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

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Abstract

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

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

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Introduction

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

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

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

(Niehaus *et al.*, 2017).

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

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

Beside gibberellins, the fungus is able to produce a wide array of secondary metabolites, including both mycotoxins, such as fumonisins, fusaric acid, and fusarins (Bacon *et al.*, 1996; Barrero *et al.*, 1991; Desjardins *et al.*, 1997), and pigments, like bikaverin and fusarubins (Balan *et al*, 1970; Studt *et al.*, 2012). Forty-seven putative gene clusters for secondary metabolites were found in the reference genome of *F fujikuroi* (Wiemann *et al.*, 2013), and a number of these have been characterized in recent years (Janevska and Tudzynscki, 2018). A number of global and local regulators control the production of secondary metabolites, but many are also able to regulate gibberellin production, and therefore pathogenicity. These include the global nitrogen regulators *area* (Tudzynski *et al.*, 1999) and *areb* (Pfannmüller *et al.*, 2017) and the component of the velvet complex *lae1* (Niehaus *et al.*, 2018).

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

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

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

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

Materials and methods

Microorganisms and seeds

The strains of *F. fujikuroi* named Augusto2, CSV1 and I1.3, previously isolated from diseased rice plants in Piedmont (Amatulli *et al.*, 2010) and maintained in the Agroinnova microorganism collection, were grown on sterile PDB for 10 days at 23°C. Afterwards, the suspensions were filtered through sterile gauze, centrifuged for 20 min at 6,000 RPM and resuspended in Ringer solution. The Burker chamber was then used to obtain concentrations of 10^5 conidia/ml. Rice seeds 'Galileo', susceptible to bakanae disease (Amatulli *et al.*, 2010) were thermally treated by dipping in water at 60°C for 5 min, immersed in a solution of 1% NaClO for 2 min and then washed three times with sterile water for 5 min. The seeds were then divided and immersed in the conidial suspension of the different strains and kept in agitation for 30 min.

Pathogenicity trials

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

RNA extraction and qPCR

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

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

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

***In vitro* assays**

Every strain was grown in PDB flasks (30 ml of medium) and YES Agar plates. The flasks and the plates were inoculated with 100 µl of a solution containing 10^5 conidia/ml, prepared following the same procedure used for the pathogenicity trials. The plates were kept at 24°C, with a 12:12 h light/dark photoperiod, a light intensity of 1 cd and a relative luminosity of 55 cd. During the fungal growth in YES Agar, the mycelial diameter was measured, and the color and texture were monitored.

Chemical extractions

Samples obtained by PDB flasks were filtered to separate the mycelium from the growth medium. Mycelium was weighed (500mg) and extracted with 1ml of methanol:water (8:2 v/v), during 1 hour in ultrasonic bath. Supernatant was centrifuged and filtered by 0.45 μm filters, after which it was placed in the vials for HPLC analysis. Regarding YES Agar plates, the extraction was carried out on the whole plates with 3ml of methanol. The solvent was spread on the whole surface and the mycelium was scratched and brought to suspension. The extract was then placed in tubes and concentrated with a Concentrator 5301 (Hamburg, Germany). The dried residue was dissolved in methanol:water (1:1 v/v) and placed in vials for HPLC analysis. Similarly to the procedure used for mycelia, 500 mg of *in vivo* sample were extracted with 1 ml of methanol:water (8:2 v/v) by ultrasonic bath for 1 hour. Supernatant was centrifuged and filtered with 0,45 μm filters, after which it was placed in vials.

HPLC-MS/MS

Liquid chromatography was performed with Varian Model 212-LC micro pumps (Hansen Way, CA, USA) coupled with a Varian 126 autosampler Model 410 Prostar. A Synergi 4u Fusion-RP 80A (100 mm × 2.0 mm, Phenomenex, Castel Maggiore, Italy) analytical column was used coupled with Fusion-RP (4 × 2.0 mm) security guard for LC separation. The chromatographic conditions were: column temperature at 45 °C; mobile phase consisting of eluent A (HCOOH 0,05% in H2O) and eluent B (CH3CN). A gradient elution was applied as follows: 0 to 20% of B in 5 minutes, from 20% to 80% of B in 15 minutes, from 80% to 100% of B in 1 minute. Five minutes of post run were necessary for column conditioning before the subsequent injection. The injection volume was 20 µl, and the flow speed was of flow of 200 µl/min.

The triple quadrupole mass spectrometer (Varian 310-MS) was operated in the negative/positive electrospray ionization mode (ESI^-/ESI^+). To select the MS/MS parameters for the analysis of metabolites by multiple reaction monitoring (MRM). For the quantification of fumonisin B4 the calibration curve of fumonisin B1 was used, since fumonisin B4 currently lacks a specific commercial standard. Two transitions were selected for each compound: GA3: 345>214 (CE 14 eV), 345>143 (CE 30 eV); FB1: 722>334 (CE 38 eV), 722>352 (CE 34 eV); FB2/FB3: 706>336 (CE 36 eV), 706>354 (CE 34 eV); FB4: 690>338 (CE 30 eV), 690>320 (CE 30 eV). The collision gas (Ar) pressure was set at 2 mbar for all of the experiments.

Sequencing, assembly and analysis

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

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

Gene prediction

Gene prediction was conducted using the version 2.31.8 of MAKER (Cantarel *et al.*, 2008). Both predictors augustus v.2.5.5 (Stanke and Waack, 2003) and SNAP v.2006-07-28 (*http://korflab.ucdavis.edu/software.html*) were used. augustus used the "--fusarium" option for gene prediction, while SNAP was trained to obtain a file.hmm specific for the three genomes. The necessary repeat libraries were constructed using the basic procedure (*http://weatherby.genetics.utah.edu/MAKER/wiki/index.php/Repeat_Library_Construction--Basic*). The external data provided to MAKER, and used for the training of SNAP, were all the EST, protein sequences and transcript sequences of *F. fujikuroi* available on NCBI. To launch MAKER, the option "-fix_nucleotides" was used, in order to allow the program to work with degenerate nucleotides present in the external data. The option “correct_est_fusion” was also activated in the control files. After the analysis, introns shorter than 10 bp, predicted by snap, were removed, and, when this caused a frameshift mutation, the prediction of the gene splicing sites was repeated with augustus v.2.5.5 (Stanke and Waack, 2003).

SNP mining

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

‘samtools mpileup -guf reference.fa augusto2.sort.bam CSV1.sort.bam I1.3.sort.bam | bcftools view -cg - | vcfutils.pl varFilter -D 200 -Q 20 - > file.vcf’

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

Analysis of polymorphisms

The SnpEff program v. 4.2 (Cingolani *et al.,* 2012) was used to evaluate the impact of the SNPs/indels identified with the SNP mining, after building a database for IMI 58289 following the manual instructions (http://snpeff.sourceforge.net/SnpEff_manual.html#databases). Afterwards, we checked if the strains Augusto2, CSV1 and I1.3 presented missense or nonsense polymorphisms in the gibberellin and fumonisin gene clusters, or in other genes involved in the biosynthesis regulation of these metabolites (see **supplementary table 3** for references). The presence of these polymorphisms was then checked in the sort.bam files with the viewer Tablet (Milne *et al.*, 2013). EffectorP 1.0 and 2.0 (Sperschneider *et al.*, 2016) were used on the secreted portion of the *F. fujikuroi* proteome (Wiemann *et al.*, 2013) to predict putative effector genes, and these genes were also checked for polymorphisms. The impact of polymorphisms of interest was predicted with Provean Protein (Choi and Chan, 2015). All the genes presenting putatively MODERATE and HIGH impact polymorphisms in either Augusto2 or CSV1, but not in both, were identified, according to the evaluation of SNPeff. These genes were annotated with BLAST2GO with default parameters, and, when they presented GO terms related to regulation of transcription, pathogenesis or metabolism, the impact of their polymorphisms was predicted with Provean Protein (Choi and Chan, 2015).

Structural variant analysis

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

Phylogenetic analysis

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

Comparing Augusto2 and CSV1

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

Results

Pathogenicity trials

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

In vitro trials

The three strains on YES Agar produced mycelia of different color, dimension and texture (**supplementary figure 1**). CSV1 and I1.3 mycelia are characterized by a reverse red-orange color, not present in Augusto2. This color is also present in the front view of the CSV1 mycelia. Growth speed was not uniform as well (**supplementary figure 2**). CSV1 grew faster at the beginning of the trial, reaching a diameter of around 40 mm at 5 days after the inoculation. Afterwards, Augusto2 and I1.3 started growing faster, reaching, after 14 days of inoculation, average diameters of 80 and 65 mm, against the 50 mm of CSV1.

Chemical analyses

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

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

Real time RT-PCRs

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

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

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

Sequencing, assembly and bioinformatic analysis

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

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

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

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

SNP mining

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

Regarding the fusaric acid gene cluster, there were some intron and intergenic polymorphisms, but no missense or nonsense polymorphisms. Unique missense and nonsense SNPs in the regulators were also searched (**supplementary table 3)**, and 1 missense SNP in the sequence of the global regulator *vea* was identified in the strains Augusto2 and I1.3. All these polymorphisms were analyzed with PROVEAN PROTEIN (Choi and Chan, 2015), and two SNPs observed in the strain I1.3, one in the polyketide synthase *fum1* and one in the transcription factor *fum21* were predicted to have a deleterious effect on the function of their protein. CSV1 and Augusto2, despite their differences in the phenotype, had most polymorphisms in common. Only 138 reference genes have a missense, nonsense or frameshift polymorphism not common to both CSV1 and Augusto2. Of this subset, 34 genes had some GO terms related to pathogenicity, metabolism or regulation of transcription, and only eight had stop, frameshift or missense mutations predicted to be deleterious by PROVEAN Protein (**supplementary table 5**). By mapping the reads on the scaffold 005 of *F. fujikuroi* strain 005 (Genbank: FMSL01000005.1), the gene FFB14_06372, encoding PKS51, a protein involved in causing stunting and withering in hosts, was not covered in reads in any of the strains, suggesting its absence in the analyzed genomes.

Structural variant analysis

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

Phylogenetic analysis

The analysis with OrthoFinder identified 14,699 orthogroups among the considered proteomes. In the resulting phylogenetic tree, *F. fujikuroi* strains Augusto2 and CSV1 seem to be closer to each other than to I1.3, although it must be noted that the support values of the tree tend to be low (**supplementary figure 3**).

Effector prediction and analysis

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

Discussion

Temperature effect

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

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

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

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

The gene encoding PKS51, associated with the *F. fujikuroi* pathotype causing stunting and withering, was not present in the three examined strains.

Fumonisin production

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

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

In the work of Matić *et al.* (2013) the fumonisin synthesis of the same three strains was analyzed, with similar results: Augusto2 produced by far the highest quantity of these mycotoxins, followed by CSV1. Interestingly, in the conditions tested in that work, strain I1.3 was able to produce a small amount of fumonisin B1.

Different phenotypes, similar genomes

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

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

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

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

The genes with predicted function-affecting polymorphisms do not seem directly correlated to the observed differences in the phenotype, and neither do the genes present in only one of the genomes. However, the protein CCT62922.1, a pisatin demethylase, was putatively affected by an inversion in CSV1, and this class of proteins is known to be a factor of virulence in both *F. oxysporum* and *F. solani* (Rocha *et al*., 2015; Wasmann and VanEtten, 1996). Conversely, CCT63174.1, an endo polygalacturonase, a virulence factor in *F. graminearum* (Paccanaro *et al*., 2017), was removed by a deletion in Augusto2 and I1.3, but not in CSV1. Another protein putatively not functioning in Augusto2 was CCT73390.1, an integral membrane protein, and some proteins of this class are factors of virulence for plant pathogens, such as integral membrane protein PTH11, which is required for pathogenicity and appressorium formation in *Magnaporthe grisea* and it exhibits host-preferential expression in *F. graminearum* (DeZwaan *et al*., 1999; Harris *et al*., 2016). Finally, CCT74990.1, related to a fructosyl amino acid oxidase, was predicted to be affected by an inversion in CSV1, but this protein was proven to be dispensable for development and growth in *Aspergillus nidulans*, whose null mutant for this gene grew normally and developed as many conidia and sexual structures as the wild-type (Jeong *et al*., 2002).

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

Conclusions

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

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

Finally, it was observed that the considered *F. fujikuroi* strains became much more virulent at higher temperatures. This observation could be linked to the effect of occurring climatic changes. The rise of average temperatures in spring may affect rice production not only with increasing losses induced by abiotic stresses, but also with the average increase of virulence of *F. fujikuroi*. While the danger posed to rice by climate change favoring abiotic stresses is known (Mohammed and Tarpley, 2009), and efforts are underway to obtain climate-resilient cultivars (Sreenivasulu *et al.*, 2015), there is little knowledge over the impact of increased temperatures on the interactions between rice and fungal pathogens.

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Tables

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

	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

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

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

Figure captions

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

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

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

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

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

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

e-Xtras

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

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

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

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

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

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

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

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

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

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

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