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1 **Different phenotypes, similar genomes: three newly sequenced *Fusarium***
2 ***fujikuroi* strains induce different symptoms in rice depending on temperature.**

3

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14

15 **Abstract**

16 Bakanae, caused by the hemibiotrophic fungus *Fusarium fujikuroi*, is one of the most
17 important diseases of rice, causing up to 75% of losses, depending on strain and
18 environmental conditions. Some strains cause elongation and thin leaves, while others
19 induce stunting and chlorotic seedlings. Differences in symptoms are attributed to
20 genetic differences in the strains. *F. fujikuroi* strains Augusto2, CSV1 and I1.3 were
21 sequenced with Illumina MySeq, and pathogenicity trials were conducted on rice cv.
22 Galileo, susceptible to bakanae. By performing gene prediction, SNP calling and
23 structural variant analysis with a reference genome, we show how an extremely limited
24 number of polymorphisms in genes not commonly associated with bakanae disease
25 can cause strong differences in phenotype. CSV1 and Augusto2 are particularly close,
26 with only 21,887 SNPs between them, but they differ in virulence, reaction to
27 temperature, induced symptoms, colony morphology and color, growth speed,
28 fumonisin and gibberellin production. Genes potentially involved in the shift in
29 phenotype are identified. Furthermore, we show how temperature variation may result
30 in different symptoms even in rice plants inoculated with the same *F. fujikuroi* strain.
31 Moreover, all the *F. fujikuroi* strains became more virulent at higher temperatures.
32 Significant differences were likewise observed in gibberellic acid production and in the
33 expression of both fungal and plant gibberellin biosynthetic genes.

34

35 **Keywords:** *Fusarium fujikuroi*, bakanae, rice, genomics, Illumina sequencing

36

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42

43 **Introduction**

44 Bakanae, caused by the hemibiotrophic fungal pathogen *Fusarium fujikuroi*
45 [teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura], is one of the most
46 important diseases of rice (Carter *et al.*, 2008; Desjardins *et al.*, 1997). Crop losses
47 due to bakanae are largely depending on climate and rice cultivars, varying from 3%
48 to 75% in certain cases (Saremi *et al.*, 2008).

49 Originally observed in Japan in 1828, bakanae disease is now present in several
50 countries in America, Europe, Asia and Africa. Researchers have used disparate
51 approaches to investigate how rice plants can resist to the disease, from RNA
52 sequencing to QTL mapping (Fiyaz *et al.*, 2016; Ji *et al.*, 2018; Matic *et al.* 2016), but,
53 despite this, to date no rice cultivar showing a complete resistance to bakanae has
54 been developed, and there is still a limited knowledge of the mechanisms of resistance
55 (Bagga and Kumar, 2000; Desjardins *et al.*, 2000).

56 The pathogen commonly induces symptoms like abnormal height, thin leaves and
57 grains entirely or partially empty, mainly due to the production of gibberellins (Niehaus
58 *et al.*, 2017). *F. fujikuroi* is also able to increase the production of these phytohormones
59 by the plant, with less susceptible cultivars showing less gibberellin production, and a
60 reduced expression of their biosynthetic gene cluster, compared to highly susceptible
61 cultivars (Kim *et al.*, 2018; Matic *et al.*, 2016; Siciliano *et al.*, 2015). Despite this, there
62 are also reports of strains inducing stunted and chlorotic seedlings (Gupta *et al.*, 2015),
63 often followed root and crown rots (Amoah *et al.*, 1995; Karov *et al.*, 2009). Due to
64 these differences, *F. fujikuroi* strains have been recently divided in two pathotypes

65 (Niehaus *et al.*, 2017).

66 Besides the ability to induce bakanae disease, some strains of *F. fujikuroi* are also
67 known for the production of fumonisins, neurotoxic mycotoxins (Desjardins *et al.*,
68 1997; Wulff *et al.*, 2010). The most studied fumonisin, FB1, is known to cause equine
69 leucoencephalomalacia and porcine pulmonary edema (Scott, 2012), and it has been
70 associated with human esophageal cancer (Chu and Li, 1994; Sydenham *et al.*, 1990)
71 and kidney and liver cancer in mouse (Creppy, 2002).

72 *Fusarium fujikuroi* has been sequenced for the first time in 2013 (Jeong *et al.*, 2013;
73 Wiemann *et al.*, 2013), but many more strains have become available in recent years
74 (Bashyal *et al.*, 2017; Chiara *et al.*, 2015; Niehaus *et al.*, 2017; Radwan *et al.*, 2018;
75 Urbaniak *et al.*, 2018). This fungus has a genome of around 45 Mb, divided among 12
76 chromosomes, with repetitive elements constituting less than 1% of the total. The gene
77 content on average varies between 13,000 and 15,000 genes, including around 1,200
78 genes encoding for secreted proteins.

79 Beside gibberellins, the fungus is able to produce a wide array of secondary
80 metabolites, including both mycotoxins, such as fumonisins, fusaric acid, and fusarins
81 (Bacon *et al.*, 1996; Barrero *et al.*, 1991; Desjardins *et al.*, 1997), and pigments, like
82 bikaverin and fusarubins (Balan *et al.*, 1970; Studt *et al.*, 2012). Forty-seven putative
83 gene clusters for secondary metabolites were found in the reference genome of *F*
84 *fujikuroi* (Wiemann *et al.*, 2013), and a number of these have been characterized in
85 recent years (Janevska and Tudzynski, 2018). A number of global and local
86 regulators control the production of secondary metabolites, but many are also able to
87 regulate gibberellin production, and therefore pathogenicity. These include the global
88 nitrogen regulators *area* (Tudzynski *et al.*, 1999) and *areb* (Pfanmüller *et al.*, 2017)

89 and the component of the velvet complex *lae1* (Niehaus *et al.*, 2018).

90 *F. fujikuroi* shows complete synteny in the fumonisin cluster with *F. verticillioides* and
91 *F. oxysporum* (Wiemann *et al.*, 2013), despite a reduced production of these
92 molecules (Stępień *et al.*, 2011; Wulff *et al.*, 2010) when compared with the above-
93 mentioned species. However, in *F. fujikuroi* the ability to produce fumonisins, and the
94 quantity produced, can vary significantly depending on the genotype and the
95 environment (Matić *et al.*, 2013).

96 Temperature is one of the most important factors influencing both the virulence of *F.*
97 *fujikuroi* strains and the production of fumonisins, but, notwithstanding, there are few
98 works investigating its effect on the rice-*F. fujikuroi* pathosystem (Saremi and Farrokhi,
99 2004; Matić *et al.*, 2017).

100 This work aims to use a combination of high-throughput sequencing, comparative
101 genomics, chemical analyses and molecular biology to investigate the effect of
102 temperature on virulence and secondary metabolite production in three *F. fujikuroi*
103 strains showing different phenotype.

104 The considered strains are Augusto2, CSV1 and I1.3, all isolated from infected rice
105 plants in northern Italy (Amatulli *et al.*, 2010).

106

107 **Materials and methods**

108 **Microorganisms and seeds**

109 The strains of *F. fujikuroi* named Augusto2, CSV1 and I1.3, previously isolated from
110 diseased rice plants in Piedmont (Amatulli *et al.*, 2010) and maintained in the
111 Agriinnova microorganism collection, were grown on sterile PDB for 10 days at 23°C.
112 Afterwards, the suspensions were filtered through sterile gauze, centrifuged for 20 min
113 at 6,000 RPM and resuspended in Ringer solution. The Burkler chamber was then

114 used to obtain concentrations of 10^5 conidia/ml. Rice seeds 'Galileo', susceptible to
115 bakanae disease (Amatulli *et al.*, 2010) were thermally treated by dipping in water at
116 60°C for 5 min, immersed in a solution of 1% NaClO for 2 min and then washed three
117 times with sterile water for 5 min. The seeds were then divided and immersed in the
118 conidial suspension of the different strains and kept in agitation for 30 min.

119

120 **Pathogenicity trials**

121 After drying for 24 h on sterile paper, the seeds were sown in sterilized substrates
122 (70% white peat and 30% clay, with pH between 5.5 and 6). The N content was
123 between 110 mg/l and 190 mg/l, P_2O_5 was of 140-230 mg/l and K_2O was 170-280 mg/l.
124 The plants were grown in two growth chambers: one was kept at 22°C and the other
125 at 31°C. Disease symptoms were monitored weekly starting one week post
126 germination (wpg). A disease index was attributed, depending on the visible
127 symptoms: 0: healthy plant; 1: reduced dimension, chlorotic leaves; 2: internode
128 elongation, significant yellowing, significant dwarfism; 3: necrosis of the crown; 4: dead
129 or not-germinated plant. Each strain was tested on 4 replicates of 30 plants. Four
130 replicates of 30 uninoculated plants were used as control. The experiment was
131 performed twice.

132

133 **RNA extraction and qPCR**

134 RNA was extracted from the basal half of the shoot of plants inoculated with each of
135 the strains, as well as from control plants, by using the RNeasy kit (Qiagen, Hilden,
136 Germany). The extracted RNA was quantified by Nanodrop (Thermo Fisher Scientific,
137 Waltham, Massachusetts, United States) and purified using the TURBO DNA-free kit
138 (Ambion, Foster City, California, United States). The samples were then checked for

139 DNA contamination by PCR. The gene used was the rice elongation factor 1-alpha.
140 After verifying the sample purity, the RNA was used to obtain the cDNA, using the
141 iScript cDNA synthesis kit (Biorad, Hercules, California, United States). The samples
142 were then used in real time qPCR (Applied Biosystems StepOnePlus, Foster City,
143 California, United States), with primers for *fum1* (fumonisin gene cluster polyketide
144 synthase, *F. fujikuroi*), *fum21* (fumonisin gene cluster transcription factor, *F. fujikuroi*),
145 *cps/ks* (gibberellin gene cluster ent-copalyl diphosphate synthase ent-kaurene
146 synthase, *F. fujikuroi*), and *gib20ox1* (Gibberellin 20 oxidase 1, rice). The PCR mix
147 were composed of 5 µl of Applied Biosystems SYBR Green Power Mix, 2 µl of cDNA,
148 0.15 µl of each primer (10 µM) and 2.4 µl of nuclease free water. The thermal cyclor
149 protocol was the following: 95°C for 10 min, followed by 40 cycles (95°C for 15 s; 60°C
150 for 60 s) and 95°C for 15 s. The ubiquitin *F. fujikuroi* gene (Wiemann *et al.*, 2013) and
151 the rice elongation factor 1-alpha (Manosalva *et al.*, 2009) were used as housekeeping
152 genes, respectively for fungal and plant genes. The sequences of the primers used
153 are reported in **supplementary table 1**.

154 The efficiency of the primers used for *fum21* and *gib20ox1* amplification was tested
155 with a standard curve built upon five serial dilutions (1:10).

156

157 ***In vitro* assays**

158 Every strain was grown in PDB flasks (30 ml of medium) and YES Agar plates. The
159 flasks and the plates were inoculated with 100 µl of a solution containing 10⁵
160 conidia/ml, prepared following the same procedure used for the pathogenicity trials.
161 The plates were kept at 24°C, with a 12:12 h light/dark photoperiod, a light intensity of
162 1 cd and a relative luminosity of 55 cd. During the fungal growth in YES Agar, the

163 mycelial diameter was measured, and the color and texture were monitored.

164

165 **Chemical extractions**

166 Samples obtained by PDB flasks were filtered to separate the mycelium from the
167 growth medium. Mycelium was weighed (500mg) and extracted with 1ml of

168 methanol:water (8:2 v/v), during 1 hour in ultrasonic bath. Supernatant was centrifuged
169 and filtered by 0.45 µm filters, after which it was placed in the vials for HPLC analysis.

170 Regarding YES Agar plates, the extraction was carried out on the whole plates with
171 3ml of methanol. The solvent was spread on the whole surface and the mycelium was

172 scratched and brought to suspension. The extract was then placed in tubes and
173 concentrated with a Concentrator 5301 (Hamburg, Germany). The dried residue was

174 dissolved in methanol:water (1:1 v/v) and placed in vials for HPLC analysis. Similarly
175 to the procedure used for mycelia, 500 mg of *in vivo* sample were extracted with 1 ml

176 of methanol:water (8:2 v/v) by ultrasonic bath for 1 hour. Supernatant was centrifuged
177 and filtered with 0,45 µm filters, after which it was placed in vials.

178

179 **HPLC-MS/MS**

180 Liquid chromatography was performed with Varian Model 212-LC micro pumps
181 (Hansen Way, CA, USA) coupled with a Varian 126 autosampler Model 410 Prostar.

182 A Synergi 4u Fusion-RP 80A (100 mm × 2.0 mm, Phenomenex, Castel Maggiore,
183 Italy) analytical column was used coupled with Fusion-RP (4 × 2.0 mm) security guard

184 for LC separation. The chromatographic conditions were: column temperature at
185 45 °C; mobile phase consisting of eluent A (HCOOH 0,05% in H₂O) and eluent B

186 (CH₃CN). A gradient elution was applied as follows: 0 to 20% of B in 5 minutes, from
187 20% to 80% of B in 15 minutes, from 80% to 100% of B in 1 minute. Five minutes of

188 post run were necessary for column conditioning before the subsequent injection. The
189 injection volume was 20 µl, and the flow speed was of flow of 200 µl/min.
190 The triple quadrupole mass spectrometer (Varian 310-MS) was operated in the
191 negative/positive electrospray ionization mode (ESI⁻/ESI⁺). To select the MS/MS
192 parameters for the analysis of metabolites by multiple reaction monitoring (MRM). For
193 the quantification of fumonisin B4 the calibration curve of fumonisin B1 was used, since
194 fumonisin B4 currently lacks a specific commercial standard. Two transitions were
195 selected for each compound: GA3: 345>214 (CE 14 eV), 345>143 (CE 30 eV); FB1:
196 722>334 (CE 38 eV), 722>352 (CE 34 eV); FB2/FB3: 706>336 (CE 36 eV), 706>354
197 (CE 34 eV); FB4: 690>338 (CE 30 eV), 690>320 (CE 30 eV). The collision gas (Ar)
198 pressure was set at 2 mbar for all of the experiments.

199

200 **Sequencing, assembly and analysis**

201 The *F. fujikuroi* strains Augusto2, CSV1 and I1.3 were sequenced by Parco
202 Tecnologico Padano using a next generation Illumina MiSeq sequencer. For each
203 strain, a paired end library was generated using the Nextera XT DNA preparation kit
204 (Illumina, San Diego, California, United States). For strain I1.3, a mate-pair library was
205 also generated using the Nextera Mate Pair kit (Illumina, San Diego, California, United
206 States), following the protocols provided by the manufacturer. Libraries were purified
207 by AMPure XP beads and normalized to ensure equal library representation in the
208 pools. Equal volumes of libraries were diluted in the hybridization buffer, heat
209 denatured and sequenced. Standard phi X control library (Illumina) was spiked into
210 the denatured HCT 116 library. The libraries and phi X mixture were finally loaded into
211 a MiSeq 250 and MiSeq 300-Cycle v2 Reagent Kit (Illumina). Base calling was
212 performed using the Illumina pipeline software. Demultiplexing was done using an

213 Illumina provided software. Trimming of adapters and removal of ambiguous bases
214 was done using Trimalore
215 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), and the resulting
216 cleaned reads were checked with fastqc
217 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) for remaining
218 contamination. For the I1.3 reads, the program “Scythe”
219 (<https://github.com/vsbuffalo/scythe>) was also used to remove remaining adapters.
220 Initially, *de novo* assembly was performed, using SPAdes version 3.7.1 (Bankevich *et*
221 *al.*, 2012), and the obtained assembly was used in a reference guided approach with
222 IMR-DENOM (<http://mtweb.cs.ucl.ac.uk/mus/www/19genomes/IMR-DENOM/>), since
223 the low sequencing coverage of Augusto2 and CSV1 made it impossible to obtain a
224 good purely *de novo* assembly (**Supplementary table 2**). The selected mapper used
225 in IMR-DENOM was bwa (Li and Durbin, 2009).

226

227 **Gene prediction**

228 Gene prediction was conducted using the version 2.31.8 of MAKER (Cantarel *et al.*,
229 2008). Both predictors augustus v.2.5.5 (Stanke and Waack, 2003) and SNAP v.2006-
230 07-28 (<http://korflab.ucdavis.edu/software.html>) were used. augustus used the “--
231 fusarium” option for gene prediction, while SNAP was trained to obtain a file.hmm
232 specific for the three genomes. The necessary repeat libraries were constructed using
233 the basic procedure
234 ([http://weatherby.genetics.utah.edu/MAKER/wiki/index.php/Repeat_Library_Constru](http://weatherby.genetics.utah.edu/MAKER/wiki/index.php/Repeat_Library_Construction--Basic)
235 *ction--Basic*). The external data provided to MAKER, and used for the training of
236 SNAP, were all the EST, protein sequences and transcript sequences of *F. fujikuroi*
237 available on NCBI. To launch MAKER, the option “-fix_nucleotides” was used, in order

238 to allow the program to work with degenerate nucleotides present in the external data.
239 The option “correct_est_fusion” was also activated in the control files. After the
240 analysis, introns shorter than 10 bp, predicted by snap, were removed, and, when this
241 caused a frameshift mutation, the prediction of the gene splicing sites was repeated
242 with augustus v.2.5.5 (Stanke and Waack, 2003).

243

244 **SNP mining**

245 The clean paired end reads of each of the three strains of interest were mapped on
246 the reference genome of *F. fujikuroi* strain IMI 58289 (Wiemann *et al.*, 2013), using
247 bwa v.0.7.12-r1039 (<http://bio-bwa.sourceforge.net>) with default options. The resulting
248 sam files were converted to sort.bam by samtools v.0.1.19-96b5f2294a
249 (<http://samtools.sourceforge.net/>), and they were used for SNP mining with the
250 following pipeline:

```
251 'samtools mpileup -guf reference.fa agosto2.sort.bam CSV1.sort.bam I1.3.sort.bam  
252 | bcftools view -cg - | vcfutils.pl varFilter -D 200 -Q 20 - > file.vcf'
```

253 Heterozygous SNPs were assumed to be derived from sequencing errors and were
254 excluded from the analysis. The SNPs were mapped on the genome with the program
255 CircosVCF (Drori *et al.*, 2017). The reads of the strains were also mapped, using the
256 same pipeline, on the scaffold 005 of the *F. fujikuroi* strain B14 (Genbank:
257 FMSL01000005.1), and Tablet (Milne *et al.*, 2013) was used to check if any reads
258 mapped on the gene FFB14_06372.

259

260 **Analysis of polymorphisms**

261 The SnpEff program v. 4.2 (Cingolani *et al.*, 2012) was used to evaluate the impact of
262 the SNPs/indels identified with the SNP mining, after building a database for IMI 58289

263 following the manual instructions
264 (http://snpeff.sourceforge.net/SnpEff_manual.html#databases). Afterwards, we
265 checked if the strains Augusto2, CSV1 and I1.3 presented missense or nonsense
266 polymorphisms in the gibberellin and fumonisin gene clusters, or in other genes
267 involved in the biosynthesis regulation of these metabolites (see **supplementary table**
268 **3** for references). The presence of these polymorphisms was then checked in the
269 sort.bam files with the viewer Tablet (Milne *et al.*, 2013). EffectorP 1.0 and 2.0
270 (Sperschneider *et al.*, 2016) were used on the secreted portion of the *F. fujikuroi*
271 proteome (Wiemann *et al.*, 2013) to predict putative effector genes, and these genes
272 were also checked for polymorphisms. The impact of polymorphisms of interest was
273 predicted with Provean Protein (Choi and Chan, 2015). All the genes presenting
274 putatively MODERATE and HIGH impact polymorphisms in either Augusto2 or CSV1,
275 but not in both, were identified, according to the evaluation of SnpEff. These genes
276 were annotated with BLAST2GO with default parameters, and, when they presented
277 GO terms related to regulation of transcription, pathogenesis or metabolism, the
278 impact of their polymorphisms was predicted with Provean Protein (Choi and Chan,
279 2015).

280

281 **Structural variant analysis**

282 The software BreakDancer v1.3.6 (Fan *et al.*, 2014) was used to identify structural
283 variants in the genome. Variants with a score lower than 80 were removed, and an
284 original python script (**supplementary file 1**) was used to identify genes localized in
285 the regions affected by the remaining variations. Genes present in an area involved in
286 a deletion are considered to be affected, as are genes that have the edge of an
287 inversion or a translocation inside their sequence. The script only works on variations

288 involving only one scaffold, and therefore structural variants affecting different
289 chromosomes were checked manually.

290

291 **Phylogenetic analysis**

292 OrthoFinder v. 2.3.3 (Emms and Kelly, 2015) was used with the option “-M msa” to
293 obtain a genome-wise phylogenetic tree based on single-copy genes, comparing the
294 *F. fujikuroi* strains Augusto2, CSV1 and I1.3 to several other annotated isolates of the
295 same species. The strains used for this analysis were: B20 (GenBank:
296 GCA_900096605.1), C1995 (GenBank: GCA_900096645.1), E282 (GenBank:
297 GCA_900096705.1), FGSC_8932 (GenBank: GCA_001023045.1), FSU48 (GenBank:
298 GCA_900096685.1), IMI58289 (GenBank: GCA_900079805.1), KSU3368 (GenBank:
299 GCA_001023065.1), KSU X-10626 (GenBank: GCA_001023035.1), m567 (GenBank:
300 GCA_900096615.1), MRC2276 (GenBank: GCA_900096635.1) and NCIM1100
301 (Genbank: GCA_900096625.1), with *Fusarium oxysporum* f. sp. *lycopersici* 4287 used
302 as outgroup (GenBank: GCA_000149955.2). STAG (Emms and Kelly, 2015) was used
303 to generate an unrooted species tree, and the root was placed with MEGA (Kumar *et*
304 *al.*, 1994) between *F. oxysporum* and the *F. fujikuroi* strains.

305

306 **Comparing Augusto2 and CSV1**

307 Proteinortho v. 5.16 (Lechner *et al.*, 2011) was used to identify genes present either
308 in CSV1 or in Augusto2, but not in both. Following this, genes unique to CSV1 were
309 blasted against the genome of Augusto2, and vice versa. Genes with a good blast hit
310 were excluded from the analysis, since they could be actually present in both strains,

311 their absence in one derived by an error from the gene predictor.

312

313 **Results**

314 **Pathogenicity trials**

315 Plants inoculated with the strains Augusto2, CSV1 and I1.3 presented widely different
316 symptoms (**figure 1**). At 22°C and 2 wpg, the symptoms of all the strains were mixed
317 between those associated to the two pathotypes identified by Niehaus *et al.* (2017)
318 some plants showed elongation, while others were stunted. At 3 wpg, CSV1 induced
319 stunting and withering, while I1.3 tended to induce more elongation and plants
320 inoculated with Augusto2 could present both types of symptoms. Disease indexes are
321 reported in **figure 2**. While all the strains showed a similar virulence at 22°C, at 31°C
322 Augusto2 and I1.3 were much more virulent, and nearly all the plants died at 2 wpg,
323 with the remaining ones showing extreme elongation.

324

325 ***In vitro* trials**

326 The three strains on YES Agar produced mycelia of different color, dimension and
327 texture (**supplementary figure 1**). CSV1 and I1.3 mycelia are characterized by a
328 reverse red-orange color, not present in Augusto2. This color is also present in the
329 front view of the CSV1 mycelia. Growth speed was not uniform as well
330 (**supplementary figure 2**). CSV1 grew faster at the beginning of the trial, reaching a
331 diameter of around 40 mm at 5 days after the inoculation. Afterwards, Augusto2 and
332 I1.3 started growing faster, reaching, after 14 days of inoculation, average diameters

333 of 80 and 65 mm, against the 50 mm of CSV1.

334

335 **Chemical analyses**

336 The results of the *in vivo* quantification of GA3 are presented in **figure 3**. At 22°C and
337 2 wpg, plants inoculated with Augusto2 contained slightly more GA3 than those
338 inoculated with the other strains. However, one week later the highest amount of GA3
339 was found in I1.3-inoculated plants. At 31°C and 2 wpg, the highest quantity of GA3
340 was found in plants inoculated with Augusto2 and I1.3, while plants affected by strain
341 CSV1 had a GA3 quantity slightly but not significantly higher than control plants. At 3
342 wpg, the quantity of GA3 in CSV1-inoculated plants decreased still, probably because
343 many plants died. Fumonisin were not present *in vivo* at a detectable level in any of
344 the plant samples.

345 Besides, GA3 and fumonisin production by the three strains were tested *in vitro*
346 **(supplementary table 4)**. CSV1 did not produce GA3 at detectable levels *in vitro* on
347 YES Agar, but it was the highest producer on PDB (10,676.7 ppb). Fumonisin were
348 produced *in vitro* by Augusto2 (181,052 ppb of FB1 on average), and much less by
349 CSV1 (22 ppb of FB1), while I1.3 did not produce these mycotoxins in any situation.

350

351 **Real time RT-PCRs**

352 The gene expression of *cps/ks* and Gibberellin 20 oxidase 1 at various time points is
353 presented in **figure 4**. At 2 wpg, *cps/ks*, a key gene of the fungal gibberellin gene
354 cluster, was mainly expressed in I1.3-inoculated plants, both at 22°C and 31°C. On
355 the contrary, at 22°C and 3 wpg, this gene was mostly expressed in strain CSV1. In
356 Augusto2, the level of expression did not change significantly in the three examined

357 conditions.

358 Regarding the gene Gibberellin 20 oxidase 1, belonging to the plant gibberellin gene
359 cluster, the expression was higher in strain I1.3 at 22°C and 2 wpg, with CSV1 and
360 Augusto2 showing similar expression. At 31°C, the RT-PCR results were similar in
361 every strain, showing low expression, while at 22°C and 3 wpg, CSV1 induced the
362 highest level of expression, followed by I1.3 and Augusto2.

363 Neither *fum1* nor *fum21* expression was detected in plants at any time point,
364 confirming the results obtained by the chemical analyses.

365

366 **Sequencing, assembly and bioinformatic analysis**

367 The results of the genome sequencing are presented in **table 1**. MiSeq Illumina
368 sequencing produced respectively 3.76, 3.9 and 2.12 millions of raw paired ends reads
369 for Augusto2, CSV1 and I1.3. For I1.3, 16.57 million reads of mate pair reads were
370 also obtained.

371 The estimated coverage, based on the 43.65 Mb length of the reference genome of
372 strain IMI 58289 (Wiemann *et al.*, 2013), was 9.68X, 10.16X and 62.34X, for Augusto2,
373 CSV1 and I1.3, respectively. Starting from these data, the reference guided approach
374 with IMR/DENOM allowed to reconstruct the 12 chromosomes of the three *F. fujikuroi*
375 strains. Using MAKER, it was possible to predict 13563, 13578 and 13690 proteins for
376 Augusto2, CSV1 and I1.3. The assemblies and their annotations were deposited in
377 GenBank: I1.3 (Accession number: CP023101 - CP023112); Augusto2 (CP023089 -
378 CP023100); CSV1 (CP023077 - CP023088).

379 By using MAKER on the *de novo* assemblies of the three strains, it was possible to
380 observe that the genes of these clusters do not appear to be in a different order in the
381 genomes. However, the short length of the *de novo* assembly scaffolds made it

382 impossible to verify the position of every gene of the clusters.

383 The genes present either in Augusto2 or in CSV1, but not in both, are listed in
384 **supplementary table 5**.

385

386 **SNP mining**

387 Compared to the reference genome of *F. fujikuroi* strain IMI 58289, 178,594, 182,179
388 and 180,779 SNPs/indels were found in Augusto2, CSV1 and I1.3, respectively
389 (**supplementary file 2**). The distribution of these polymorphisms in the three strains
390 is shown in **figure 5**, and their position on the reference genome is presented in **figure**
391 **6**. The vast majority of the polymorphisms (over 80%; 148,623 SNPs/indels) are
392 shared by the three strains. The differences between the analyzed strains and the
393 reference strain were evaluated with SNPeff (Cingolani *et al.*, 2012), and the results
394 of this analysis are presented in **table 2**. None of the strains presented a unique
395 polymorphism in the gibberellin gene cluster, not even at intergenic level (data not
396 shown). With “unique polymorphism”, a mutation not common to all the three strains
397 is meant. In the fumonisin gene clusters, on the other hand, there were a number of
398 polymorphisms upstream and downstream the genes, together with various unique
399 missense polymorphisms. In particular, in *I1.3* strain, there were 4 missense
400 polymorphisms in the transcription factor *fum21* and 2 in the polyketide synthase *fum1*.
401 One missense polymorphism in each of these two genes was also present in the
402 strains CSV1 and Augusto2.

403 Regarding the fusaric acid gene cluster, there were some intron and intergenic
404 polymorphisms, but no missense or nonsense polymorphisms. Unique missense and
405 nonsense SNPs in the regulators were also searched (**supplementary table 3**), and
406 1 missense SNP in the sequence of the global regulator *vea* was identified in the

407 strains Augusto2 and I1.3. All these polymorphisms were analyzed with PROVEAN
408 PROTEIN (Choi and Chan, 2015), and two SNPs observed in the strain I1.3, one in
409 the polyketide synthase *fum1* and one in the transcription factor *fum21* were predicted
410 to have a deleterious effect on the function of their protein. CSV1 and Augusto2,
411 despite their differences in the phenotype, had most polymorphisms in common. Only
412 138 reference genes have a missense, nonsense or frameshift polymorphism not
413 common to both CSV1 and Augusto2. Of this subset, 34 genes had some GO terms
414 related to pathogenicity, metabolism or regulation of transcription, and only eight had
415 stop, frameshift or missense mutations predicted to be deleterious by PROVEAN
416 Protein (**supplementary table 5**). By mapping the reads on the scaffold 005 of *F.*
417 *fujikuroi* strain 005 (Genbank: FMSL01000005.1), the gene FFB14_06372, encoding
418 PKS51, a protein involved in causing stunting and withering in hosts, was not covered
419 in reads in any of the strains, suggesting its absence in the analyzed genomes.

420

421 **Structural variant analysis**

422 The results of breakdancer are presented in **supplementary file 3**. 107 deletions, 21
423 inversions and 3 intra-chromosomal translocations were identified, putatively affecting
424 the function of 66 genes in at least one of the strains.

425

426 **Phylogenetic analysis**

427 The analysis with OrthoFinder identified 14,699 orthogroups among the considered
428 proteomes. In the resulting phylogenetic tree, *F. fujikuroi* strains Augusto2 and CSV1
429 seem to be closer to each other than to I1.3, although it must be noted that the support

430 values of the tree tend to be low (**supplementary figure 3**).

431

432 **Effector prediction and analysis**

433 A list of 323 putative effectors was obtained by running EffectorP 1.0 and 2.0 on the
434 *F. fujikuroi* secretome (Wiemann *et al.*, 2013) and cross-referencing results
435 (Sperschneider *et al.*, 2016). Two of the identified genes had missense polymorphisms
436 present in CSV1 but not in Augusto2: FFUJ_01956 and FFUJ_11601. Analysis with
437 PROVEAN Protein, however, predicted that these two polymorphisms did not have an
438 effect on the protein function.

439

440 **Discussion**

441 **Temperature effect**

442 This work investigates the effect of temperature in the rice-*F. fujikuroi* pathosystem:
443 three newly sequenced *F. fujikuroi* strains induce different symptoms in rice depending
444 on the temperature. Pathogenicity trials were conducted with three strains of different
445 virulence, and in every case, the pathogen was much more virulent at 31°C than at
446 22°C. In fact, at 31°C the strains I1.3 and Augusto2 were so virulent that most of the
447 plants were dead after 2 wpg.

448 Niehaus *et al.* (2017) showed how there are at least two pathotypes of *F. fujikuroi*, one
449 associated with bakanae-like symptoms and gibberellin production, the other inducing
450 withering and stunting. The pathotypes are thought to be diverse from a phylogenetic,
451 symptomatic and metabolomic point of view. However, in the current study, strains
452 phylogenetically close to each other were capable of inducing both types of symptoms.
453 At 22°C and 2 wpg, the symptoms of all the strains are mixed, with some plants
454 showing elongation and some stunting (**figure 1**). However, at this time point, I1.3

455 showed a high expression of both *cps/ks* and Gibberellin 20 Oxidase 1 (**figure 4**),
456 belonging respectively to the fungal and plant gibberellin gene clusters, and this
457 induced one week later “bakanae-like” symptoms in all the plants. Conversely, CSV1
458 and Augusto2 did not express strongly the gibberellin gene clusters, and therefore the
459 induced symptoms were mostly dwarfism, with no elongation in CSV1 and slight
460 elongation in Augusto2 (**figure 1**). This is corroborated by the HPLC-MS analysis:
461 plants inoculated with strain I1.3 contained a higher concentration of GA3 at 3 wpg,
462 while one week before the quantities were similar for every strain (**figure 3**). On the
463 other hand, at 31°C, the surviving plants inoculated with Augusto2 and I1.3 showed
464 elongation (**figure 1**), and they had a very high content in GA3 (**figure 3**), while CSV1
465 mostly induced stunting, and contained less GA3. In addition, the expression of
466 CPS/KS and Gibberellin 20 oxidase 1 was low in CSV1, and one week later the GA3
467 level was even less. The very low number of surviving plants did not permit to perform
468 analysis at 31°C and 3 wpg for Augusto2 and I1.3, but they both showed a low
469 expression of Gibberellin 20 oxidase 1 at 2 wpg, though a significant expression of
470 *cps/ks* was measured in I1.3. Even at 2 wpg, most of the plants were dead, so the
471 significantly greater expression of *cps/ks* in I1.3 at 2 wpg is due to the survival of few
472 plants which showed a high expression level.

473 In conclusion, it seems that, despite their proximity from a phylogenetical point of view
474 (**figures 5 and 7**), I1.3 induces a “bakanae-like” phenotype at all temperatures, and
475 CSV1 is characterized by low GA3 production and stunting, while Augusto2 is actually
476 capable of changing the induced symptoms depending on the temperature, being
477 closer to I1.3 at 31°C and a mix of both phenotypes at 22°C.

478 The gene encoding PKS51, associated with the *F. fujikuroi* pathotype causing stunting

479 and withering, was not present in the three examined strains.

480

481 **Fumonisin production**

482 Fumonisin are mycotoxins whose consumption produces a vast array of effects on
483 animals, including nephrotoxicity and hepatotoxicity (Bolger *et al.*, 2001), as well as
484 neurotoxicity and cardiotoxicity (Scott, 2012). Fumonisin or fumonisin transcripts
485 were not detected *in vivo*, neither with HPLC-MS nor with real time PCRs, but this was
486 expected, given the fact that this pathogen produces minimal amounts of these
487 metabolites (Wiemann *et al.*, 2013). However, fumonisins were detected *in vitro* for
488 strains Augusto2 and CSV1. I1.3 did not produce fumonisins at a detectable level
489 neither *in vivo* nor *in vitro*, likely as an effect of the putatively important polymorphisms
490 that this strain has in the transcription factor *fum21* and the polyketide synthase *fum1*,
491 since both genes are essential for the correct functioning of the gene cluster
492 (Alexander *et al.*, 2009).

493 A study of Cruz *et al.* (2013) found no relationship in *F. fujikuroi* between pathogenicity
494 and the ability to produce fumonisins (Cruz *et al.*, 2013), while Niehaus *et al.* (2017)
495 observed that the deletion of the fumonisin PKS caused a reduction in virulence, but
496 only in stunting-inducing strains. Our data correlate well with these studies: none of
497 our strains produced fumonisins in detectable quantity *in vivo* and, while this has
498 probably a negligible effect on the virulence of I1.3 and Augusto2, it may impact the
499 virulence of CSV1, which induced stunting at both the tested temperatures and
500 presented a lower virulence than the other two isolates.

501 In the work of Matic *et al.* (2013) the fumonisin synthesis of the same three strains was
502 analyzed, with similar results: Augusto2 produced by far the highest quantity of these
503 mycotoxins, followed by CSV1. Interestingly, in the conditions tested in that work,

504 strain I1.3 was able to produce a small amount of fumonisin B1.

505

506 **Different phenotypes, similar genomes**

507 The three sequenced *F. fujikuroi* strains were isolated from the same geographic area,
508 but their phenotype was very different. CSV1 and Augusto2 are particularly close from
509 an evolutionary point of view (**supplementary figure 3**), with only 21,887 SNPs
510 between them, but they differ in virulence, reaction to temperature, induced symptoms,
511 colony morphology and color, growth speed, fumonisin and gibberellin production.
512 Given the low sequencing coverage used, the amount of SNPs was probably
513 underestimated, but the high percentage of shared polymorphisms (93% of the total
514 for CSV1 and 95% for Augusto2) is a further proof of the low evolutionary distance
515 between the two strains.

516 The most common genes involved in pathogenesis and gibberellin production were
517 checked for polymorphisms, but no SNPs that could explain these variations were
518 found. Even if Augusto2 and I1.3 had a missense SNP in *vea*, a regulator of secondary
519 metabolism associated to fumonisin and fusarin production, there is currently no
520 evidence linking fusarins to the development of the disease. Fumonisin production is
521 believed to have no relationship with pathogenicity of elongation-inducing *F. fujikuroi*
522 as well (Cruz *et al.*, 2013; Niehaus *et al.*, 2017), and none of the considered strains
523 produced fumonisins at a detectable level *in vivo*. Two putative effectors presented
524 missense polymorphisms in CSV1 and not in the other strains, but a prediction
525 analysis with PROVEAN Protein showed that it is unlikely for these differences to have
526 an impact on the protein function.

527 The differences between the genomes of Augusto2 and CSV1 were further
528 investigated by checking missense, frameshift or nonsense SNPs present in either

529 CSV1 or Augusto2, but not both. The genes presenting these polymorphisms were
530 filtered by checking for GO terms related to metabolism, pathogenicity or gene
531 regulation, and the missense SNPs were evaluated with PROVEAN Protein,
532 discarding those with a putative neutral effect. The remaining genes are listed in
533 **supplementary table 5**, which contains also the genes putatively affected by a
534 structural variant either in Augusto2 or in CSV1, but not in both. An other source of
535 phenotype variation could be the absence or presence of certain genes in the
536 genomes, though only 14 genes were present either in Augusto2 or CSV1, but not in
537 both (**supplementary table 5**).

538 The genes with predicted function-affecting polymorphisms do not seem directly
539 correlated to the observed differences in the phenotype, and neither do the genes
540 present in only one of the genomes. However, the protein CCT62922.1, a pisatin
541 demethylase, was putatively affected by an inversion in CSV1, and this class of
542 proteins is known to be a factor of virulence in both *F. oxysporum* and *F. solani* (Rocha
543 *et al.*, 2015; Wasmann and VanEtten, 1996). Conversely, CCT63174.1, an endo
544 polygalacturonase, a virulence factor in *F. graminearum* (Paccanaro *et al.*, 2017), was
545 removed by a deletion in Augusto2 and I1.3, but not in CSV1. Another protein
546 putatively not functioning in Augusto2 was CCT73390.1, an integral membrane
547 protein, and some proteins of this class are factors of virulence for plant pathogens,
548 such as integral membrane protein PTH11, which is required for pathogenicity and
549 appressorium formation in *Magnaporthe grisea* and it exhibits host-preferential
550 expression in *F. graminearum* (DeZwaan *et al.*, 1999; Harris *et al.*, 2016). Finally,
551 CCT74990.1, related to a fructosyl amino acid oxidase, was predicted to be affected
552 by an inversion in CSV1, but this protein was proven to be dispensable for
553 development and growth in *Aspergillus nidulans*, whose null mutant for this gene grew

554 normally and developed as many conidia and sexual structures as the wild-type
555 (Jeong *et al.*, 2002).

556 Besides these proteins, a number of the genes presented in **supplementary table 5**
557 are currently uncharacterized, and their activity could contribute to the differences
558 observed between Augusto2 and CSV1

559

560 **Conclusions**

561 This study presents a comparative genomics analysis of three *F. fujikuroi* strains
562 isolated in northern Italy, the largest European production area of rice. The strains
563 showed remarkable difference in the phenotype, despite being very close from an
564 evolutionary point of view, suggesting that a few key mutations in a small number of
565 genes can dramatically alter the phenotype induced by the pathogen. A few candidate
566 genes that may explain these phenotypic differences were identified.

567 The species *F. fujikuroi* was recently divided in two phylogenetically separated
568 pathotypes (Niehaus *et al.*, 2017), which induce respectively bakanae symptoms or
569 stunting and withering. However, in this study it has been observed that minimal
570 genetic differences can induce symptom modifications, and some strains may be able
571 to induce both types of phenotypes, depending on environmental factors such as
572 temperature.

573 Finally, it was observed that the considered *F. fujikuroi* strains became much more
574 virulent at higher temperatures. This observation could be linked to the effect of
575 occurring climatic changes. The rise of average temperatures in spring may affect rice
576 production not only with increasing losses induced by abiotic stresses, but also with
577 the average increase of virulence of *F. fujikuroi*. While the danger posed to rice by
578 climate change favoring abiotic stresses is known (Mohammed and Tarpley, 2009),

579 and efforts are underway to obtain climate-resilient cultivars (Sreenivasulu *et al.*,
580 2015), there is little knowledge over the impact of increased temperatures on the
581 interactions between rice and fungal pathogens.

582

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858 **Tables**

859

860 **Table 1:** Data regarding the genome reference-guided assembly and annotation of
861 strains Augusto2, CSV1 and I1.3 of *Fusarium fujikuroi*.

862

	Augusto2	CSV1	I1.3
Genome Size	~43.7 Mb	~43.7 Mb	~45.6Mb
Sequencing coverage	9.7X	10.2X	62.3X
Number of contigs	12	12	12
Number of Large contigs (>100 Kb)	12	12	12
N50 (base pairs)	4,218,434	4,212,448	4,426,414
GC content	47.49%	47.51%	47.2%
Number of genes	13,563	13,578	13,690
Annotated genes	10,073	10,080	9,838

863

864 **Table 2:** Number and putative effect of polymorphisms detected in the strains CSV1,
865 Augusto2 and I1.3 of *Fusarium fujikuroi*. The reference used for the SNP calling was
866 the genome of *F. fujikuroi* strain IMI 58289.

867

Strain	CSV1	Augusto2	I1.3
Number of polymorphisms	182,179	178,594	180,779
SNPs	176,34	172,722	174,933
Insertions	3,061	3,08	3,082
Deletions	2,778	2,778	2,764
Variant rate	1/241 bases	1/245 bases	1/242 bases

Predicted polymorphism effect

Silent	41,476	41,457	41,375
Missense	27,404	27,385	27,356
Nonsense	376	373	373

Polymorphism impact

High	973	973	949
Moderate	27,589	27,565	27,551
Low	43,318	43,301	43,228
Negligible	714,739	708,481	712,012

868

869

870 **Figure captions**

871

872 **Figure 1:** Rice plants (cv. Galileo) inoculated with *F. fujikuroi* strains CSV1, Augusto2
873 or I1.3.

874 **Figure 2:** Disease indexes of rice plants (cv. Galileo) inoculated with the 3 studied
875 strains of *F. fujikuroi*.

876 **Figure 3:** GA3 quantity in rice plants (cv. Galileo) inoculated with the 3 studied strains
877 of *F. fujikuroi*. Analysis done at 2 or 3 weeks since germination, at 22°C and 31°C.
878 The error bars represent the standard deviation. Values followed by the same letter
879 are not statistically different by Duncan's multiple range test ($p < 0.05$). This test was
880 executed in an independent manner for the samples at 22 °C and those at 31 °C.

881 **Figure 4:** Gene expression of *cps/ks*, a gene of *F. fujikuroi* gibberellin cluster (**A**), and
882 of gibberellin 20 oxidase 1, a gene of rice gibberellin cluster (**B**). Data obtained by
883 reverse transcriptase real time PCR. The error bar is the standard deviation. Values
884 followed by the same letter are not statistically different by Duncan's multiple range
885 test ($p < 0.05$).

886 **Figure 5:** Venn graphic showing the distribution of polymorphisms among the *F.*
887 *fujikuroi* strains Augusto2, CSV1 and I1.3. The genome of strain IMI 58289 was used
888 as reference in the SNP calling. Image obtained with the software at the following link:
889 <http://bioinformatics.psb.ugent.be/webtools/Venn/>

890 **Figure 6:** The figure shows the localization of polymorphisms in the *F. fujikuroi* strains
891 Augusto2, CSV1 and I1.3 on the reference genome of strain IMI 58289. The external
892 ring shows the polymorphisms of strain Augusto2, the central one shows those of
893 CSV1 and the internal one shows the ones of I1.3. Image obtained with CircosVCF
894 (Drori *et al.*, 2017).

895 **e-Xtras**

896 **Supplementary figure 1:** Front and reverse view of *F. fujikuroi* strains Augusto2,
897 CSV1 and I1.3, growing on YES Agar plates at different time points. The inoculation
898 on the plates was done with 100 µl of a suspension of $5 \cdot 10^5$ conidia/ml

899 **Supplementary figure 2:** Diameter of colonies of *F. fujikuroi* strains Augusto2, CSV1
900 and I1.3, growing on YES Agar plates. The inoculation on the plates was done with
901 100 µl of a suspension of $5 \cdot 10^5$ conidia/ml.

902 **Supplementary figure 3:** The tree describes the phylogeny of the strains Augusto2,
903 CSV1 and I1.3 of *F. fujikuroi*, in relation to other strains of the same species. *Fusarium*
904 *oxysporum* f. sp. *lycopersici* 4287 was used as outgroup (GenBank:
905 GCA_000149955.2). The tree was obtained by using the programs OrthoFinder 2.3.3
906 (Emms and Kelly, 2015) and STAG (Emms and Kelly, 2018). The root was placed with
907 MEGA (Kumar *et al.*, 1994) between *F. oxysporum* and the *F. fujikuroi* strains.

908

909 **Supplementary table 1:** Primers used for the reverse transcription real time PCRs.

910 **Supplementary table 2:** Data regarding the *de novo* assembly of strains Augusto2,
911 CSV1 and I1.3 of *Fusarium fujikuroi*.

912 **Supplementary table 3:** Genes of the gibberellin and fumonisin gene clusters, and
913 regulators checked for polymorphisms in the three strains.

914 **Supplementary table 4:** HPLC-MS quantification of GA3, fumonisin B1, fumonisin
915 B2, fumonisin B3 and fumonisin B4 in *F. fujikuroi* strains growing on PDB and YES
916 Agar media.

917 **Supplementary table 5:** Sheet 1 contains genes present either in strain CSV1 or
918 Augusto2, but not both. The putative function of their closest blast hit is included in the
919 table, as is their absence or presence in the strain I1.3 genome. Sheet 2 contains all
920 genes presenting a deleterious missense polymorphism or a nonsense or frameshift
921 polymorphism either in Augusto2 or CSV1, but not in both. Only genes described by
922 at least a GO term related to regulation of transcription, pathogenesis or metabolism
923 were included. The position of the polymorphism, the amino acid commonly found in
924 that position and the protein putative function are indicated. Sheet 3 contain
925 information about the genes putatively affected by structural variants identified with
926 BreakDancer (Fan *et al.*, 2014). Genes were considered to be putatively affected when
927 they had a deletion inside their sequence, or when the extremity of an inversion or
928 translocation was localized inside their sequence.

929

930 **Supplementary file 1:** Python3 script used to identify genes putatively affected by
931 structural variations identified with BreakDancer (Fan *et al.*, 2014).

932 **Supplementary file 2:** vcf file obtained from the SNP calling of the reference genome
933 of *F. fujikuroi* strain IMI 58289.

934 **Supplementary file 3:** results of BreakDancer (Fan *et al.*, 2014), run with default
935 parameters.