

1 **A highly multiplexed assay to monitor pathogenicity,**
2 **fungicide resistance and gene flow in the fungal wheat**
3 **pathogen *Zymoseptoria tritici***

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20 **Abstract**

21
22 Crop pathogens pose severe risks to global food production due to the rapid rise of resistance to pesticides
23 and host resistance breakdowns. Predicting future risks requires monitoring tools to identify changes in
24 the genetic composition of pathogen populations. Here we report the design of a microfluidics-based
25 amplicon sequencing assay to multiplex 798 loci targeting virulence and fungicide resistance genes, and
26 randomly selected genome-wide markers for the fungal pathogen *Zymoseptoria tritici*. The fungus causes
27 one of the most devastating diseases on wheat showing rapid adaptation to fungicides and host resistance.
28 We optimized the primer design by integrating polymorphism data from 632 genomes of the same
29 species. To test the performance of the assay, we genotyped 192 samples in two replicates. Analysis of
30 the short-read sequence data generated by the assay showed a fairly stable success rate across samples to
31 amplify a large number of loci. The performance was consistent between samples originating from pure
32 genomic DNA as well as material extracted directly from infected wheat leaves. In samples with mixed
33 genotypes, we found that the assay recovers variations in allele frequencies. We also explored the
34 potential of the amplicon assay to recover transposable element insertion polymorphism relevant for
35 fungicide resistance. As a proof-of-concept, we show that the assay recovers the pathogen population
36 structure across French wheat fields. Genomic monitoring of crop pathogens contributes to more
37 sustainable crop protection and yields.

38 **Introduction**

39 Approximately 30 percent of all crop diseases are caused by fungi [1]. Plant pathogenic fungi affect crops
40 at various life cycle stages and plant tissues, including seeds, root and leaf development, and
41 inflorescence [2–5]. Yield reductions by pathogenic fungi cause food insecurity and economic losses
42 [6,7]. Crop protection is primarily achieved through the application of a variety of fungicides and
43 resistance breeding [8,9]. However, fungal pathogens have evolved resistance to all major fungicides
44 currently in use [10]. In addition, efforts to breed resistant crop varieties have repeatedly been defeated
45 by rapid evolutionary change in pathogen populations allowing them to circumvent resistance
46 mechanisms [9]. Predicting future breakdowns in fungicide efficacy and crop resistance remains
47 challenging. Fungicide resistance is monitored across the European continent by analyzing mutations in
48 known target genes related to the fungicide mode of action [11,12]. However, the rise of pathogen strains
49 defeating crop resistance is not comprehensively monitored. Notable exceptions include the screening of
50 rust fungi [13–16]. Notably, MARPLE (mobile and real-time plant disease) is a genomics-informed
51 monitoring tool developed to quickly detect wheat rust fungal pathogens *in situ* using using Nanopore
52 sequencing [17]. To reduce damage caused by plant pathogens, a timely and accurate detection of both
53 fungicide resistance mutations and mutations associated with the defeat of crop resistance is essential.

54
55 Fungal plant pathogen populations that evolved resistance to specific fungicides harbor numerous
56 mutations in or nearby the genes encoding the targets of the chemical compounds [10,18–20]. Similarly,
57 pathogen populations virulent on previously resistant crop varieties have often mutated or deleted a
58 specific set of genes that encode proteins recognized by the plant immune system [21–24]. Fungicide
59 resistance has traditionally been detected using *in vitro* fungicide sensitivity assays [25,26]. Such analyses
60 require the isolation and culturing of individual fungal strains that can then be tested for growth on media
61 containing different fungicide concentrations. The fungicide dose that effectively inhibits growth by 50%
62 is determined for comparison among samples (*i.e.*, EC50) [25,27]. The method is laborious and limited to

63 fungal species that can be cultured in absence of the host. With advances in molecular techniques, a
64 number of genetic screening methods have been developed including Sanger sequencing, TaqMan assays
65 based on fluorescently-tagged, allele-specific probes [28]. In general, such screening approaches are
66 labor-intensive and have low potential for multiplexing large numbers of individual loci. Virulence
67 surveillance of fungal plant pathogens has been implemented using simple sequence repeat (SSR)
68 markers [29,30] to distinguish the virulent Ug99 race from other *P. graminis* f. sp. *tritici* lineages
69 [30,31]. However, these SSR makers have been less useful in distinguishing different Ug99 race group
70 members [32]. Besides, virulence monitoring was also performed using loop-mediated isothermal
71 amplification (LAMP), see e.g. for the wilt *Fusarium oxysporum* f. sp. *lycopersici* (Fol) [33]. However,
72 LAMP assays can be expensive given costs of individual probes.

73
74 The advent of next generation sequencing (NGS) approaches has removed a series of limitations in
75 pathogen monitoring. The most general application of NGS techniques is whole genome sequencing
76 (WGS) that can be used to detect single nucleotide polymorphisms (SNPs) and structural variation [34].
77 Applications of WGS have contributed to the mapping and characterization of virulence and resistance
78 factors primarily through genome-wide association mapping [10,23,35,36]. Low-cost, high-throughput
79 methods based on NGS include reduced representation sequencing genotyping methods such as
80 restriction-site-associated DNA sequencing (RAD-seq) and Genotyping-by-Sequencing (GBS), both
81 methods rely on restriction enzymes to reduce genome size and complexity and exploring SNPs adjacent
82 to restriction enzyme sites [37,38]. However, such genotyping approaches assess only mutations near
83 restriction enzyme cut sites. Applications in fungal pathogens include fine-grained population structure
84 analyses, assessments of recombination rates, mapping of quantitative traits as well as the ability to
85 establish virulence profiles for clonal pathogens [39–44]. The analysis of individual regions involved in
86 fungicide resistance has been improved by the recent development of a PacBio long-read sequencing
87 assay based on the multiplex amplification of target genes in fungal wheat pathogen *Zymoseptoria tritici*.
88 The main advantage is the ability to generate long-reads capturing significant haplotype information of

89 individual strains revealing a series of alterations conferring increased resistance in response to different
90 commercial fungicides. However, due to the varying amplicon sizes generated by this assay two separate
91 multiplex PCRs were required to separate shorter and longer amplicons [26]. High degrees of
92 multiplexing for amplicons and samples were recently achieved using two parallel approaches for animal
93 and plant species. Genotyping-in-thousands by sequencing (GT-seq) is based on multiplex PCR targeted
94 amplicon sequencing to simultaneously genotype thousands of loci and hundreds of samples in a single
95 Illumina sequencing run [45]. A limitation of this approach is the extended time required for its
96 development (about ~4 months according to [46]). One challenge to overcome is imbalanced amplification
97 of individual loci and samples. Such bias can be reduced by the use of Fluidigm microfluidics assays,
98 which physically separate sets of amplicons and samples [47]. The fungal pathogen *Z. tritici* causes one of
99 the economically most important wheat diseases called Septoria tritici blotch (STB) [48]. The pathogen
100 has emerged at the onset of wheat domestication in the Middle East [49] and has since spread to all
101 wheat-producing areas of the world [50]. Populations have evolved resistance to all commercially used
102 fungicides and repeatedly across continents [23]. Major routes to resistances included the rise of
103 mutations in genes encoding the targets of the fungicide, in particular in *CYP51* encoding the target of
104 azoles [8,10]. Furthermore, upregulation of the transporter gene *MFS1* due to the insertion of transposable
105 elements in the promoter region contributed to azole resistance [20]. The rise of succinate dehydrogenase
106 inhibitor (SDHI) resistance mutations are the most recent of the observed gains in resistance (Fungicide
107 Resistance Action Committee, FRAC, 2021). In parallel to the rapid evolution to resist fungicides, *Z.*
108 *tritici* has also surmounted most known resistance factors segregating among wheat cultivars [51].
109 Association mapping in *Z. tritici* has recently revealed specific mutations underlying the gain of virulence
110 on previously resistant wheat cultivars including cultivars carrying the resistance gene *Stb6* and others
111 [35,52,53]. Recently, Amezrou et al. (unpublished) identified an additional 58 candidate pathogenicity
112 related genes based on association mapping on 12 wheat differential cultivars. The genes linked to gains
113 of virulence are typically referred to as effector genes and show rapid evolutionary change in populations
114 of *Z. tritici* [22,52,53]. Gene flow among *Z. tritici* populations is leading to significant weak

115 differentiation at the continental scale and high local diversity [50,53,54]. Monitoring of fungicide
116 resistance mutations is mainly achieved through the sequencing of target genes including the recent
117 development of long-read sequencing assays [26]. A joint monitoring of pathogenicity related mutations
118 and genetic diversity is lacking though.

119

120 Here, we report the design and validation of a microfluidics based multiplex targeted amplicon
121 sequencing assay that allows the simultaneous monitoring of mutations in fungicide resistance genes and
122 effector genes associated with a wide range of host resistance factors. In addition, we enable the
123 monitoring of hundreds of equally spaced polymorphisms along chromosomes to identify recent changes
124 in the genetic composition of pathogen populations. We validate the performance of the assay using
125 replication, sensitivity analyses to low input DNA, mixed samples as well as the performance on DNA
126 directly obtained from infected wheat leaves.

127 **Results**

128 *Marker design based on whole-genome sequenced individuals across species*

129 We used whole-genome sequencing datasets of 632 *Z. tritici* isolates collected in Oceania (Australia, New
130 Zealand), the United States, Switzerland, France, and Israel to identify segregating SNPs and improve the
131 design of a total of 798 amplicons of ~200 bp of length (except for the *MFS1* and *ZtSDHC3* loci). The
132 short and largely identical amplicon lengths improve PCR efficiency and balance among loci. Known
133 polymorphism within the species was used to mask sites to avoid primer mismatches and amplification
134 drop-outs (Fig. 1A). We designed 25 amplicons across genes associated with fungicide resistance
135 including *CYP51*, alternative oxidase (*AOX*), beta-tubulin (*TUB1*), *SDH1-4* genes including *ZtSDHC3*, as
136 well as *cytochrome b* (*CYTB*) (Table B in the File S1). For each gene, we prioritized amplicons covering
137 non-synonymous substitution if available. Due to the complexity of the transposable element insertion
138 polymorphism in the promoter region of the transporter gene *MFS1*, we designed a total of 16 primer
139 pairs for amplicons matching known sequence variants near three insertion sites [20] (Table B in File S1).
140 For loci associated with pathogenicity on diverse cultivars, we retained a set of 67 amplicons successfully
141 passing primer design (Table B in File S1). We also randomly selected SNPs at ~50 kb distances to
142 monitor the genetic make-up of populations for a total of 691 designed amplicons across all chromosomes
143 (Table B in File S1). The random SNP set also included by chance the previously selected fungicide
144 resistance gene *cytochrome b* (*CYTB*).

145

146 *Assessment of loci quality across the targeted sequencing assay*

147 We performed targeted sequencing of all 798 loci based on the Fluidigm Juno system in a single run
148 using microfluidics (Fig. 1B). The 192 samples included four sets of pure DNA from different isolates
149 mixed in equal proportions, ten samples including each DNA of the same three isolates in different
150 proportions, and 178 samples constituted from extracted leaf material from different wheat fields across
151 France mostly (*i.e.* $n = 172$), Belgium, Ireland and the United Kingdom. The complete set of samples was

152 replicated once for the amplification and Illumina sequencing step. The total sequencing output over both
153 replicates was 2,418,905,407 read pairs and 338.89 Gb. For 31 samples, the amplification and Illumina
154 sequencing procedures failed in either one of the two replicates of each sample, therefore the failed
155 replicates were eliminated. Across a replicate run (*i.e.* FC2), samples produced between 5,976-
156 173,049,611 read pairs with numbers broadly consistent between the two replicate runs (Fig. 2A). We
157 found that the mapping rate against the reference genome ranged from 96.93-100% among most sample
158 replicates (Fig. 2B).

159
160 To assess the faithful amplification of individual loci, we first focused on the four samples with mixtures
161 of pure fungal DNA of 26 to 30 isolates. Combining the two replicates, we used eight samples to evaluate
162 sequencing read coverage across the 782 amplicons designed outside of the *MFS1* region. We found 17
163 loci with a read depth of 0. The highest read depth was 1,779,927 for an effector locus on chromosome 1.
164 For the set of genome-wide, equally spaced amplicons on core chromosomes, we accepted the locus if the
165 read counts were between 20,000 and 100,000 in the retained samples (Fig. 2C). We considered this read
166 count range to reflect the loci consistently amplifying across samples and not showing evidence for
167 duplications. With this filter, we discarded 149 loci falling outside of the read count range (Fig. 2D). For
168 randomly selected markers on accessory chromosomes, we expected lower amplification success because
169 not all isolates of the species carry the locus. We retained loci with a read count between 10,000 and
170 100,000 in the set of reference samples leading to the rejection of 21 loci (Fig. 2C-D). For randomly
171 selected mitochondrial markers, we found read counts ranging from 203,502 to 1,372,965 in the set of
172 reference samples reflecting the high copy number of mitochondria compared to the nuclear genome. All
173 12 randomly selected mitochondrial loci were kept. For effector loci, the number of mapped reads ranged
174 from 502 to 1,779,927 reads indicating significant variation in the amplification success and possibly
175 copy number (Fig. 2C). We retained all 67 designed amplicons due to the general interest in
176 polymorphism at such loci (Fig. 2D). For resistance gene loci, the number of mapped reads ranged from
177 2,986-1,372,965 reads (Fig. 2C). As for effector gene loci, all 24 designed amplicons were retained (Fig.

178 2D). In addition, we retained the amplicon for the mitochondrial resistance locus of *CYTB* with a read
179 count of 1,372,965. In summary, we retained 521 high-quality loci representing 75% of the randomly
180 selected markers designed for genetic structure analyses, as well as all 67 effector and 24 fungicide
181 resistance loci (Fig. 2D).

182

183 *Reproducibility among replicate assays and recovery of allele frequencies*

184 To assess the reproducibility of the sequencing assay, we repeated the amplification and sequencing
185 procedure two times. We found that the number of read pairs recovered for each sample were positively
186 correlated between replicates ($r = 0.78$, $p\text{-value} < 0.0001$; Fig. 3A). We also found a positive correlation
187 in the mapping rate of reads recovered from the same samples ($r = 0.85$, $p\text{-value} < 0.0001$; Fig. 3B). To
188 investigate effects on allele frequencies assessed for mixed samples, we compared the pooled DNA of
189 population 41 sample. We used allele frequencies estimated from read depth for the reference and
190 alternative allele at SNP loci. Reference allele frequencies at 201 SNP loci calculated in both replicates of
191 each sample were highly correlated ($r = 0.89$, $p\text{-value} < 0.0001$) with outliers corresponding to poorly
192 covered loci in either one of the two replicates of the same sample (Fig. 3C).

193

194 Furthermore, we analyzed allele frequencies in ten samples (*i.e.* G1-G10) constituted from a mix of pure
195 DNA from the same three isolates in different proportions (INRA10-FS1006, INRA10-FS1022, IPO-
196 09455; Fig. 4; Table A in Supplementary File S1). We used existing whole genome sequencing and SNP
197 calling data for the three isolates to assess polymorphism across the genome [55]. Using the known
198 dilutions of pure DNA, we established the expected frequencies of reference alleles (*i.e.* matching the
199 allele present in the reference genome IPO323) or alternative alleles across loci. Then, we analyzed
200 mapped reads from the targeted sequencing assay from the mixed samples G1-G10 across all amplicons
201 to identify the proportion of reads matching the reference allele (Fig. 4). If the targeted sequencing assay
202 faithfully amplified DNA in mixed samples, the expected reference allele frequency in the mixed samples
203 should match the recovered proportion of reads matching the reference allele. Across the ten different

204 mixed samples, the match in reference allele frequencies was high in most samples (linear regression with
205 $R^2 > 0.55$ in 7 out of 10 mixtures). The mixed sample G1 showed no association between DNA dilutions
206 and recovered allele frequencies and two additional samples (G5 and G6) showed weak associations ($R^2 =$
207 0.27-0.45).

208

209 *SNP monitoring in fungicide resistance genes*

210 We investigated the amplification success for the amplicons covering the *CYP51* locus using eight sets of
211 mixed pure fungal DNA samples with read counts ranging from 10,115 to 31,323 reads. The genotyping
212 of infected wheat leaf samples from the field revealed that the target SNPs were indeed polymorphic. For
213 *CYP51* and the other fungicide resistance associated genes such as *TUB1*, *AOX*, *SDH2* and *SDH3* the
214 dominant genotype per wheat leaf varied among samples (see File S1, Tables D and E). The reference
215 genome isolate IPO323 is generally susceptible to different fungicide classes. Hence, the allele carried by
216 the reference genome is likely associated with higher susceptibility. Consistent with recent gains in
217 fungicide resistance, mutations in the beta-tubulin and *CYP51* locus tended to be different from the
218 reference genome (*i.e.* the alternative allele, Table E in File S1). Loci without recent strong recent gains
219 more likely retained the IPO323 genotype (*i.e.* reference allele, Table E in File S1).

220

221 *Amplicons for the promoter region of MFS1*

222 The amplicons designed for the promoter region of *MFS1* are matching known haplotypes differing in
223 their insertion of transposable element sequences. Due to the sequence complexity, we chose to first
224 cluster sequencing reads into individual amplicons instead of directly mapping reads to a *MFS1*
225 haplotype. Analyzing the 10 samples with different DNA mixtures of three isolates including replicates,
226 we identified 10 sequence clusters with at least 22 reads (lowest number observed in sample G3). We
227 used BLAST to retrieve the subset ($n = 10$) of the clustered sequences matching the *MFS1* promoter
228 region. The sequences matched positions from 1-4946 bp (for sample G9) on the consensus *MFS1*
229 sequence with all being upstream of the coding sequence as expected (Fig. 5A). We did not recover any

230 amplicon matching forward and/or reverse primer positions based on the amplicon design (Fig. 5B).
231 However, all amplicons did not match the expected amplicon length most likely due to the complexity of
232 the underlying sequence. Furthermore, the pooled amplification of multiple primer pairs matching the
233 promoter region has likely produced chimeric amplicons in some contexts. We used the retrieved
234 amplicons matching the promoter region to form clusters of near identical BLAST matches based on
235 alignment length and positions. We identified 10 well supported amplicon clusters showing variation in
236 abundance among the analyzed samples (See Table C in File S1).

237

238 *Genetic differentiation in French and European wheat field populations*

239 We used the 158 wheat leaf samples infected by *Z. tritici* collected from fields across France with at least
240 five genotyped samples per location and additional samples from Belgium, Ireland and the United
241 Kingdom to assess the genetic structure using the genome-wide marker set (Supplementary Figure 1 in
242 File S2). Based on a principal component analysis of 85 genome-wide SNPs, we found no clear
243 differentiation among samples originating from different countries (Fig. 6A). Focusing on the genetic
244 differentiation among French regions ($n = 82$ genome-wide SNPs for the French populations only), we
245 found some modest differentiation of genotypes from wheat fields in Midi-Pyrénées and Champagne
246 (Fig. 6B). However, the overall differentiation of the field samples was low with the first and second
247 principal component explaining only ~4%.

248

249 **Discussion**

250
251 We developed a microfluidics-based amplicon sequencing assay combining the advantages of high-
252 throughput sequencing and multiplex PCR. We assessed the performance of 798 loci to reliably and
253 sensitively genotype randomly selected genome-wide markers, as well as pathogenicity and fungicide
254 resistance-related genes in a diverse set of *Z. tritici* samples. We show that a large portion of the designed
255 markers can be amplified consistently across samples, used to monitor the emergence of relevant
256 mutations and provide an assessment of allele frequencies in mixed samples. The set of genome-wide
257 markers provides means to assess the genetic structure of the pathogen directly from field collected wheat
258 leaves.

259
260 Within-species polymorphism can lead to amplification failures due to mismatching primers. We
261 considered this issue particularly relevant for the wheat pathogen *Z. tritici* as the species harbors
262 genetically highly diverse populations within single fields [53]. As expected, we detected a high number
263 of SNPs in regions intended for amplicon design leading to the rejection of amplicon candidates prior to
264 the genotyping stage. Furthermore, we noticed targeted regions with weak amplification success. The
265 poor performance of some primer pairs is most likely explained by a combination of factors. First, we
266 ignored low-frequency SNPs at the masking stage to be able to proceed to the amplicon design for more
267 loci. Second, our species-wide genomic survey of SNPs may have missed polymorphisms present in the
268 assayed samples. The filtering thresholds can be adjusted and more genome sequencing datasets could be
269 included in future amplicon design efforts. Despite some failed attempts at amplifying individual loci, we
270 obtained high degrees of sequencing read coverage for most loci. Most samples yielded hundreds to
271 thousands of reads for each locus. Such deep coverage across the amplicon assay provides a detailed
272 picture of genotypic diversity particularly for mixed samples directly obtained from infected leaves. A
273 major limitation with the multiplexed amplicon sequencing assay is the shortness of the amplified
274 sequence (~200 bp). The short amplicon length ensures a high degree of multiplexing by providing stable

275 amplification across the entire assay. However, longer amplicons would be needed to recover entire
276 sequences (*i.e.* haplotypes) of the azole resistance locus *CYP51* or several effector genes of interest. A
277 potential solution would be to design overlapping amplicons to cover an entire locus. However, this
278 approach was unsuccessful *e.g.* for the effector gene *AvrStb6* providing no sufficiently conserved sections
279 inside or adjacent to the coding sequence for an overlapping amplicon design. Limitations in amplicon
280 length and haplotype resolution can be overcome using long-read sequencing as developed to monitor
281 fungicide resistance loci in *Z. tritici* [26]. Long-read sequencing may also help to overcome issues with
282 amplifying the highly polymorphic promoter region of *MFS1*. Long amplicons could capture the entire
283 promoter region instead of focusing on individual insertion points. However, long-read approaches are
284 not well-suited to amplify hundreds of loci consistently across many samples. Ultimately, a combination
285 of different approaches performing highly multiplexed short reads sequencing and separate long-read
286 sequencing for the most complex loci will be required.

287
288 A versatile pathogen genotyping assay should perform well with low-input pure fungal DNA as well as
289 mixed samples containing DNA both from multiple strain genotypes and the host (*i.e.* wheat plants). We
290 find that the assay replicated well across most of the tested sample types both in terms of the number of
291 recovered reads per sample as well as the proportion of reads that could be mapped to the *Z. tritici*
292 reference genome. Besides, we found that in mixed samples (*i.e.* containing more than one genotype), the
293 assay reproduces well the allele frequencies across the two independent genotyping runs. We also
294 assessed the ability of the assay to recover allele frequencies of mixtures of known isolates. Using known
295 genotypes of three isolates as a control, the amplicon assay recovered well the allele frequencies in most
296 tested mixtures. The weak performance of some individual mixtures is likely due to errors during
297 handling rather than a general issue of reproducing allele frequencies. The accurate recovery of allele
298 frequencies in a mixed sample is clearly contingent on sufficient sequencing depth though and we have
299 evaluated the performance only at loci with ≥ 50 mapped reads. The genotyping of transposable element
300 insertions in the *MFS1* promoter region was not conclusive. The overlapping amplicons and very high

301 levels of sequence polymorphism prevented a clear assignment of amplicons to transposable element
302 insertion genotypes, but our data opens up a path for a more comprehensive design strategy to capture
303 inserted sequences.

304

305 The microfluidics-based multiplex PCR targeted amplicon sequencing requires bioinformatics analyses
306 both for the design of the amplicons and for the genotyping after a successful run. Nearly all designed
307 amplicons and associated primer sequences could be used also outside of a microfluidics protocol. A
308 technically less demanding version of our approach is typically referred to as GT-seq, which consists in
309 amplifying loci in large pools of primer pairs and indices to distinguish samples [45]. Given the short
310 amplicons, using individual primer pairs for targeted qPCR assays would also be possible. The number of
311 recovered loci for targeted amplicon sequencing remains below untargeted approaches such as RAD-seq
312 and GBS. Untargeted reduced-representation approaches provide however only genome-wide information
313 on genetic differentiation. This may be informative *e.g.* for virulence profiles in clonal pathogens [43],
314 however this approach is unsuitable to recover genotypes at specific loci. Targeted amplification such as
315 the microfluidics based multiplex PCR performs also well in mixed samples. RAD-seq and GBS are
316 unlikely to perform well if substantial proportions of plant DNA are present, because large plant genomes
317 will typically contain many more restriction sites compared to fungal genomes.

318

319 The developed microfluidics-based targeted amplicon assay allows a cost-effective and reproducible
320 monitoring of hundreds of loci to track mutations at pathogenicity loci and fungicide resistance evolution
321 in field populations. The integration of genome-wide markers greatly enhances the quality of pathogen
322 monitoring by providing information about patterns of gene flow. Our study revealed only weak
323 differentiation across Western European countries and among French regions consistent with high levels
324 of gene flow and genetic diversity [55]. Knowledge of genetic structure can help identify recent
325 movements of the pathogen due to natural or human-mediated dispersal. The rapid rise in resistance of *Z.*
326 *tritici* populations after the application of fungicides can more effectively be monitored due to the large

327 number of loci that can be assayed simultaneously. Furthermore, tracking mutations at effector loci opens
328 new opportunities to track adaptation to different wheat cultivars across regions. With the availability of
329 whole genome sequencing data for an increasing number of crop pathogens, the targeted amplicons could
330 be expanded to simultaneously or separately genotype other major pathogens including rusts to improve
331 the surveillance and management of crop diseases globally.

332

333

334 **Methods**

335 *Genome sequences used for the design of the assay*

336 The amplicons were designed based on known polymorphisms within the species. Polymorphic sites were
337 used both to select SNPs to amplify but also to mask polymorphisms to avoid primer binding mismatches.
338 For this, we used whole genome sequencing information from 632 *Z. tritici* isolates collected across the
339 global distribution range of wheat. Isolates included six different populations with a sample size of 29-
340 178. A total of 88 isolates were collected in Australia including Tasmania in 2001 and 2015 [22].
341 Additional isolates from Oceania included 75 isolates collected in New Zealand in 2013 and 2015 [55]. A
342 total of 154 isolates were collected in Oregon, USA, in 1990 and 2015 [22]. 178 isolates were in wheat
343 fields near Zurich in Switzerland in 1999 and 2016 [22,53] and 29 isolates were isolated in the Nahal Oz
344 region in Israel in 1992 [22]. Finally, 108 isolates were retrieved from a panel of French isolates [35].

345

346 *SNP calling and identification of polymorphisms for the amplicon design*

347 We performed read alignment and SNP discovery for the generated genomic datasets, as previously
348 described [22,35]. In summary, we trimmed raw Illumina reads using Trimmomatic v. 0.38 [56] and
349 mapped retained reads to the reference genome IPO323 [57] using bowtie v2.3.5 [58]. We used the
350 Genome Analysis Toolkit (GATK) v4.0.1 [59] including the HaplotypeCaller tool to identify candidate
351 SNPs. We filtered for a set of high-quality polymorphisms using the GATK VariantFiltration tool and
352 vcftools v.0.1.15 [60]. A more extensive description of the filtering procedures and validations are
353 available [61].

354

355 *Polymorphism selection for neutral markers, pathogenicity and fungicide resistance genes*

356 Effector candidate genes were retrieved from GWAS focused to identify candidate effectors interacting
357 with major wheat resistance genes (Amezrou et al., unpublished)[52,61]. We included 65 candidate
358 effector genes showing a significant association for symptom development on at least one wheat cultivar.

359 We designed at least one amplicon overlapping the most significantly associated SNP in each of the
360 effector genes. If a significantly associated SNP could not be reproduced in the worldwide isolate
361 collection, a random nearby SNP (within ~200 bp) was selected as the target for the amplicon design. If a
362 different SNP was selected, we filtered for SNPs with a minor allele count of 5 and a minimal genotyping
363 rate of 80%. For the effector gene *AvrStb6*, we designed two additional amplicons to cover polymorphism
364 in the coding sequence. To monitor fungicide resistance gene mutations, we covered 25 genes related to
365 fungicide resistance in *Z. tritici* populations including the mitochondrial genes *CYTB* and *AOX*, the
366 nuclear genes beta tubulin 1 (carbendazim resistance), *CYP51* (azole resistance), as well as *SDH1*, *SDH2*,
367 *SDH3* and *SDH4* (SDHI resistance). The amplicons covered resistance mutations if known for the
368 species. If no mutation was previously documented in *Z. tritici*, the amplicon covered randomly selected
369 SNPs in the coding sequence. Similar to the procedure for effector loci, if a known SNP associated with
370 fungicide resistance could not be recovered, we selected a SNP within ~100 bp (minor allele count of 3,
371 minimum genotyping rate 80%). The broader inclusion of polymorphisms for filtering was possible due
372 to the generally lower degree of detected variants in resistance genes. We defined an additional amplicon
373 to target the paralog of *SDH3* (*ZtSDHC3*) [62]. For this, we analyzed the paralog sequence discovered in
374 the pangenome of *Z. tritici* [63].

375
376 Multidrug fungicide resistance in *Z. tritici* is mediated by transposable element insertions in the promotor
377 region of the transporter *MFS1*. We designed 16 amplicons covering three previously reported
378 transposable element insertions and haplotypes [20]. The amplicons were designed to either amplify if an
379 insertion was present or not. Amplicons were designed on a consensus sequence of previously described
380 haplotypes [20]. In addition to polymorphisms related to pathogenicity and fungicide resistance, we
381 randomly selected equally spaced polymorphisms along all 21 chromosomes to capture neutral population
382 structure. For this, we selected 691 SNPs with a minor allele frequency of 5% and a minimal genotyping
383 rate of 80%. SNPs were selected at a distance of 50 kb (if available) using the *--thin* option in *vcftools*. In
384 summary, a total of 798 amplicons were designed for pathogenicity, fungicide resistance as well as gene

385 flow tracking across *Z. tritici* populations. See Table B in File S1 for details on all selected effector and
386 fungicide resistance genes as well as whole genome neutral markers.

387

388 *Amplicon design*

389 For genome-wide markers and markers in effector and fungicide resistance genes (except *ZtSDHC3* and
390 *MFS1*), we extracted a 401 bp sequence from the reference genome centered on the SNP to target. The
391 extracted sequence was centered around the target SNP, which was marked by IUPAC code and
392 parentheses according to company instructions. The sequence was then used to define primers amplifying
393 a ~200 bp stretch of DNA including the target SNP. The amplicon length was limited to ~200 bp to
394 ensure efficient and balanced amplification across loci. To improve amplification success across a broad
395 range of *Z. tritici* genotypes, we masked known polymorphic sites on the sequence containing the targeted
396 SNP to prevent accidental primer design in known polymorphic regions. We used bcftools v1.9 [64] to
397 mask non-target sites showing evidence for polymorphism in the panel of 632 analyzed isolates using the
398 -I option of the consensus command and re-wrote sequences with samtools v1.9 [65]. For resistance and
399 pathogenicity loci, we used a minor allele count of 3 and 5, respectively to consider the polymorphism for
400 masking. For genome-wide markers, we used a minor allele frequency cut-off of 5%. If the resulting
401 sequence contained more than 10% masked sites, the amplicon was not considered further. Additional
402 sequences were excluded by Fluidigm Inc. if the sequences failed to yield adequate primer candidates for
403 the desired ~200 bp amplicons. If the initial amplicon design had failed, we repeated the procedure for
404 effector loci but relaxed the filter to consider only SNPs with a minor allele count of ≥ 25 .

405

406 *Samples included for the validation of the amplicon sequencing assay*

407 We assessed the performance of the microfluidics assay using different sets of samples collected from
408 wheat fields in Europe. Four samples included equimolar DNA mixtures of 26 to 30 isolates obtained by
409 culturing single spore isolates from field-collected wheat leaves. Three single spore isolates identified as
410 INRA10-FS1006, INRA10-FS1022 and IPO-09455 were collected in 2009 and 2010 in the Ile-de-France

411 region and were used to create DNA mixtures in ten different proportions (samples G1-G10). Finally, 178
412 samples were obtained by extracting DNA directly from infected wheat leaves collected in different
413 regions of France, Belgium, Ireland and United Kingdom (Table A in File S1). No permit is required to
414 collect naturally infected wheat leaves.

415

416 *DNA extractions and microfluidics assay*

417 DNA extractions to test the microfluidics assay were performed using the following procedures. For pure
418 cultures and directly from infected wheat leaves using DNeasy® Plant Mini Kit (Qiagen, Hilden,
419 Germany). DNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher, Waltham, Massachusetts,
420 USA). We followed the Fluidigm Inc. (San Francisco, California, USA) Juno™ targeted amplicon
421 sequencing protocol according to the manufacturer's protocol. As input DNA, we used the following 1.5-
422 200 ng of total amount (See Table A in Supplementary File S1). We performed the entire microfluidics
423 procedure twice independently on different Juno LP 192.24 integrated fluidic circuits plate (IFC).
424 Libraries were prepared following the manufacturer's protocol. Target amplicons were generated for each
425 sample and pools of primers using PCR on a specialized thermocycler (Juno system; Fluidigm). Illumina
426 sequencing was performed in paired-end mode to generate 100 bp reads on the NovaSeq™6000 platform
427 at Integragen Inc. (Evry, France) and produced 363.89 Gb of raw sequencing data for both independent
428 chips combined.

429

430 *Amplicon sequence data analyses*

431 We used Trimmomatic v0.38 [56] with the following parameters: LEADING:3 TRAILING:3
432 SLIDINGWINDOW:4:15 MINLEN:36. Due to the short amplicon length compared to the read lengths,
433 we used FLASH v1.2.11 [66] to merge forward and reverse reads per pair into single pseudo-reads.
434 Finally, pseudo-reads were aligned to the IPO323 reference genome using bowtie2 v2.3.5 [57,58]. We
435 assessed individual read counts at each analysis step using MultiQC v.1.7 [67]. After individual
436 genotyping using the GATK HaplotypeCaller tool, we performed multi-sample genotype calling using

437 CombineGVCFs and GenotypeGVCFs [68]. Variant sites were removed if these met the following
438 conditions: $QD < 5$, $MQ < 20$, $-2 > ReadPosRankSum > 2$, $-2 > MQRankSum > 2$, $-2 > BaseQRankSum$
439 > 2 .

440

441 The DNA mixtures (G1-G10) contained three isolates INRA10-FS1006, INRA10-FS1022 and IPO-09455
442 with existing SNP genotyping information [55]. Isolates in mixed samples were diluted in different
443 proportions to cover a range of isolate mixtures. To assess the reproducibility of allele frequencies of the
444 mixed DNA samples, we analyzed mapped reads at each SNP genotyped using the amplicon sequencing
445 assay. Expected proportions of reference alleles (matching the reference genome IPO323) were inferred
446 in mixed samples using the known genotypes of the isolates. Only amplicon sequencing loci with a
447 minimum read coverage of 50 were considered to reduce noise in allele frequency assessments. For
448 amplicons targeting the promoter region of *MFS1*, we first used seqtk [69] to subsample 10,000,000 reads
449 from large merged paired-end reads FASTQ files and we then performed a clustering analysis of Illumina
450 reads to obtain read sets originating from the same locus. We used CD-HIT-EST [70] with an identity
451 threshold set to 100% to cluster sequencing reads. For each cluster, the representative sequence identified
452 by CD-HIT-EST was aligned to the *MFS1* promoter consensus sequence using BLASTn 2.12.0 [71].
453 Only BLASTn best hits with a bit score above 100 and identity $> 90\%$ were kept. To identify clusters of
454 nearly identical hits based on position and identity, we performed *k*-means clustering with the R packages
455 {factoextra}[72], {clustertend} [73], {cluster} [74], {NbClust} [75]. For each sample, we identified the
456 optimal number of clusters ($K = 1-10$) by performing a silhouette analysis [76].

457

458 *Data visualization and population genetic analyses*

459 Data analyses were performed using R 4.0.4 [77]. The R packages included in {tidyverse} [78] were
460 used for summarizing and plotting coverage across loci, visualizing retained SNPs, the outcomes of
461 different filtering stages and genotyping. We used bcftools v1.9 [64] to calculate allele frequencies at
462 SNP loci. The allele frequency correlation between both flow cells chips was analyzed with the R

463 package {report} [79] and visualized using {ggpubr} [80] and {ggplot2} [81]. To analyze genetic
464 diversity and population structure, we performed a principal component analysis (PCA) using the R
465 packages {vcfR} [82], {adegenet}[83], {ade4} [84] and {ggplot2} [81]. For population analyses, we
466 focused only on the second replicate (flow cell) and genome-wide SNPs without effector and resistance
467 gene loci to reflect neutral population structure. Loci were filtered for a minor allele frequency of 0.05
468 and allowing for 20% missing data (--max-missing 0.8).

469

470 **Data availability:** Raw sequencing data is available on the NCBI Sequence Read Archive (SRA) under
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472

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482

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487 **References**

- 488
- 489 1. Jain A, Sarsaiya S, Wu Q, Lu Y, Shi J. A review of plant leaf fungal diseases and its
490 environment speciation. *Bioengineered*. 2019;10: 409–424.
491 doi:<https://doi.org/10.1080/21655979.2019.1649520>
- 492 2. Gonzalez M, Pujol M, Metraux J, Gonzalez-Garcia V, Bolton MD, BORRÁS HIDALGO
493 O. Tobacco leaf spot and root rot caused by *Rhizoctonia solani* Kühn. *Molecular plant*
494 *pathology*. 2011;12: 209–216. Available: <https://doi.org/10.1111/j.1364-3703.2010.00664.x>
- 495 3. Serrato-Diaz L, Rivera-Vargas L, French-Monar R. First report of *Neofusicoccum*
496 *mangiferae* causing rachis necrosis and Inflorescence blight of mango (*Mangifera indica*) in
497 Puerto Rico. *Plant Disease*. 2014;98: 570–570. doi:10.1094/PDIS-08-13-0878-PDN
- 498 4. He C, Zhang Y, Zhou W, Guo Q, Bai B, Shen S, et al. Study on stripe rust (*Puccinia*
499 *striiformis*) effect on grain filling and seed morphology building of special winter wheat
500 germplasm Huixianhong. *Plos one*. 2019;14: e0215066. doi:10.1371/journal.pone.0215066
- 501 5. Zhang X, Wang R, Ning H, Li W, Bai Y, Li Y. Evaluation and management of fungal-
502 infected carrot seeds. *Scientific Reports*. 2020;10: 1–8. doi:10.1038/s41598-020-67907-5
- 503 6. Avery SV, Singleton I, Magan N, Goldman GH. The fungal threat to global food security.
504 *Fungal biology*. 2019;123: 555–557. doi:10.1016/j.funbio.2019.03.006
- 505 7. Horbach R, Navarro-Quesada AR, Knogge W, Deising HB. When and how to kill a plant
506 cell: infection strategies of plant pathogenic fungi. *Journal of plant physiology*. 2011;168:
507 51–62. doi:10.1016/j.jplph.2010.06.014
- 508 8. Corkley I, Fraaije B, Hawkins N. Fungicide resistance management: Maximizing the
509 effective life of plant protection products. *Plant Pathology*. 2022;71: 150–169.
510 doi:10.1111/ppa.13467
- 511 9. Nelson R, Wiesner-Hanks T, Wisser R, Balint-Kurti P. Navigating complexity to breed
512 disease-resistant crops. *Nature Reviews Genetics*. 2018;19: 21–33. doi:10.1038/nrg.2017.82
- 513 10. Mohd Assaad N, McDonald BA, Croll D. Multilocus resistance evolution to azole
514 fungicides in fungal plant pathogen populations. *Molecular ecology*. 2016;25: 6124–6142.
515 doi:10.1111/mec.13916
- 516 11. Brent KJ, Hollomon DW. Fungicide resistance: the assessment of risk (Vol. 2). *Global Crop*
517 *Protection Federation Brussels, Belgium*; 1998.
- 518 12. Klappach BK, Stammler G, Bryson R, Semar M, Mehl BA, Steiger D, et al. Succinate
519 Dehydrogenase Inhibitor (SDHI) Working Group. Meeting on January (Vol. 21, p. 22);
520 2020. p. 22. Available: [https://www.frac.info/docs/default-source/working-groups/sdhi-](https://www.frac.info/docs/default-source/working-groups/sdhi-fungicides/sdhi-meeting-minutes/minutes-of-the-2020-sdhi-meeting-21-22th-of-january-2020-with-recommendations-for-2020.pdf?sfvrsn=5918499a_2)
521 [fungicides/sdhi-meeting-minutes/minutes-of-the-2020-sdhi-meeting-21-22th-of-january-](https://www.frac.info/docs/default-source/working-groups/sdhi-fungicides/sdhi-meeting-minutes/minutes-of-the-2020-sdhi-meeting-21-22th-of-january-2020-with-recommendations-for-2020.pdf?sfvrsn=5918499a_2)
522 [2020-with-recommendations-for-2020.pdf?sfvrsn=5918499a_2](https://www.frac.info/docs/default-source/working-groups/sdhi-fungicides/sdhi-meeting-minutes/minutes-of-the-2020-sdhi-meeting-21-22th-of-january-2020-with-recommendations-for-2020.pdf?sfvrsn=5918499a_2)

- 523 13. Park R, Fetch T, Hodson D, Jin Y, Nazari K, Prashar M, et al. International surveillance of
524 wheat rust pathogens: progress and challenges. *Euphytica*. 2011;179: 109–117.
525 doi:10.1007/s10681-011-0375-4
- 526 14. Olivera Firpo P, Newcomb M, Flath K, Sommerfeldt Impe N, Szabo L, Carter M, et al.
527 Characterization of *Puccinia graminis* f. sp. *tritici* isolates derived from an unusual wheat
528 stem rust outbreak in Germany in 2013. *Plant Pathology*. 2017;66: 1258–1266.
529 doi:<https://doi.org/10.1111/ppa.12674>
- 530 15. Cook NM, Chng S, Woodman TL, Warren R, Oliver RP, Saunders DG. High frequency of
531 fungicide resistance-associated mutations in the wheat yellow rust pathogen *Puccinia*
532 *striiformis* f. sp. *tritici*. *Pest Management Science*. 2021;77: 3358–3371.
533 doi:10.1002/ps.6380
- 534 16. Fontyn C, Zippert A-C, Delestre G, Marcel TC, Suffert F, Goyeau H. Is virulence
535 phenotype evolution driven exclusively by Lr gene deployment in French *Puccinia triticina*
536 populations? *Plant Pathology*. 2022;00: 1–14. doi:10.1111/ppa.13599
- 537 17. Radhakrishnan GV, Cook NM, Bueno-Sancho V, Lewis CM, Persoons A, Mitiku AD, et al.
538 MARPLE, a point-of-care, strain-level disease diagnostics and surveillance tool for
539 complex fungal pathogens. *BMC biology*. 2019;17: 1–17. doi:10.1186/s12915-019-0684-y
- 540 18. Bowyer P, Mosquera J, Anderson M, Birch M, Bromley M, Denning DW. Identification of
541 novel genes conferring altered azole susceptibility in *Aspergillus fumigatus*. *FEMS*
542 *microbiology letters*. 2012;332: 10–19. doi:10.1111/j.1574-6968.2012.02575.x
- 543 19. Deising HB, Reimann S, Pascholati SF. Mechanisms and significance of fungicide
544 resistance. *Brazilian Journal of Microbiology*. 2008;39: 286–295. doi:10.1590/S1517-
545 838220080002000017
- 546 20. Omrane S, Audéon C, Ignace A, Duplaix C, Aouini L, Kema G, et al. Plasticity of the
547 MFS1 promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*.
548 *MSphere*. 2017;2: e00393-17. doi:10.1128/mSphere.00393-17
- 549 21. Kashiwa T, Suzuki T, Sato A, Akai K, Teraoka T, Komatsu K, et al. A new biotype of
550 *Fusarium oxysporum* f. sp. *lycopersici* race 2 emerged by a transposon-driven mutation of
551 avirulence gene AVR1. *FEMS Microbiology Letters*. 2016;363: fnw132.
552 doi:10.1093/femsle/fnw132
- 553 22. Hartmann FE, Sánchez-Vallet A, McDonald BA, Croll D. A fungal wheat pathogen
554 evolved host specialization by extensive chromosomal rearrangements. *The ISME journal*.
555 2017;11: 1189–1204. doi:<https://doi.org/10.1038/ismej.2016.196>
- 556 23. Hartmann FE. Using structural variants to understand the ecological and evolutionary
557 dynamics of fungal plant pathogens. *New Phytologist*. 2022;234: 43–49.
558 doi:10.1111/nph.17907

- 559 24. Olivera PD, Villegas D, Cantero □ Martínez C, Szabo LJ, Rouse MN, Luster DG, et al. A
560 unique race of the wheat stem rust pathogen with virulence on Sr31 identified in Spain and
561 reaction of wheat and durum cultivars to this race. *Plant Pathology*. 2022;71: 873–889.
562 doi:<https://doi.org/10.1111/ppa.13530>
- 563 25. Saville A, Graham K, Grünwald NJ, Myers K, Fry WE, Ristaino JB. Fungicide sensitivity
564 of US genotypes of *Phytophthora infestans* to six oomycete-targeted compounds. *Plant*
565 *Disease*. 2015;99: 659–666. doi:10.1094/PDIS-05-14-0452-RE
- 566 26. Samils B, Andersson B, Edin E, Elfstrand M, Rönneburg T, Bucur D, et al. Development of
567 a PacBio long-read sequencing assay for high throughput detection of fungicide resistance
568 in *Zymoseptoria tritici*. *Frontiers in microbiology*. 2021;12: 692845. Available:
569 <https://10.3389/fmicb.2021.692845>
- 570 27. Wang J, Bradley CA, Stenzel O, Pedersen DK, Reuter-Carlson U, Chilvers MI. Baseline
571 sensitivity of *Fusarium virguliforme* to fluopyram fungicide. *Plant disease*. 2017;101: 576–
572 582. doi:<https://doi.org/10.1094/PDIS-09-16-1250-RE>
- 573 28. Schleinitz D, DiStefano JK, Kovacs P. Targeted SNP genotyping using the TaqMan®
574 assay. *Disease gene identification*. Springer; 2011. pp. 77–87. Available:
575 https://doi.org/10.1007/978-1-61737-954-3_6
- 576 29. Zhong S, Leng Y, Friesen T, Faris J, Szabo L. Development and characterization of
577 expressed sequence tag-derived microsatellite markers for the wheat stem rust fungus
578 *Puccinia graminis* f. sp. *tritici*. *Phytopathology*. 2009;99: 282–289.
579 doi:<https://doi.org/10.1094/PHTO-99-3-0282>
- 580 30. Visser B, Herselman L, Park RF, Karaoglu H, Bender CM, Pretorius ZA. Characterization
581 of two new *Puccinia graminis* f. sp. *tritici* races within the Ug99 lineage in South Africa.
582 *Euphytica*. 2011;179: 119–127. doi:<https://doi.org/10.1007/s10681-010-0269-x>
- 583 31. Jin Y, Szabo L, Pretorius Z, Singh R, Ward R, Fetch Jr T. Detection of virulence to
584 resistance gene Sr24 within race TTKS of *Puccinia graminis* f. sp. *tritici*. *Plant Disease*.
585 2008;92: 923–926. doi:<https://doi.org/10.1094/PDIS-92-6-0923>
- 586 32. Singh RP, Hodson DP, Jin Y, Lagudah ES, Ayliffe MA, Bhavani S, et al. Emergence and
587 spread of new races of wheat stem rust fungus: continued threat to food security and
588 prospects of genetic control. *Phytopathology*. 2015;105: 872–884. doi:10.1094/PHTO-01-
589 15-0030-FI
- 590 33. Ayukawa Y, Hanyuda S, Fujita N, Komatsu K, Arie T. Novel loop-mediated isothermal
591 amplification (LAMP) assay with a universal QProbe can detect SNPs determining races in
592 plant pathogenic fungi. *Scientific reports*. 2017;7: 1–9. doi:10.1038/s41598-017-04084-y
- 593 34. Cao K, Zhou Z, Wang Q, Guo J, Zhao P, Zhu G, et al. Genome-wide association study of
594 12 agronomic traits in peach. *Nature Communications*. 2016;7: 13246.
595 doi:10.1038/ncomms13246

- 596 35. Zhong Z, Marcel TC, Hartmann FE, Ma X, Plissonneau C, Zala M, et al. A small secreted
597 protein in *Zymoseptoria tritici* is responsible for avirulence on wheat cultivars carrying the
598 *Stb6* resistance gene. *New Phytologist*. 2017;214: 619–631.
599 doi:<https://doi.org/10.1111/nph.14434>
- 600 36. Pereira D, McDonald BA, Croll D. The genetic architecture of emerging fungicide
601 resistance in populations of a global wheat pathogen. *Genome biology and evolution*.
602 2020;12: 2231–2244. doi:10.1093/gbe/evaa203
- 603 37. Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, et al. Rapid SNP
604 discovery and genetic mapping using sequenced RAD markers. *PloS one*. 2008;3: e3376.
605 doi:10.1371/journal.pone.0003376
- 606 38. Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, et al. A robust,
607 simple genotyping-by-sequencing (GBS) approach for high diversity species. *PloS one*.
608 2011;6: e19379. doi:<https://doi.org/10.1371/journal.pone.0019379>
- 609 39. Croll D, Lendenmann MH, Stewart E, McDonald BA. The impact of recombination
610 hotspots on genome evolution of a fungal plant pathogen. *Genetics*. 2015;201: 1213–1228.
611 doi:10.1534/genetics.115.185280
- 612 40. Talas F, McDonald BA. Genome-wide analysis of *Fusarium graminearum* field populations
613 reveals hotspots of recombination. *BMC genomics*. 2015;16: 1–12. doi:10.1186/s12864-
614 015-2166-0
- 615 41. Lendenmann MH, Croll D, Palma-Guerrero J, Stewart EL, McDonald BA. QTL mapping of
616 temperature sensitivity reveals candidate genes for thermal adaptation and growth
617 morphology in the plant pathogenic fungus *Zymoseptoria tritici*. *Heredity*. 2016;116: 384–
618 394. doi:10.1038/hdy.2015.111
- 619 42. Stewart EL, Croll D, Lendenmann MH, Sanchez Vallet A, Hartmann FE,
620 Palma Guerrero J, et al. Quantitative trait locus mapping reveals complex genetic
621 architecture of quantitative virulence in the wheat pathogen *Zymoseptoria tritici*. *Molecular*
622 *plant pathology*. 2018;19: 201–216. doi:10.1111/mpp.12515
- 623 43. Aoun M, Kolmer JA, Breiland M, Richards J, Brueggeman RS, Szabo LJ, et al.
624 Genotyping-by-sequencing for the study of genetic diversity in *Puccinia triticina*. *Plant*
625 *Disease*. 2020;104: 752–760. doi:10.1094/PDIS-09-19-1890-RE
- 626 44. Wyka S, Mondo S, Liu M, Nalam V, Broders K. A large accessory genome and high
627 recombination rates may influence global distribution and broad host range of the fungal
628 plant pathogen *Claviceps purpurea*. *PloS one*. 2022;17: e0263496.
629 doi:<https://doi.org/10.1371/journal.pone.0263496>
- 630 45. Campbell NR, Harmon SA, Narum SR. Genotyping in Thousands by sequencing
631 (GT²seq): A cost effective SNP genotyping method based on custom amplicon
632 sequencing. *Mol Ecol Resour*. 2015;15: 855–867. doi:10.1111/1755-0998.12357

- 633 46. Meek MH, Larson WA. The future is now: Amplicon sequencing and sequence capture
634 usher in the conservation genomics era. *Molecular Ecology Resources*. 2019;19: 795–803.
635 Available: <https://doi.org/10.1111/1755-0998.12998>
- 636 47. Kessler C, Brambilla A, Waldvogel D, Camenisch G, Biebach I, Leigh DM, et al. A robust
637 sequencing assay of a thousand amplicons for the high-throughput population monitoring
638 of Alpine ibex immunogenetics. *Molecular Ecology Resources*. 2022;22: 66–85.
639 doi:<https://doi.org/10.1111/1755-0998.13452>
- 640 48. Fones H, Gurr S. The impact of *Septoria tritici* Blotch disease on wheat: An EU
641 perspective. *Fungal genetics and biology*. 2015;79: 3–7. doi:10.1016/j.fgb.2015.04.004
- 642 49. Stukenbrock EH, Banke S, Javan-Nikkhah M, McDonald BA. Origin and domestication of
643 the fungal wheat pathogen *Mycosphaerella graminicola* via sympatric speciation. *Molecular
644 biology and evolution*. 2007;24: 398–411. doi:10.1093/molbev/msl169
- 645 50. Zhan J, Pettway RE, McDonald BA. The global genetic structure of the wheat pathogen
646 *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial
647 diversity, regular recombination, and gene flow. *Fungal Genetics and Biology*. 2003;38:
648 286–297. doi:10.1016/s1087-1845(02)00538-8
- 649 51. Brown JK, Chartrain L, Lasserre-Zuber P, Saintenac C. Genetics of resistance to
650 *Zymoseptoria tritici* and applications to wheat breeding. *Fungal Genetics and Biology*.
651 2015;79: 33–41. doi:10.1016/j.fgb.2015.04.017
- 652 52. Meile L, Croll D, Brunner PC, Plissonneau C, Hartmann FE, McDonald BA, et al. A fungal
653 avirulence factor encoded in a highly plastic genomic region triggers partial resistance to
654 *septoria tritici* blotch. *New Phytologist*. 2018;219: 1048–1061.
655 doi:<https://doi.org/10.1111/nph.15180>
- 656 53. Singh NK, Karisto P, Croll D. Population-level deep sequencing reveals the interplay of
657 clonal and sexual reproduction in the fungal wheat pathogen *Zymoseptoria tritici*. *Microbial
658 genomics*. 2021;7. doi:10.1099/mgen.0.000678
- 659 54. Linde CC, Zhan J, McDonald BA. Population structure of *Mycosphaerella graminicola*:
660 from lesions to continents. *Phytopathology*. 2002;92: 946–955.
661 doi:10.1094/PHYTO.2002.92.9.946
- 662 55. Feurtey A, Lorrain C, McDonald MC, Milgate A, Solomo P, Warren R, et al. A thousand-
663 genome panel retraces the global spread and climatic adaptation of a major crop pathogen.
664 *Genomics*; 2022. doi:10.1101/2022.08.26.505378
- 665 56. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence
666 data. *Bioinformatics*. 2014;30: 2114–2120. doi:10.1093/bioinformatics/btu170
- 667 57. Goodwin SB, Ben M'Barek S, Dhillon B, Wittenberg AH, Crane CF, Hane JK, et al.
668 Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals

- 669 dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS genetics*.
670 2011;7: e1002070. doi:10.1371/journal.pgen.1002070
- 671 58. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nature methods*.
672 2012;9: 357–359. doi:10.1038/nmeth.1923
- 673 59. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy Moonshine A,
674 et al. From FastQ data to high confidence variant calls: the genome analysis toolkit best
675 practices pipeline. *Current protocols in bioinformatics*. 2013;43: 11–10.
676 doi:10.1002/0471250953.bi1110s43
- 677 60. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, et al. The variant call
678 format and VCFtools. *Bioinformatics*. 2011;27: 2156–2158.
679 doi:10.1093/bioinformatics/btr330
- 680 61. Hartmann FE, McDonald BA, Croll D. Genome wide evidence for divergent selection
681 between populations of a major agricultural pathogen. *Molecular ecology*. 2018;27: 2725–
682 2741. doi:10.1111/mec.14711
- 683 62. Steinhauer D, Salat M, Frey R, Mosbach A, Luksch T, Balmer D, et al. A dispensable
684 paralog of succinate dehydrogenase subunit C mediates standing resistance towards a
685 subclass of SDHI fungicides in *Zymoseptoria tritici*. Lee Y-H, editor. *PLoS Pathog*.
686 2019;15: e1007780. doi:10.1371/journal.ppat.1007780
- 687 63. Badet T, Oggenfuss U, Abraham L, McDonald BA, Croll D. A 19-isolate reference-quality
688 global pangenome for the fungal wheat pathogen *Zymoseptoria tritici*. *BMC biology*.
689 2020;18: 1–18. doi:https://doi.org/10.3929/ethz-b-000400871
- 690 64. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and
691 population genetical parameter estimation from sequencing data. *Bioinformatics*. 2011;27:
692 2987–2993. doi:https://doi.org/10.1093/bioinformatics/btr509
- 693 65. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. 1000 genome project
694 data processing subgroup. The sequence alignment/map format and SAMtools.
695 *Bioinformatics*. 2009;25: 2078–2079. doi:https://doi.org/10.1093/bioinformatics/btp352
- 696 66. Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome
697 assemblies. *Bioinformatics*. 2011;27: 2957–2963. doi:10.1093/bioinformatics/btr507
- 698 67. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for
699 multiple tools and samples in a single report. *Bioinformatics*. 2016/06/16 ed. 2016;32:
700 3047–3048. doi:10.1093/bioinformatics/btw354
- 701 68. Van der Auwera GA, O’Connor BD. *Genomics in the cloud: using Docker, GATK, and*
702 *WDL in Terra*. O’Reilly Media; 2020. Available:
703 <https://play.google.com/store/books/details?id=vsXaDwAAQBAJ>

- 704 69. Shen W, Le S, Li Y, Hu F. SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q
705 File Manipulation. PLOS ONE. 2016;11: 1–10. doi:10.1371/journal.pone.0163962
- 706 70. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-generation
707 sequencing data. Bioinformatics. 2012;28: 3150–3152.
708 doi:<https://doi.org/10.1093/bioinformatics/bts565>
- 709 71. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool.
710 Journal of molecular biology. 1990;215: 403–410. doi:10.1016/S0022-2836(05)80360-2
- 711 72. Kassambara A, Mundt F. actextra: Extract and Visualize the Results of Multivariate Data
712 Analyses. 2020. Available: <https://CRAN.R-project.org/package=actextra>
- 713 73. Wright K, YiLan L, RuTong Z. clustertend: Check the Clustering Tendency. 2022.
714 Available: <https://CRAN.R-project.org/package=clustertend>
- 715 74. Maechler M, Rousseeuw P, Struyf A, Hubert M, Hornik K. Cluster: cluster analysis basics
716 and extensions. R package version. 2012;1: 56. Available: [https://CRAN.R-](https://CRAN.R-project.org/package=cluster)
717 [project.org/package=cluster](https://CRAN.R-project.org/package=cluster)
- 718 75. Charrad M, Ghazzali N, Boiteau V, Niknafs A. {NbClust}: An {R} Package for
719 Determining the Relevant Number of Clusters in a Data Set. Journal of Statistical Software.
720 2014;61: 1--36. doi:10.18637/jss.v061.i06
- 721 76. Rousseeuw PJ. Silhouettes: a graphical aid to the interpretation and validation of cluster
722 analysis. Journal of computational and applied mathematics. 1987;20: 53–65.
723 doi:10.1016/0377-0427(87)90125-7
- 724 77. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for
725 Statistical Computing. 2021.
- 726 78. Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, et al. Welcome to
727 the Tidyverse. Journal of open source software. 2019;4: 1686. Available:
728 <https://doi.org/10.21105/joss.01686>
- 729 79. Makowski D, Ben-Shachar MS, Patil I, Lüdtke D. Automated Results Reporting as a
730 Practical Tool to Improve Reproducibility and Methodological Best Practices Adoption.
731 CRAN. 2021. Available: <https://github.com/easystats/report>
- 732 80. Kassambara A. ggpubr: “ggplot2” Based Publication Ready Plots. 2020. Available:
733 <https://CRAN.R-project.org/package=ggpubr>
- 734 81. Wickham H. ggplot2: elegant graphics for data analysis. Springer-Verlag New York; 2016.
735 Available: <https://ggplot2.tidyverse.org>
- 736 82. Knaus BJ, Grünwald NJ. vcfr: a package to manipulate and visualize variant call format
737 data in R. Molecular ecology resources. 2017;17: 44–53. doi:10.1111/1755-0998.12549

- 738 83. Jombart T, Ahmed I. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data.
739 Bioinformatics. 2011/09/16 ed. 2011;27: 3070–3071.
740 doi:<https://doi.org/10.1093/bioinformatics/btr521>
- 741 84. Dray S, Dufour A-B. The ade4 Package: Implementing the Duality Diagram for Ecologists.
742 J Stat Soft. 2007;22: 1–20. doi:[10.18637/jss.v022.i04](https://doi.org/10.18637/jss.v022.i04)
- 743

744 **Figure legends**

745

746 **Figure 1: Schematic overview of the targeted amplicon assay design.** A) Design of individual
747 amplicons (~200 bp) with primers designed to not overlap known polymorphic sites. B) Schematic
748 overview of the microfluidic chambers of a Fluidigm Juno chip accommodating up to 192 samples and 24
749 pools of primers (each up to 100 primer pairs). Following amplification in microfluidic wells, barcoded
750 products are pooled and finalized for Illumina sequencing. C) Genotypes of individual samples (pure or
751 mixed individuals) are assessed by analyzing mapped reads at each locus in the genome. Markers were
752 designed for three different categories including effector genes, genes encoding targets of fungicides and
753 genome-wide evenly spaced markers.

754

755 **Figure 2: Sequencing data recovered for the amplicon assay and loci assessment.** A) Read pairs
756 recovered per sample and replicate. Each sample was amplified and sequenced two times (two different
757 microfluidic flow cells). B) Ranking of percent mapped reads to the reference genome per sample
758 (including both replicates if available). C) Number of reads mapped per locus for the three different
759 categories of markers. The read numbers correspond to the total obtained from four pooled samples
760 performed in replicates. D) Summary of loci retained after read number filtering. Only genome-wide
761 markers were removed if they failed filtering criteria. E) Overview of retained markers per category
762 across the 21 chromosomes and mitochondrion.

763

764 **Figure 3: Consistency between replicate runs of the amplicon assay.** A) Read numbers per sample and
765 B) percentage of reads mapped to the reference genome. C) Comparison of alternative allele frequencies
766 within samples between the two replicate runs for each sample.

767

768 **Figure 4: Evaluation of mixed sample analyses.** Ten samples (G1-G10) contained mixed DNA of three
769 different isolates (INRA10-FS1006, INRA10-FS1022, IPO-09455) varying in proportions. Genotypes of
770 each of the isolates were retrieved from whole-genome sequencing of pure isolates and assigned as
771 reference alleles (*i.e.* matching the allele present in the reference genome IPO323) or alternative alleles.
772 Using known genotypes of the three isolates, reference allele proportions were defined according to the
773 dilutions in mixed samples G1-G10. Amplicon sequencing data of mixed samples was screened for all
774 genotyped SNPs to assess the proportion of the reference allele among all mapped Illumina reads. Only
775 SNPs with a minimum read coverage of 50 were used. Regression R^2 were calculated based on a linear
776 model.

777

778 **Figure 5: Analyses of amplicons designed on polymorphic transposable element insertions**
779 **upstream of the multidrug transporter gene *MgMFS1*.** A) Overview of the location of amplicons
780 designed for each of three transposable element insertion site (1-3). Multiple amplicons were designed for
781 each insertion site. The aligned reads are shown for positions near the coding sequence of *MgMFS1* for
782 sample G9 (only positions with >10 reads mapping are shown). B) After read clustering for sample G9,
783 consensus sequences were blasted against positions near the coding sequence of *MgMFS1*. The horizontal
784 bars indicate the extent of a BLASTn alignment with colors indicating the percent identity of the
785 alignment. The vertical position indicates the number of sequences that were clustered for the aligned
786 consensus sequence.

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Figure 6: Population structure analyses based on genome-wide markers genotyped on leaf-extracted assemblies of *Zymoseptoria tritici* strains. A) Principal component analysis of wheat leaf samples collected in France, Belgium, Ireland and the United Kingdom and B) the subset of wheat leaf samples collected in France colored by region.

796 **Supporting Information files**

797

798 **File S1: Supplementary Tables A-E.**

799

800 Table A: Samples and sample mixtures included in the microfluidics assay.

801

802 Table B: Designed amplicons targeting neutral markers, fungicide resistance and effector genes.
803 Loci check: coverage based assessment of amplification success (see methods). If an originally
804 targeted locus was not recovered in the species-wide SNP call set used for the amplicon design, a
805 nearby SNP was chosen (see last columns for newly selected loci).

806

807 Table C: Clustering of reads using CD-HIT-EST followed by mapping to the MFS1 promoter
808 region. Similar blast hits were grouped into K-means based clusters.

809

810 Table D: Dominant genotype recovered for wheat leaf samples at fungicide resistance loci. The
811 reference allele refers to the allele known from the reference genome isolate IPO323. Sample
812 genotypes are given as 1 and 0 for reference and alternative allele, respectively.

813

814 Table E: Dominant genotype recovered for wheat field samples across fungicide resistance loci.
815 The reference allele refers to the allele known from the reference genome isolate IPO323.

816

817

818 **File S2: Supplementary Figure 1.**

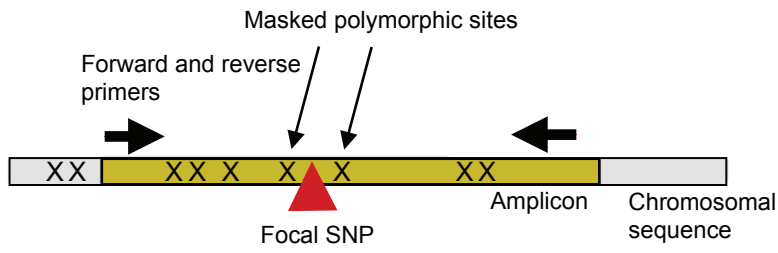
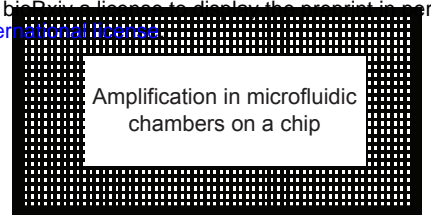
819

820 Figure 1: Wheat leaf samples collected in France, Belgium, Ireland and the United Kingdom
821 separated by the cultivar of origin or unknown cultivar ("NA"). See File S1 (Table A) for details
822 on the sample origins.

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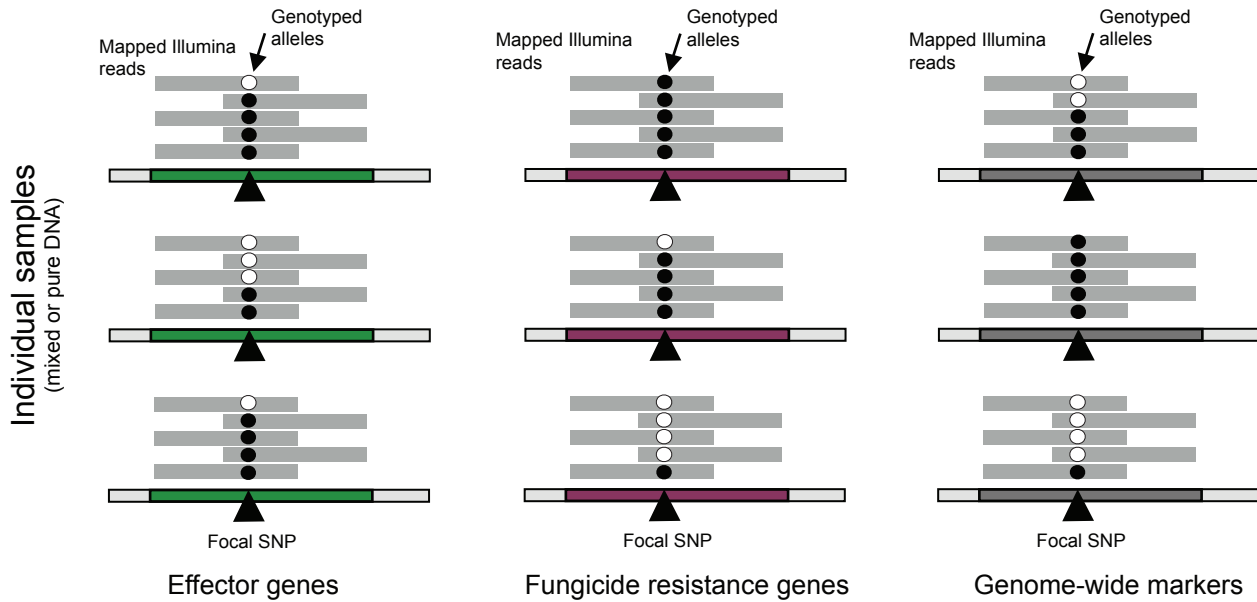
bioRxiv preprint doi: <https://doi.org/10.1101/2022.07.18.500446>; this version posted November 12, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

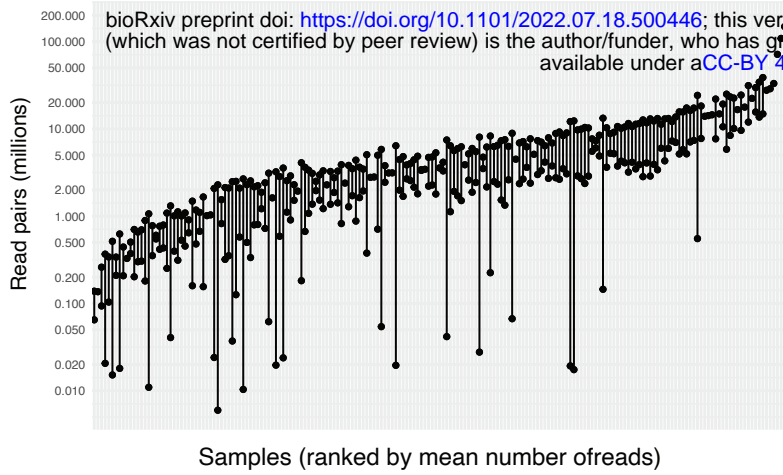
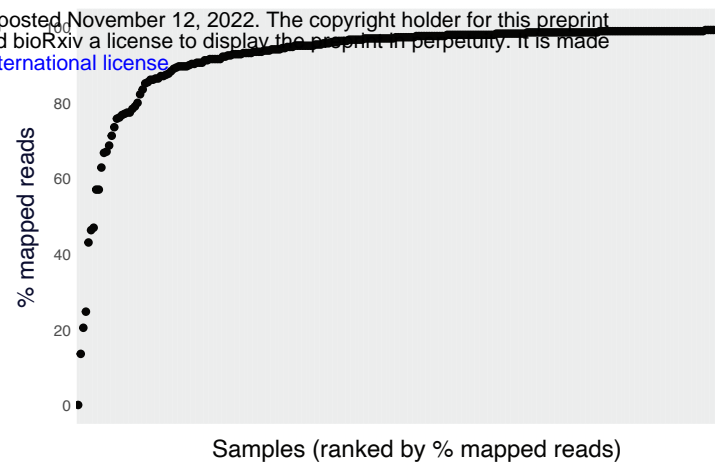
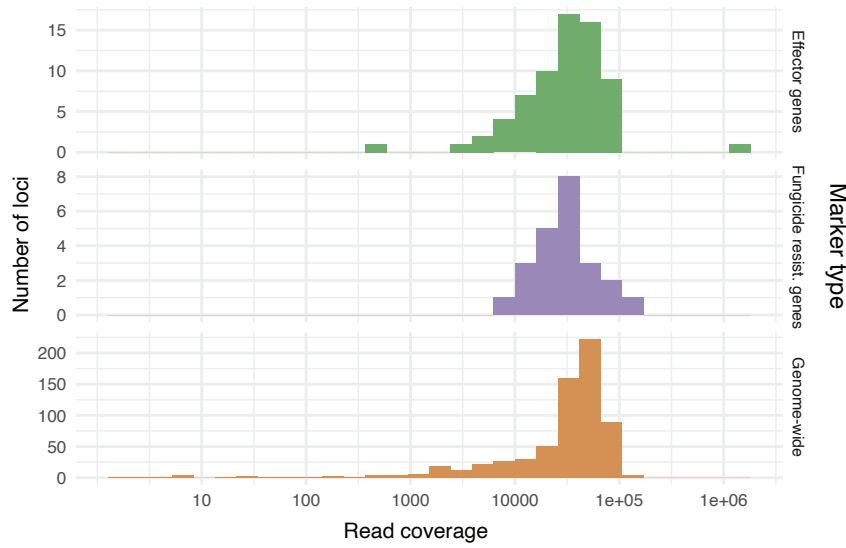
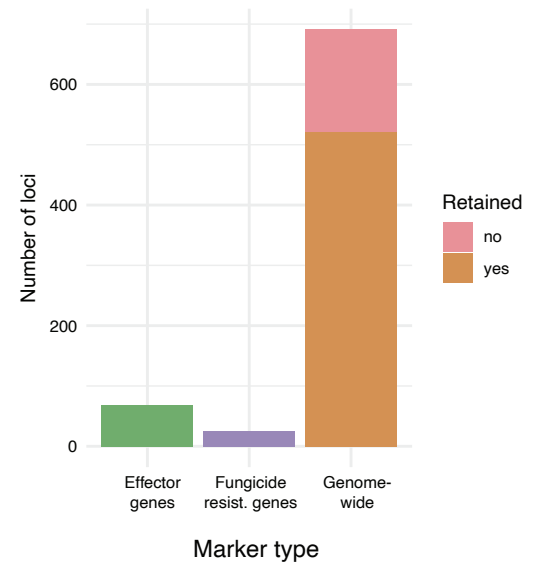
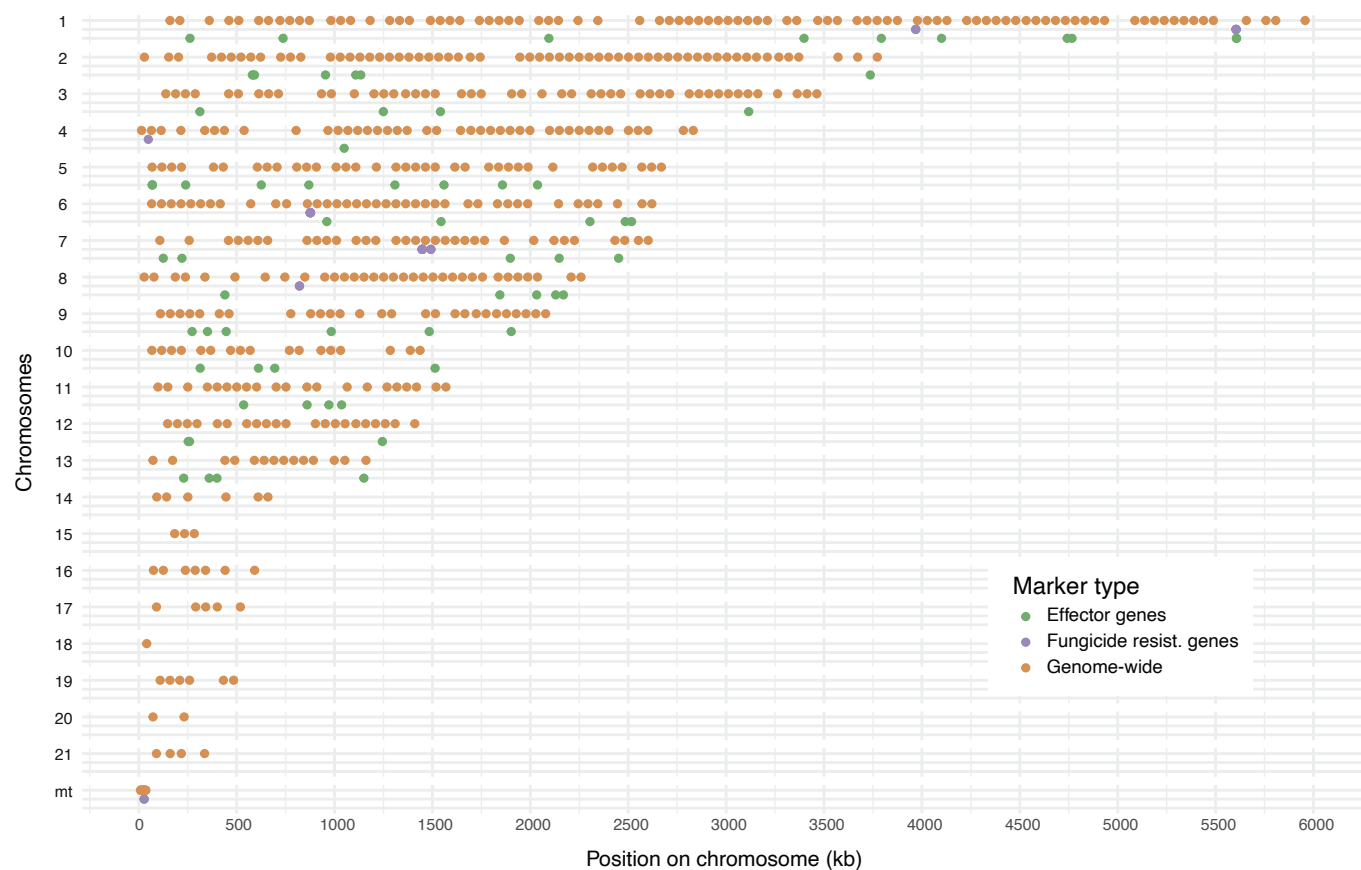
**B**

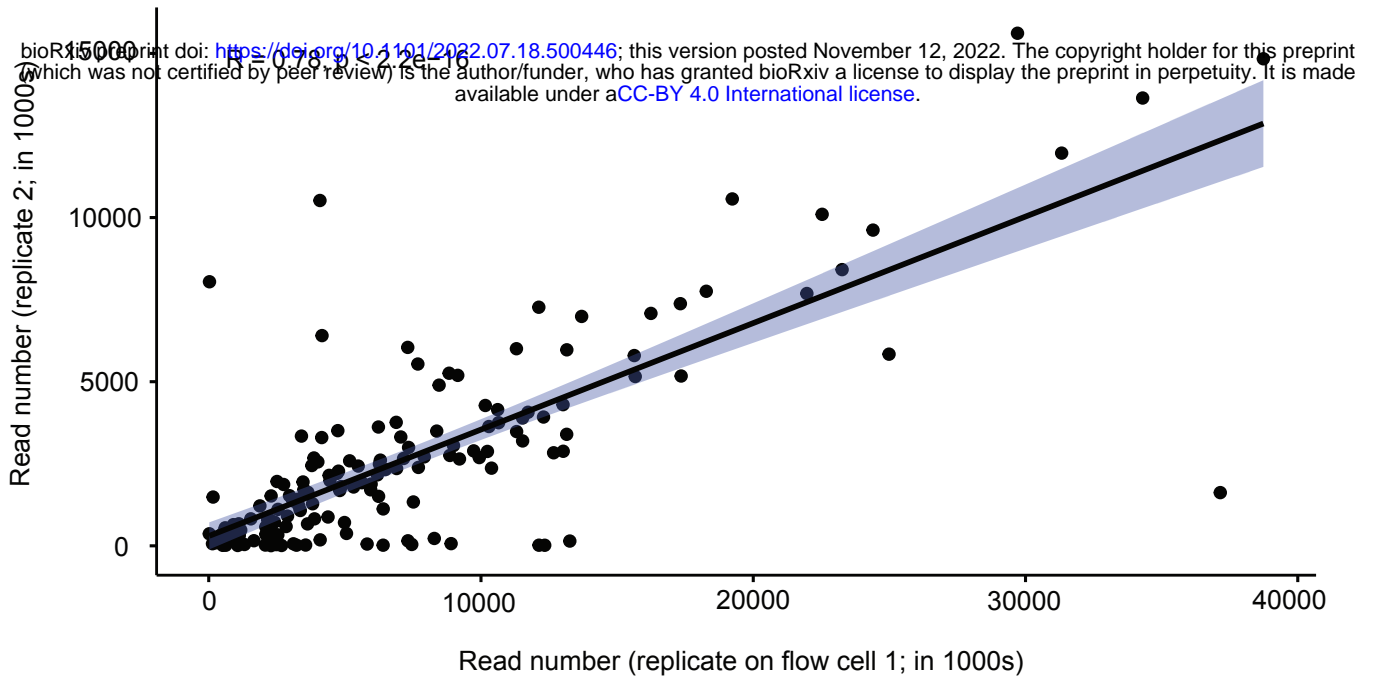
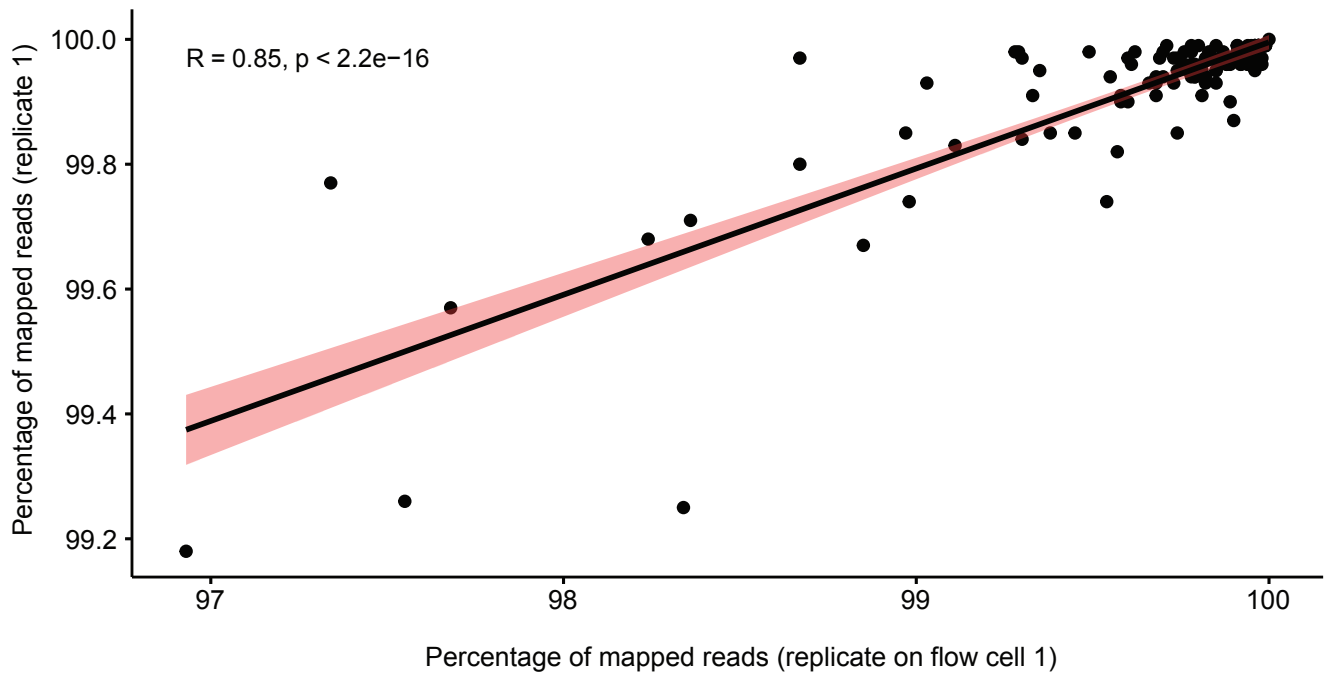
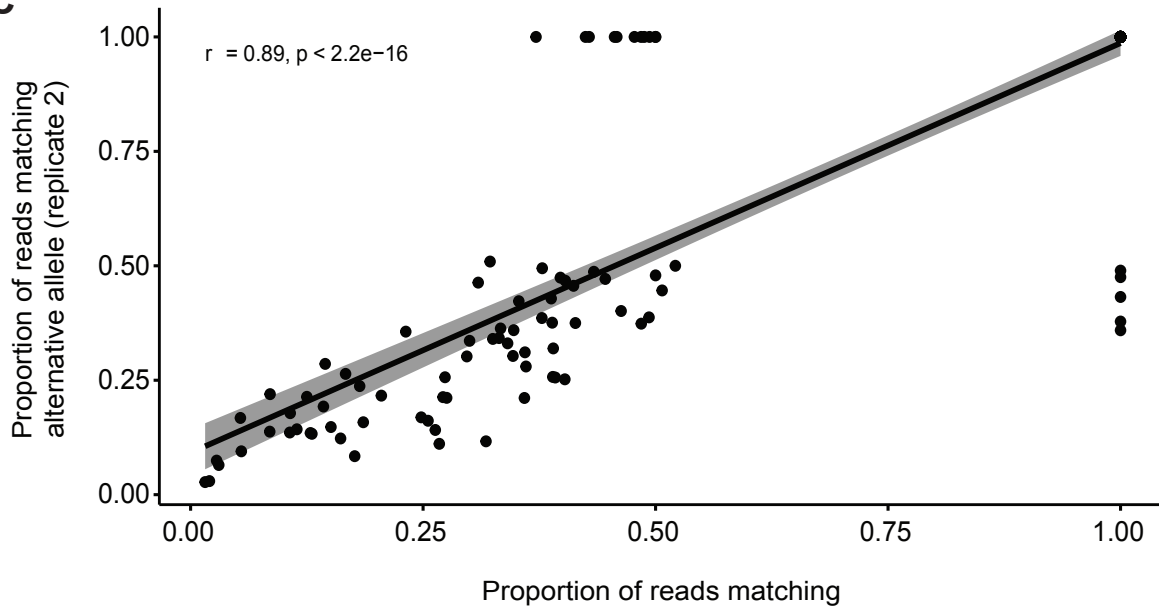
Assayed: 24 pools with 798 primer pairs in total
24 pools of up to 100 primer pairs per pool possible

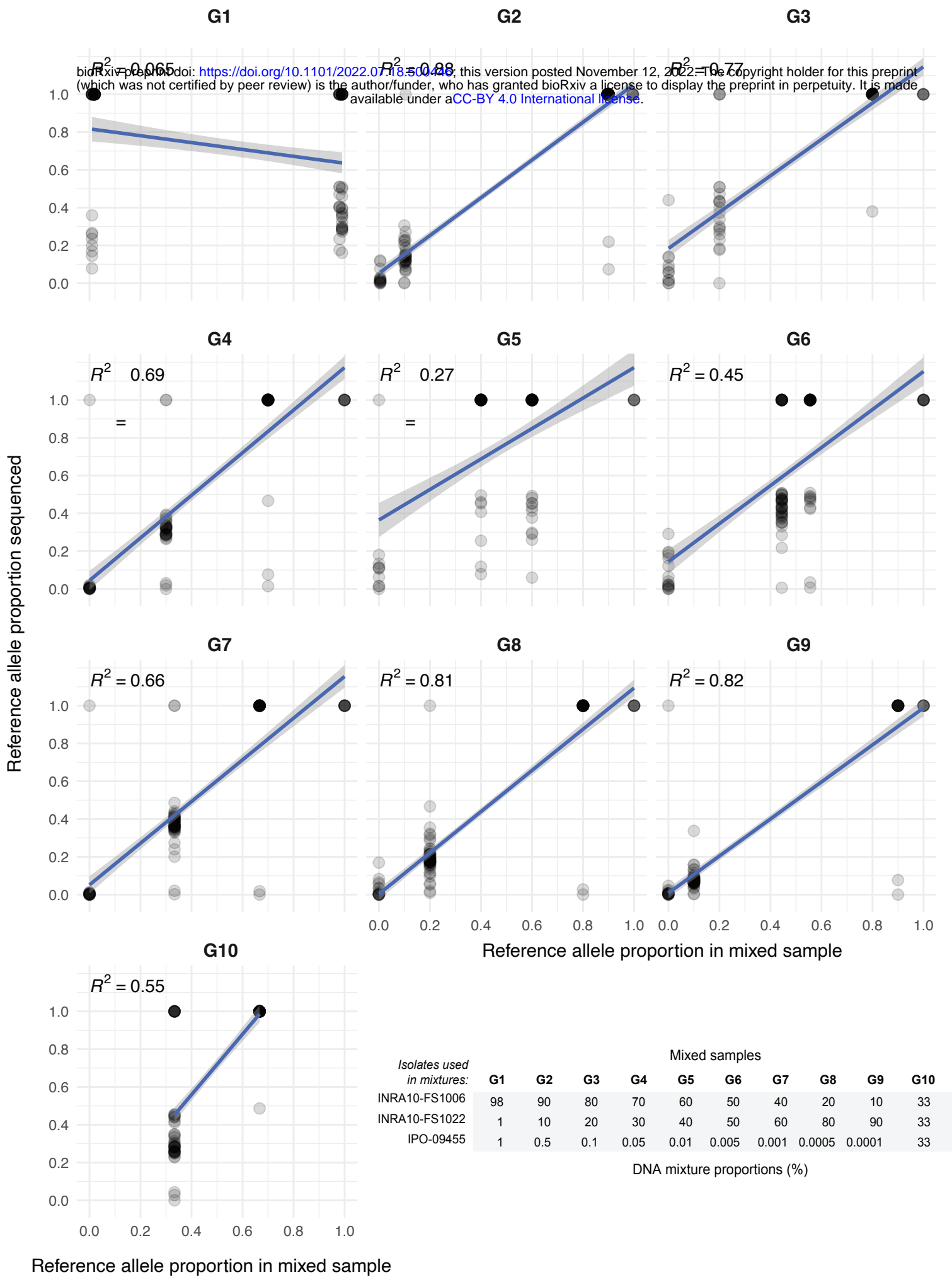
Collected Adapter Ligation & Quality Control

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Illumina sequencing

C

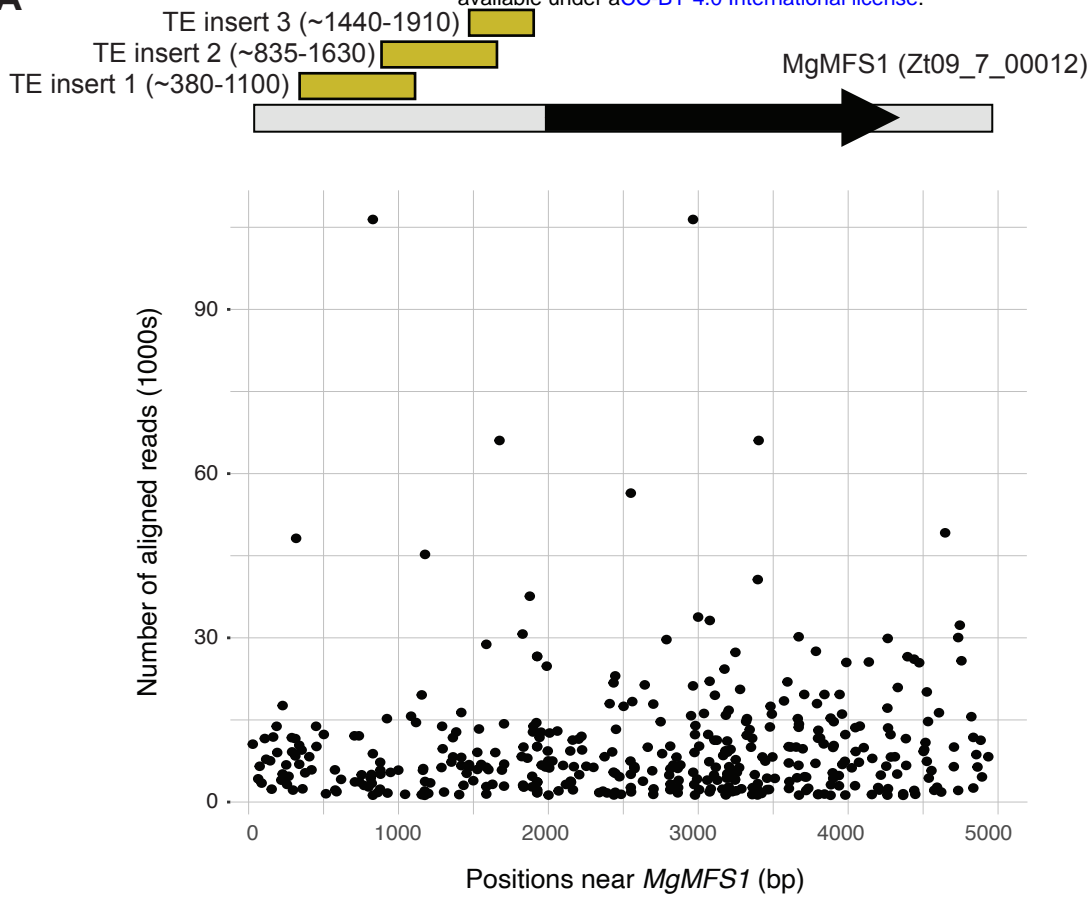
A**B****C****D****E**

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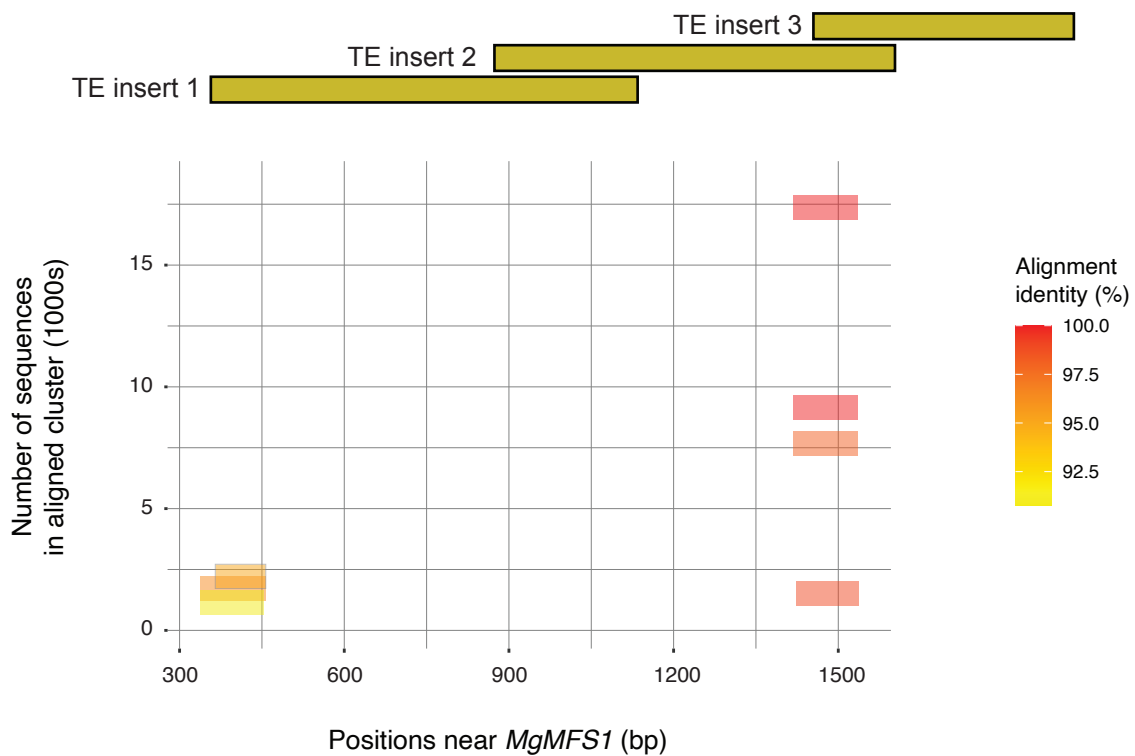


Reference allele proportion in mixed sample

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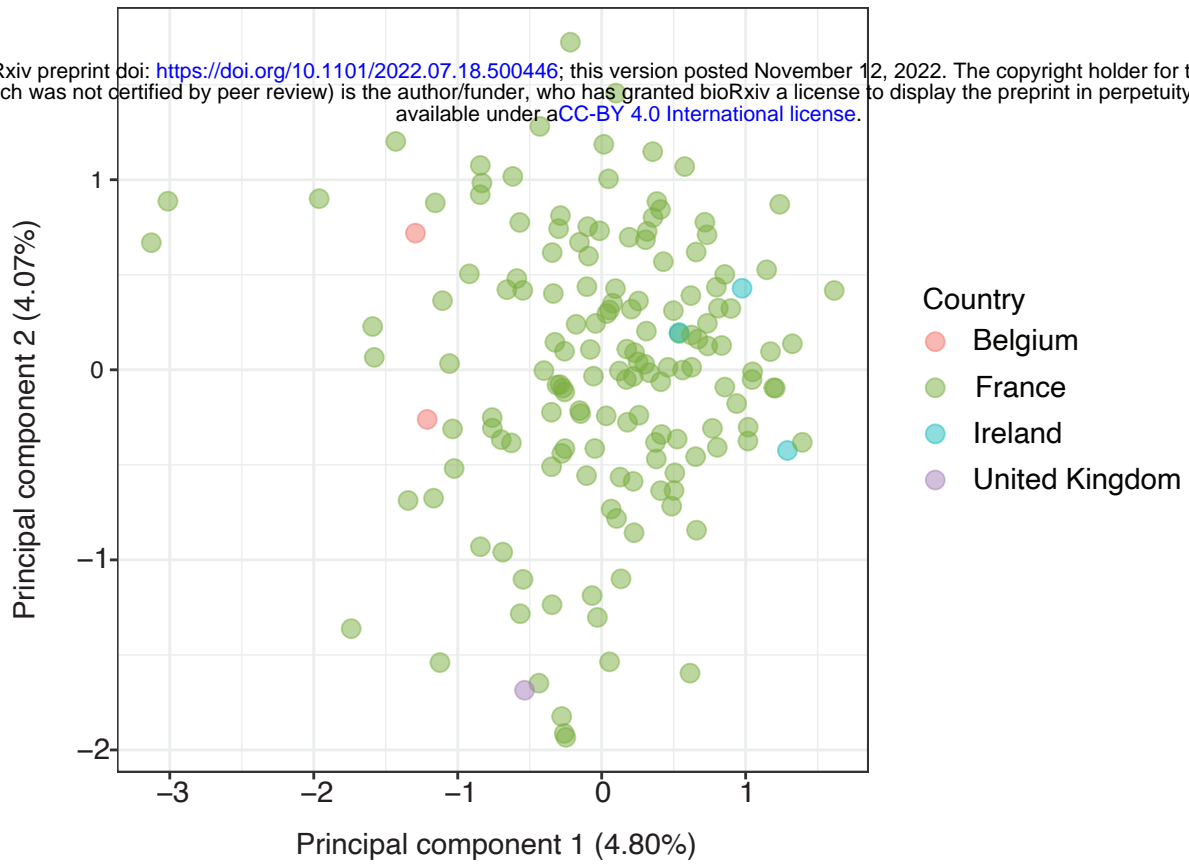


B



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**B**