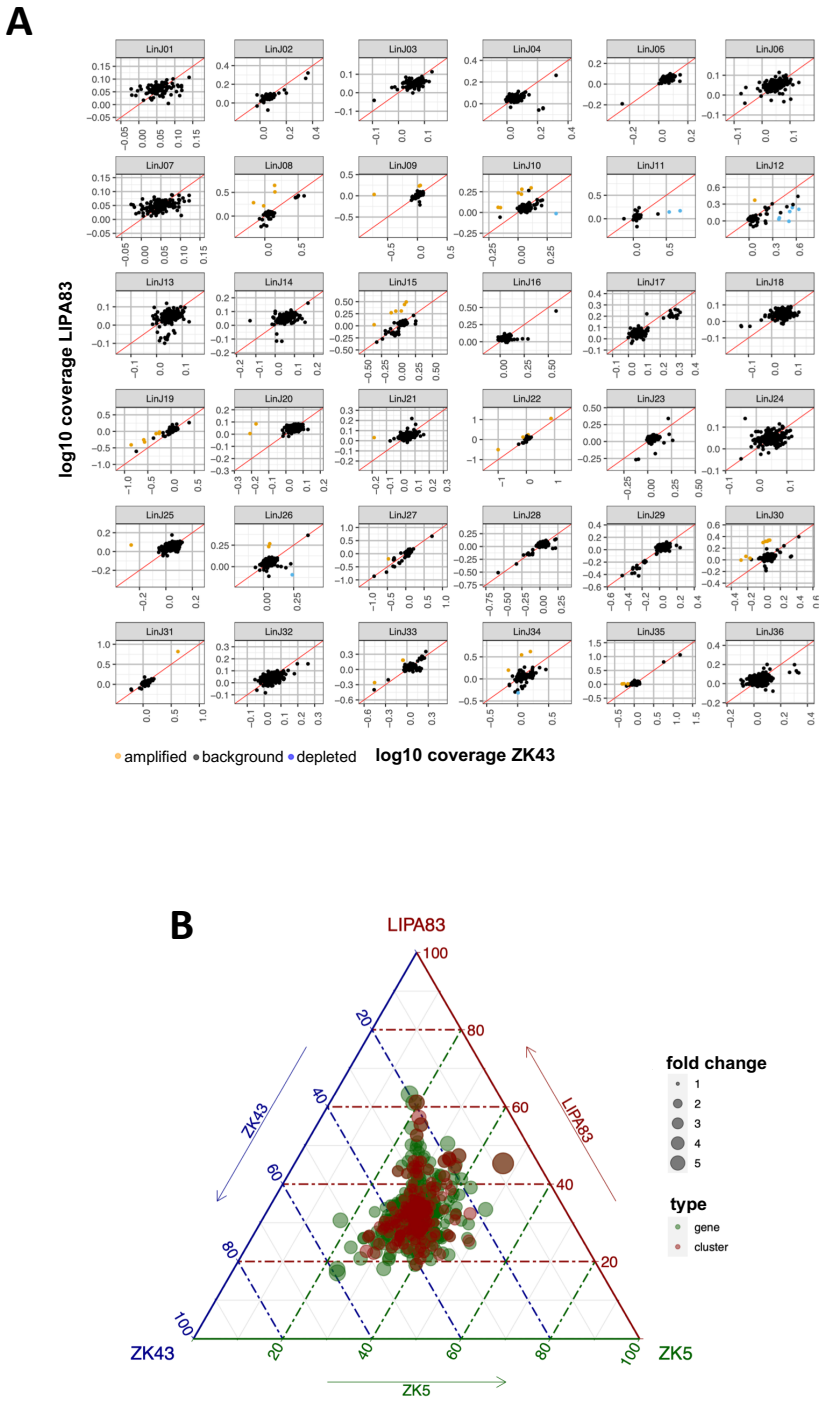
411 Bin coverage ratio visualizations. The figure shows alternative representations produced for the

412 genomic bin normalized sequencing coverage ratio of samples ZK43 and LIPA83. Same layout

413 as **Fig. 2B**. (**A**) Whole genome overview. (**B**) All the individual chromosomes separately. The

414 plot shows the example of chromosome 33.

415 **Fig. S2**:

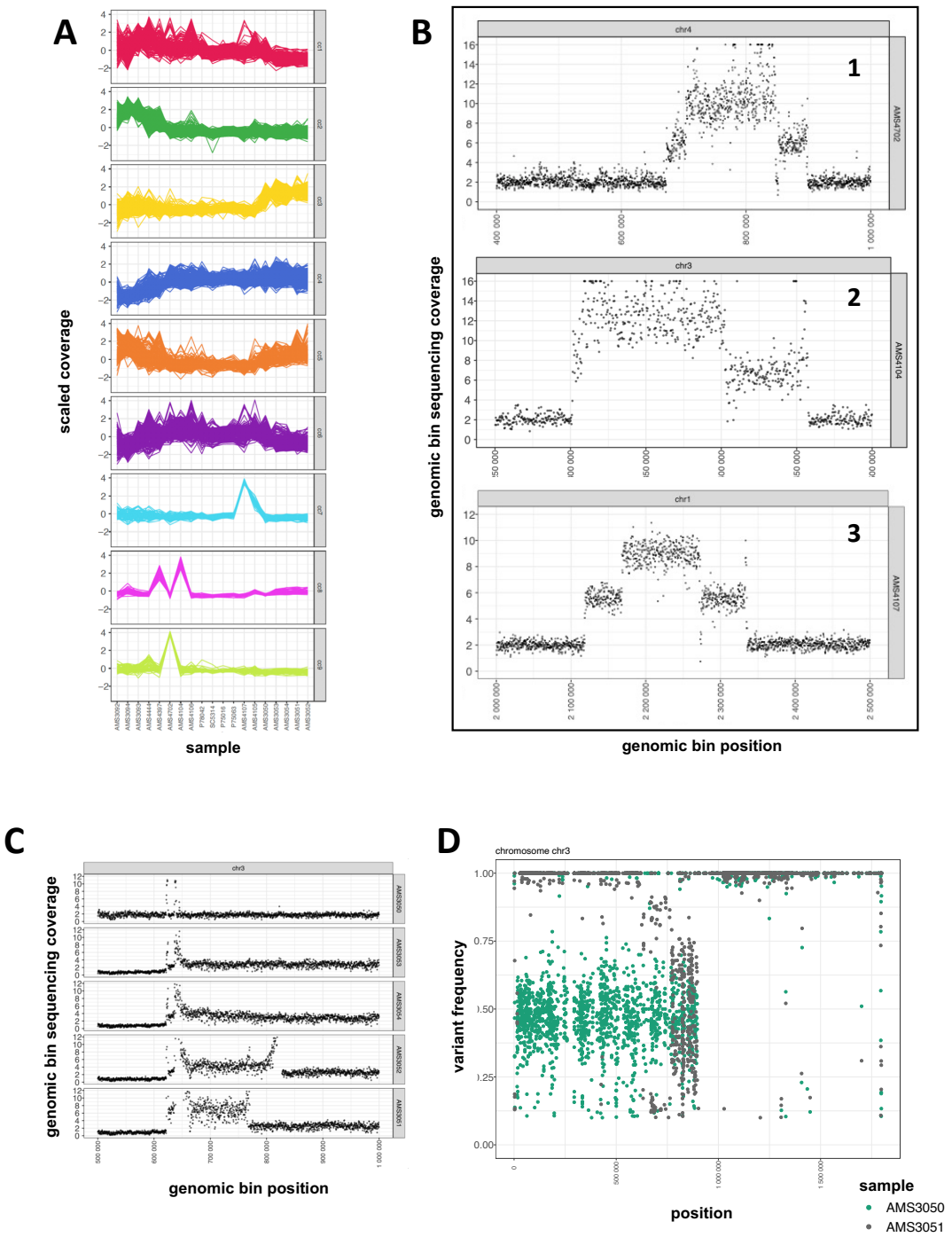


416

417 Two-ways and three-ways gene coverage comparisons. (**A**) Log10 normalized sequencing
418 coverage comparison of genes in ZK43 (x-axis) and LIPA83 (y-axis). Same layout a **Fig. 2D**
419 but representing the different chromosomes in individual panels. (**B**) Ternary plot showing the
420 relative abundance of genes (green dots) and gene clusters (red dots) in samples LIPA83, ZK43

421 and ZK28. The axes report the fraction of the genes and genes clusters normalized sequencing
422 coverage in the three strains. Each given point in the plot adding up to 100. Genes with equal
423 copy number are shown in the center of the ternary plot, while copy number variations are
424 visualized by shifts of the dots out of the center. The dots size reflects the fold change variation
425 between the maximum and the minimum observed normalized coverage value.

426 **Fig. S3:**

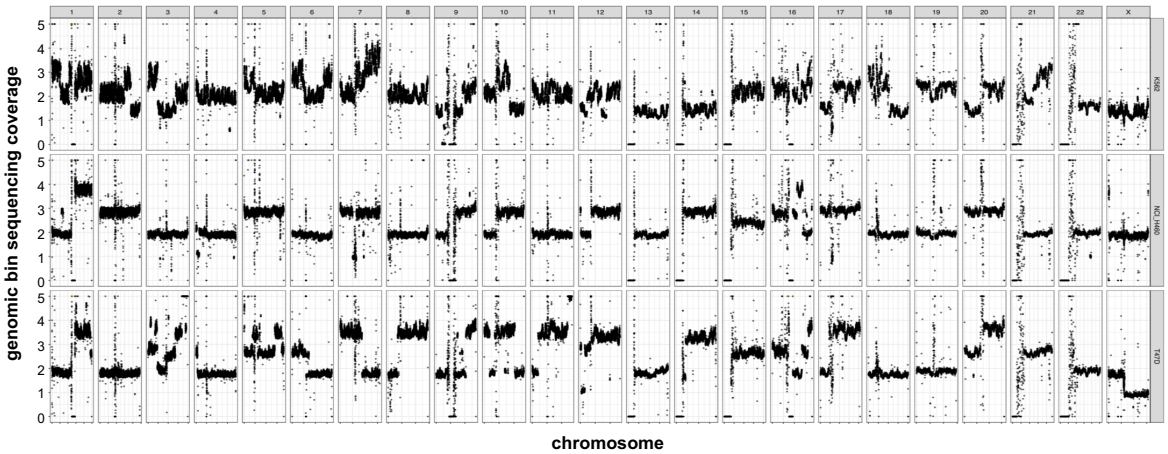


427

428 Copy number variations of evolutionary adapted *Candida albicans* strains. (**A**) Gene CNV
429 correlation clusters. The x and y axes indicate respectively the samples and the scaled
430 normalized sequencing coverage of the genes in each cluster. Different panels indicate different
431 correlation clusters. (**B**) Scatterplots showing the bin normalized sequencing coverage (y-axis)

432 of three genomic regions (chr4:400,000-1,000,000; chr3:1,250,000-1,500,000; chr1:2,000,000-
433 2,500,000) in three different samples AMS4702, AMS4104 and AMS4107. The x-axis shows
434 the genomic position. Dots represent genomic bins. (**C**) Scatterplot showing the bin normalized
435 sequencing coverage of a genomic region (chr3:500,000-1,000,000) in five different samples
436 represented as separate panels. From top to bottom the samples are: AMS3050, AMS3053,
437 AMS3054, AMS3052 and AMS3051. The x and y axes show respectively the genomic position
438 and the normalized sequencing coverage. Dots represent bins. (**D**) Comparative analysis of
439 chromosome 3 SNVs. AMS3050 SNVs are shown in green, while AMS3051 SNVs are shown
440 in grey. The x and the y axes show respectively the genomic position and the variant allele
441 frequency.

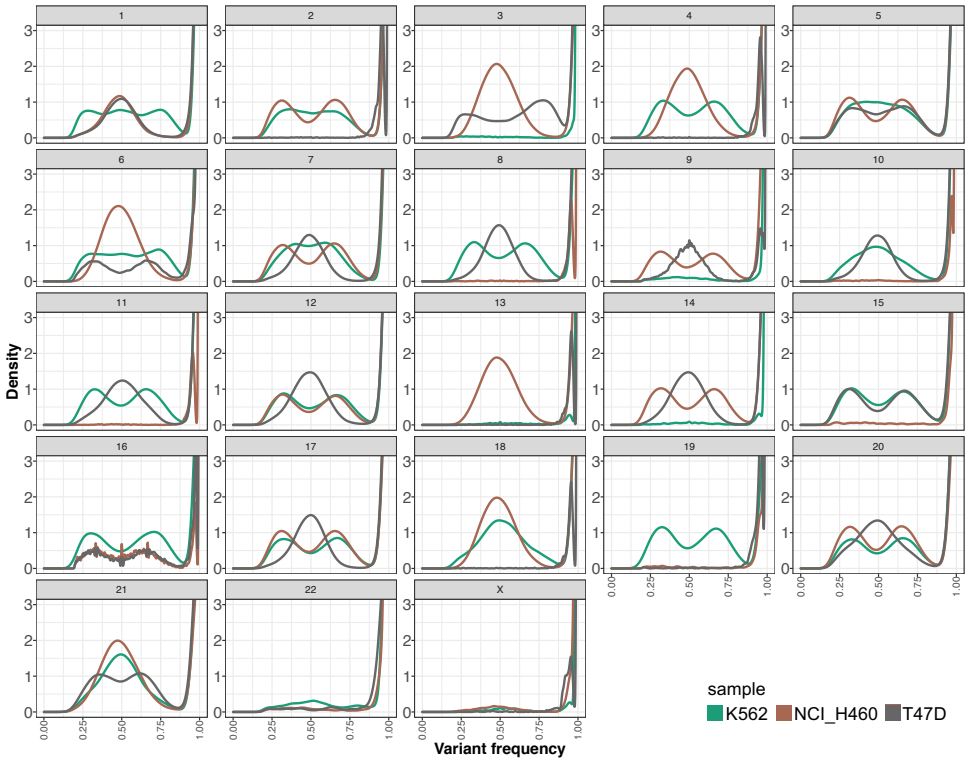
442 **Fig. S4:**



443

444 Genomic coverage analysis of three cancer cell lines. The x-axis indicates the genomic position.
445 The y-axis indicates the normalized genomic bin sequencing coverage values. Dots demonstrate
446 the genomic bins. To ease visualization, coverage values greater than 5 are reported as 5.
447 Different panels show different chromosomes. The three panel rows indicate top to bottom the
448 following samples: K562, NCI_H460 and T47D.

449 **Fig. S5:**



450

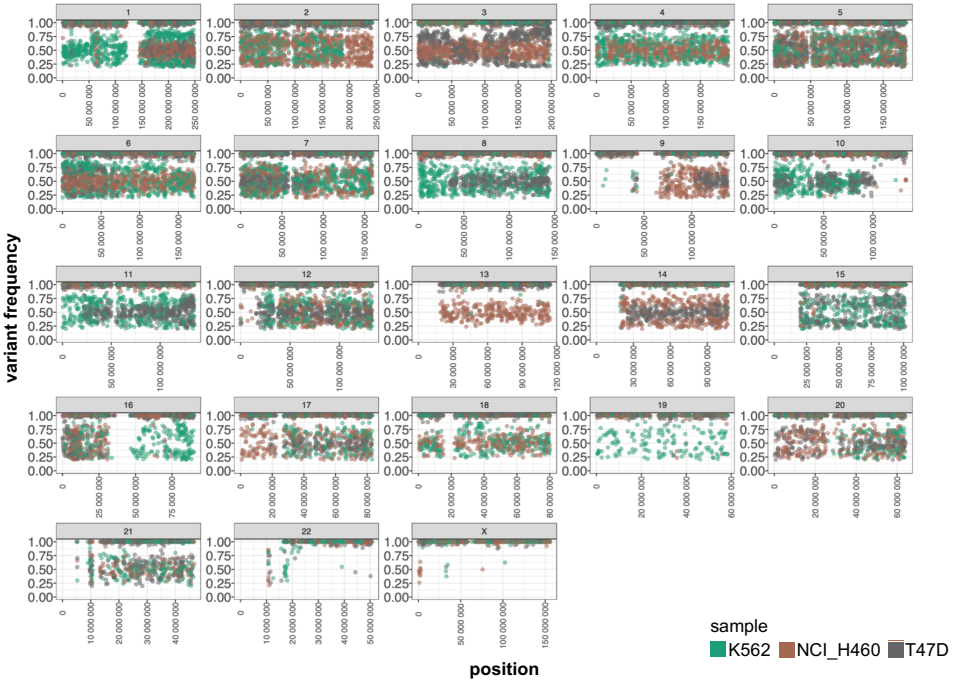
451 SNV frequency density plot. The x-axis shows the variant allele frequency. The y-axis the

452 estimated kernel density between 0 and 3. Green, brown, and grey slopes indicate respectively

453 samples K562, NCI_H460 and T47D.

454

455 **Fig. S6:**



456

457 SNV heterogeneity of cancer cell lines. The x-axis indicates the genomic position. The y-axis

458 indicates the variant allele frequency. The dots indicate SNVs and they are coloured according

459 to the sample. K562, green; NCI_H460, brown; T47D, grey. Different panels show different

460 chromosomes.

461

462 ## Methods:

463 **GIP and giptools.** All results presented in this study were generated using GIP and giptools
464 version 1.0.9. GIP code is maintained and freely distributed at the github page:
465 https://github.com/giovannibussotti/GIP. giptools container is accessible from the Singularity
466 cloud at https://cloud.sylabs.io/library/giovannibussotti/default/giptools. The GIP
467 configuration files (**Supplementary Data 2**) and the giptools command options used to
468 generate all results (**Supplementary Data 3**) are provided. The full documentation of GIP and
469 giptools including a description of all options is available from
470 https://gip.readthedocs.io/en/latest/.

471 **Read alignment.** WGS reads were downloaded from the Sequence Read Archive (SRA) (58)
472 and the European Nucleotide Archive (ENA) (59) repositories and the Encyclopedia of DNA
473 Elements (ENCODE) dashboard (60) (**Table S3**). For *Leishmania infantum* the
474 GCA_900500625 genome reference and gene annotations available from the ENSEMBL
475 protists (61) server (release-48) were used. For *Candida albicans* the assembly 21 of the
476 SC5314 strain genome reference and gene annotations available from the *Candida* Genome
477 Database (CGD) (62) were used. For *Plasmodium vivax* the P01 reference genome and gene
478 annotations available from PlasmoDB (63) (release-50) were used. For the cancer cell lines the
479 human genome GRCh38 primary assembly and gene annotations available from ENSEMBL
480 (release-102) were used. The repetitive elements of reference genomes were soft-masked by
481 GIP using Red (64). WGS reads were mapped by GIP using BWA-mem (version 0.7.17)
482 (65,66) run with option -M to label shorter split hits as secondary. Then the alignment files
483 were sorted, indexed and reformatted by GIP using Samtools (version 1.8) (67). Finally, read
484 duplicates were removed by GIP using Picard MarkDuplicates
485 (http://broadinstitute.github.io/picard) (version 2.18.9) with the option

486 "VALIDATION_STRINGENCY=LENIENT." In the four considered datasets, WGS reads
487 were aligned against full assemblies, including unsorted contigs if present. However just the
488 canonical assembled chromosomes were considered for all downstream analyses (`chrs` option,
489 **Supplementary Data 2**). A minimum read alignment MAPQ score was adopted to select genes
490 for cluster analysis, and to call for SNVs and SVs (`MAPQ` option, **Supplementary Data 2**).
491 Altogether a total of 6,306,951,266 reads were aligned to the respective reference genomes.
492 The `giptools overview` module was run to gather the alignment statistics as estimated by
493 Picard CollectAlignmentSummaryMetrics (**Table S8**).

494 **Genomic bins and genes quantification.** GIP was used to evaluate the mean sequencing
495 coverage and the mean read MAPQ of genomic bins and genes. For genomic bins, GIP
496 partitioned the input genomes into adjacent windows of user defined lengths (`binSize` option,
497 **Supplementary Data 2**). The coverage GC-content score bias was corrected (`CGcorrect`
498 option, **Supplementary Data 2**) fitting a LOESS regression with a 5-fold cross validation to
499 optimize the model span parameter. A larger window length was utilized to bin the reference
500 genomes for Circos plot representations (`binSizeCircos` option, **Supplementary Data 2**). In
501 **Fig. 1** ("Genomic bins" and "Gene CNVs" plots), **Fig. 2**, **Fig. S1**, **Fig. S2** and **Fig. 4** bins and
502 genes coverage scores were normalized by median chromosome coverage to highlight
503 amplifications or depletions with respect to the chromosome copy number. In **Fig. 1**
504 ("Structural variants" plot), **Fig. S3B-C**, **Fig. 3E-G**, and **Fig. S4A** bins and genes coverage
505 scores were normalized by median genome coverage to account for sequencing library size
506 differences. GIP evaluated statistically significant copy number variant bins and genes (**Fig. 1**
507 "Genomic bins" and "Gene CNVs" plots) using a p-value threshold of 0.001
508 (`covPerBinSigOPT` and `covPerGeSigOPT` options, **Supplementary Data 2**). Estimated p-
509 values for bins and genes CNVs were corrected for multiple testing using the Benjamini –
510 Yekutieli (`--padjust BY `) and the Benjamini – Hochberg (`--padjust BH`) methods. The somy

511 scores shown in **Fig. 1** (karyotype plot) and **Fig. S4B** were computed multiplying the median
512 genome coverage normalized bin coverage by 2. GIP enabled the CNV analysis of genes
513 sharing high sequence identity by clustering the nucleotide sequences of the genes with low
514 mean MAPQ score into groups with cd-hit-est (version 4.8.1) (68) with options `-s 0.9 -c 0.9 -
515 r 0 -d 0 -g 1`. Then for each gene cluster GIP computed the mean gene coverage normalized by
516 median chromosome coverage (**Fig. 2E**, **Fig S2B**).

517 **Gene ontology and metabolic pathway enrichment.** The FungiDB online tool (Release 52,
518 20 May 2021) (69) was used to evaluate the functional enrichment of network clusters genes.
519 For the gene ontology analysis, the biological process (BP), molecular function (MF) and
520 cellular compartment (CC) terms enrichments were tested, considering both computed and
521 curated evidences and a p-value cutoff of 0.05. For the metabolic pathway enrichment, both
522 KEGG (70) and MetaCyc (71) pathway sources were considered with a p-value cutoff of 0.05.
523 Terms and pathways with Benjamini – Hochberg adjusted p-values < 0.05 were considered
524 statistically significant.

525 **Sequencing coverage density estimates.** GIP was used to convert the read alignment files
526 (.bam format) in binary data files reflecting sequencing coverage (.bigWig format). The
527 coverage file were produced using bamCoverage from the deepTools2 suite (72) (version 3.5.1)
528 with options "--normalizeUsing RPKM --ignoreDuplicates --binSize 10 --smoothLength 30"
529 (`bigWigOPT` option, **Supplementary Data 2**). The coverage track of sample PD0689_C was
530 visualized with IGV using the `Bar Chart`, `Autoscale` and windowing function `Mean`
531 options.

532 **Single-nucleotide variant analysis.** GIP was used to call SNVs using Freebayes (version
533 1.3.2) (`freebayesOPT` option, **Supplementary Data 2**) and filter its output
534 (`filterFreebayesOPT` option, **Supplementary Data 2**). Filters included the minimum allele

535 frequency (`--minFreq`), the minimum number of reads supporting the alternative alleles (`--
536 minAO`) and minimum mean mapping quality of for the reads supporting the reference (`--
537 minMQMR`) or the alternative allele (`--minMQM`). A higher number of reads supporting the
538 variants was requested for predictions positioned inside simple repeats of the same nucleotide
539 (homopolymers) (`--minAOhomopolymer`). The homopolymers were defined as the DNA
540 region spanning ±5 bases from the SNV (`--contextSpan 5`), with over 40% of identical
541 nucleotides (--homopolymerFreq 0.4 `). Further, GIP discarded SNVs with sequencing
542 coverage above or below 4 median absolute deviations (MADs) from the median chromosome
543 coverage (`--MADrange`). snpEff (version 4.3t) (73) was used to predict the impact of SNVs
544 on coding sequence. The predicted effects that GIP considered synonymous mutations are:
545 "synonymous_variant", "stop_retained_variant" and "start_retained". The predicted effects that
546 GIP considered non-synonymous mutations are: "missense_variant", "start_lost",
547 "stop_gained", "stop_lost" and "coding_sequence_variant". The phylogenetic tree was
548 computed by the giptools module `phylogeny` using IQtree2 (version 2.1.2) (74,75) with
549 options `--seqtype DNA --alrt 1000 -B 1000`. The Venn-diagram comparison considered the
550 strains QS0044_C, QS0001_C, QS0037_C, QS0016_C and SGH_358 that were sampled from
551 different locations in Ethiopia, respectively Habala, Badowacho, Arbaminch, Hawassa and
552 Jimma. The strains were selected to have comparable average genome coverage (23). To infer
553 the tree GIP considered the set of filtered SNV and adopted the IUPAC ambiguous notation for
554 the positions with allele frequency below 70%. The tree was visualized by giptools using the
555 ggtree R-package (76).

556 **Analysis of structural variants.** GIP was used to detect structural variants including insertions,
557 tandem duplications, deletions, inversions and break-end translocations with DELLY (version
558 0.8.7) (11). To reduce incorrect predictions the DELLY output was additionally filtered
559 (`filterDellyOPT` option, **Supplementary Data 2**). GIP discarded poor predictions with

560 DELLY label "LowQual" (`--rmLowQual`) and low median MAPQ score of mapping reads (`-
561 -minMAPQ`). SVs positioned in proximity of chromosome ends were removed (--
562 chrEndFilter) to limit false predictions caused by potential misassembled regions close to the
563 telomeric ends. To ease visualization and limit the analysis only to best supported SVs GIP
564 limited the output only to the top predictions (`--topHqPercentIns`, `--topHqPercentDel`, `--
565 topHqPercentDup` and `--topHqPercentInv`) based on the SV support score as in **Formula 1**,
566 where *DV*, *DR*, *RV*, and *RR* are respectively the number of high-quality variant pairs, reference
567 pairs, variant junction reads, and reference junction reads.

568 $$\frac{\boldsymbol{DV + RV}}{\boldsymbol{DV + RV + DR + RR}} * \mathbf{100}$$

569 **Formula 1:** SV support score.

570 The predicted structural variants were represented with Circos (version 0.69-9) (77).

571 **Data and materials availability.** All data is available in the main text or the supplementary
572 materials.

573

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579 Software, G.B.; Formal Analysis, G.B., and G.F.S.; Investigation, G.B. and G.F.S.; Writing –
580 Original Draft, G.B. and G.F.S.; Writing – Review & Editing, G.B. and G.F.S.; Supervision,
581 G.B. and G.F.S.; Funding Acquisition, G.F.S. and G.B.

582 **Competing interests:** Authors declare no competing interests.

583

584 Supplementary Tables:

585 **Table S1:** Genomic bin coverage ratio of sample LIPA83 over ZK43.

586 **Table S2:** Gene coverage ratio of sample LIPA83 over ZK43.

587 **Table S3:** Sample information.

588 **Table S4:** *Candida albicans* gene CNVs.

589 **Table S5:** Gene correlation clusters in *Candida albicans*.

590 **Table S6:** Network analysis of gene CNVs in *Candida albicans*.

591 **Table S7:** Gene ontology and metabolic pathway enrichment analyses.

592 *Footnote: Terms and pathways with Benjamini – Hochberg adjusted p-values < 0.05 are*

593 *labeled in red.*

594 **Table S8:** Mapping statistics.

595

596 Supplementary Data:

597 **Supplementary Data 1:** Cancer cell lines SNVs.

598 **Supplementary Data 2:** GIP configuration files.

599 **Supplementary Data 3:** giptools commands.

600

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