

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Conventional and Real-Time PCR for the identification of *Fusarium fujikuroi* and *Fusarium proliferatum* from diseased rice tissues and seeds

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/118712> since 2016-10-18T12:26:25Z

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

1
2
3
4
5
6
7

The final publication is available at Springer via <http://dx.doi.org/10.1007/s10658-012-9998-0>

8 **Conventional and Real-Time PCR for the identification of *Fusarium fujikuroi* and *Fusarium***
9 ***proliferatum* from diseased rice tissues and seeds.**

10

11 Maria Teresa Amatulli¹, Davide Spadaro^{1*}, Maria Lodovica Gullino¹ & Angelo Garibaldi¹

12

13 ¹ Agroinnova, Centre of Competence for the Innovation in the Agro-Environmental Field,
14 University of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco (To), Italy

15

16 Corresponding author: Davide Spadaro, davide.spadaro@unito.it, Phone: +39-011-6708942, Fax:
17 +39-011-6709307.

18

19 **Keywords:** diagnostics, bakanae disease, *TEF* gene, *Oryza sativa*.

20

21 **Abstract**

22 *Fusarium fujikuroi* is a species of the *Gibberella fujikuroi* species complex (GFSC) and the causal
23 agent of bakanae disease on rice. Even if *F. fujikuroi* is the most abundant *Fusarium* species found
24 on rice, other species can also be isolated from rice, such as *F. proliferatum*. Multiple alignment of
25 translation elongation factor (*TEF*) gene sequences of different *Fusarium* spp., showed a deletion of
26 six nucleotides in *F. fujikuroi* sequence and a two nucleotide polymorphism in the same region of *F.*
27 *proliferatum* sequence. These elements of variability were used to develop a conventional and Real-
28 Time PCR assay for diagnosis. The species specific primer pairs (Fuji1F/TEF1R and
29 Proli1F/TEF1R) gave a product of 179 and 188 bp for *F. fujikuroi* and *F. proliferatum* respectively.
30 Primer specificity was confirmed by analyzing the DNA of the most representative species of the
31 GFSC and 298 strains of *Fusarium* spp. isolated from rice plants and seeds in Italy. The specific
32 primers were also successfully used to detect fungal presence directly from infected rice tissues and
33 seeds, providing a rapid tool for the early detection of pathogen contamination.

34

35 **Introduction**

36 *Fusarium fujikuroi* Nirenberg [teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura]
37 is an important rice pathogen and a member of the polyphyletic taxon, *Gibberella fujikuroi* species
38 complex (GFSC). This fungus is seed-borne and the causal agent of bakanae diseases of rice in a
39 number of rice growing areas (Carter et al. 2008). Chemical seed dressing has been the most
40 common way to control rice bakanae for a long time. In some countries, such as Japan and China,
41 seeds are soaked in water suspensions containing carbendazim, prochloraz, trifumezol, thiram or
42 other fungicides (Zhou et al., 1994). Due to the reduction in pesticide availability, in recent years
43 the disease incidence has increased also in Italy (Amatulli et al. 2010), becoming a serious
44 problem especially for the seed companies which often have to sell certified rice seed, free of *F.*
45 *fujikuroi*. The most evident symptom of the disease is yellowing and abnormal elongation of
46 infected seedlings, which led to the Japanese name *bakanae*, meaning “foolish seedling.” In older
47 plants, the roots, crowns, stems, leaf sheaths and panicles can be infected (Desjardins et al. 1997).
48 Although it is known that *F. fujikuroi* is the cause of bakanae disease of rice, other *Fusarium* spp.
49 also occur on rice including two other species of the GFSC, *Fusarium verticillioides* (Sacc.)
50 Nirenberg (tel. *G. moniliforme* Wineland) and *Fusarium proliferatum* (Matsushima) Nirenberg (*G.*
51 *intermedia* (Kuhlman) Samuels, Nirenberg & Seifert) that have been isolated on diseased plants in
52 different parts of the world (Desjardins et al. 1997; Voigt et al., 1995) and considered for many
53 years to be involved in the development of the disease. In particular, *F. proliferatum* is
54 morphologically indistinguishable from *F. fujikuroi* and can only be distinguished by making tests
55 of sexual cross-fertility (Leslie and Zeller 1997) or through DNA sequencing. Phylogenetically, *F.*
56 *proliferatum* and *F. fujikuroi* belong to the Asian clade of the GFSC (O’Donnell et al., 1998) and
57 are very closely related, although their karyotypes are different (Leslie and Summerell 2006). Both
58 species can produce a broad range of mycotoxins, such as fumonisin B₁, moniliformin, beauvericin,
59 fusaric acid, fusarin (Leslie and Summerell 2006). Moreover *F. proliferatum* is also able to produce

60 fusaproliferin (Ritieni et al. 1995). A correct identification of these fungal species and an early
61 detection on rice seeds could provide a very useful tool for understanding the disease epidemiology
62 and developing strategies to control the occurrence of bakanae disease and possible *Fusarium*-
63 toxins contamination. In recent decades, molecular techniques based on DNA analysis have been
64 widely used to identify and discriminate among isolates within a species and to develop rapid,
65 sensitive, and accurate detection methods. These methods have revealed great diversity among
66 *Fusarium* species, underestimated by earlier morphological criteria (O'Donnell et al. 2009; Obanor
67 et al. 2010). In particular, PCR-based techniques can provide species-specific primers, and are a
68 powerful diagnostic method for distinguishing fungal taxa at the species level (Edwards et al. 2002),
69 both from pure fungal cultures and from plants or food (Mulè et al. 2004b). Species-specific
70 primers to use in PCR assays have been generated from sequences of different origin: RAPDs
71 (Moeller et al. 1999) the internal transcribed spacer regions of rDNA (Moukhamedov et al. 1994),
72 the calmodulin gene (Mule et al. 2004a; 2004b) and mycotoxin biosynthetic genes (Bakan et al.
73 2002; Sreenivasa et al. 2008). The *TEF* gene, which encodes an essential part of the protein
74 translation machinery, has been selected as a good single-locus identification tool in *Fusarium*
75 because it shows high sequence polymorphism among closely related species, even compared to the
76 intron-rich portions of protein-coding genes such as calmodulin, beta-tubulin and histone H3, and
77 non-orthologous copies of the gene have not been detected in the genus (Geiser et al. 2004). For this
78 reason, the *TEF* gene sequence was selected in order to obtain reliable DNA variability for
79 distinguishing such species. The first aim of this work was to develop species-specific primers for
80 distinguishing *F. proliferatum* and *F. fujikuroi* from one another and from other *Fusarium* spp.
81 Moreover, to obtain a rapid tool for the early detection of the pathogen contamination, a PCR
82 method for the identification of these pathogens from rice seeds and tissues was developed.

83

84 **Materials and Methods**

85

86 **Fungal cultures and plant materials**

87 Fungal strains used for *TEF* amplification and gene sequence analyses are listed in Table 1. All
88 strains were grown on Potato Dextrose Agar (PDA, Merck) for 10 days at 25°C for subsequent
89 DNA extraction.

90 For artificial inoculation of rice seeds, one strain of *F. fujikuroi* (M1149) and one of *F. proliferatum*
91 (M6580) were used for preparing conidial suspensions. Briefly, 15-day-old fungal cultures were
92 flooded with sterile water. The resulting suspensions were filtered through two layers of sterile
93 cotton lint and brought to a final concentration of 10^6 spores ml⁻¹ with sterile distilled water.

94 Ten lots of naturally contaminated rice seeds were used for direct DNA extraction and for plating in
95 Komada medium (Komada, 1975) to confirm the molecular results. One hundred seeds per
96 repetitions and three repetitions per lot were tested. Other lots of rice seeds (cv. Galileo and cv.
97 Carnaroli) were treated with hot water for 15 minutes at 60°C (Forsberg et al., 2003), to remove any
98 surface contaminants, before being artificially inoculated by soaking for 20 minutes in the spore
99 suspension of *F. fujikuroi* and *F. proliferatum*. Then they were air dried for 60 minutes. Some
100 inoculated seeds were kept for total DNA extraction from seeds and for plating in Komada medium,
101 while others were sown in plastic pots (10x10 cm) containing sterile soil (60% peat, 40% sand) for
102 DNA extraction from diseased plants. Samples of inoculated plants were collected 20 days after
103 seed germination. The greenhouse temperature was maintained at 24°-26°C during the day and 16-
104 18°C during the night. As negative controls, hot water treated and non inoculated rice seeds,
105 together with healthy rice seedlings, were used.

106

107 **DNA extraction from fungal culture, rice tissues and seeds**

108 Total genomic DNA was extracted from about 100 mg of mycelium and from 100 mg of rice stem
109 seedlings by using the NucleoSpin Plant II Kit (Macherey Nagel GmbH and Co., USA) according to
110 the manufacturer's instructions, adding 10 µL of proteinase K (10 mg l⁻¹) and 10 µL of RNAse A
111 (12 mg l⁻¹) to PL1 buffer in each tube. Total genomic DNA from rice seeds was extracted according

112 to the protocol of Simpson et al. (2000), based on milling, freeze-drying and extraction in CTAB
113 buffer.

114

115 **TEF amplification**

116 Amplifications of the elongation factor 1 α gene were done by using specific primers (O'Donnell et
117 al. 1998): ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (5'-
118 GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'). The reaction mixture of 20 μ l contained 1 μ l of
119 buffer 10X, 1 U of Taq DNA polymerase (Qiagen, USA), 0.5 mM each of dATP, dCTP, dGTP, and
120 dTTP, 0.5 mM of each primer, and 1.5 mM of MgCl₂. Amplification was carried out in a T-
121 Gradient thermal cycler (Biometra, Germany) using the following steps: 95°C for 3 min, 35 cycles
122 with denaturation at 95°C for 1 min, 60°C for 1 min, 72°C for 3 min and a final extension at 72°C
123 for 5 min. Five μ l of the PCR products were separated by electrophoresis in 1.5% agarose gel
124 (Agarose D-1 LOW EEO Eppendorf, Italy) containing 0.001% of SYBR Safe DNA gel stain
125 (Invitrogen, USA) for 30 min at 3.3 V in 1 X TAE buffer.

126

127 **Sequence analyses and primer design**

128 The PCR products were purified using QIAquick PCR purification kit (QIAGEN) according to the
129 manufacturer's instructions and were sequenced in both directions by BMR Genomics Center
130 (Padova, Italy). Sequencing data were aligned with the program DNA baser V2.71.0 (Heracle
131 Software Lilienthal, Germany) and multiple sequence alignment was constructed with the program
132 MEGA version 4 (Tamura et al. 2007). Based on elements of variability, specific forward primers
133 for *F. fujikuroi* and *F. proliferatum* and a common reverse primer were designed. Primer quality
134 was checked by using PRIMER3 (Whitehead Institute for Biomedical Research) and specificity by
135 primer sequences BLASTn analyses (Table 2).

136

137 **Primer specificity and sensitivity**

138 Conventional PCR was used for evaluating Fuji1F/TEF1R and Proli1F/TEF1R efficiency with
139 DNA extracted from pure cultures of 256 strains of *F. fujikuroi* and *F. proliferatum* isolated from
140 Italian rice and kept in the microorganism collection of Agroinnova. Each 20 µl PCR reaction
141 contained 1 µl of DNA template (10 ng), 10 mM each deoxynucleoside triphosphate, 2 µl of 10x
142 buffer, 2 µM of each primer and 1.0 U of high fidelity Fast Taq (Roche). The reaction was carried
143 out in a TGradient thermal cycler (Biometra) programmed with the following protocol: 95°C for 5
144 min, 35 cycles of denaturation at 94°C for 40 s, annealing at 63°C for 40 s, extension at 72°C for 40
145 s and a final extension at 72°C for 5 min. Five µl of PCR products from each reaction were
146 electrophoresed in a 2.5% agarose gel and then stained with SYBR Safe (Invitrogen). The
147 specificity of the primer pairs were assessed by PCR with DNA from the other species of the *G.*
148 *fujikuroi* complex and other *Fusarium* spp. (Table 1) and on total genomic DNA extracted from
149 diseased rice tissues, and artificially inoculated or naturally contaminated rice seeds.

150

151 **Real-time PCR**

152 Sybr Green real-time PCR was used for evaluating Fuji1F/Fuji1R and Proli1F/Proli1R specificity
153 on the genomic DNA of *F. fujikuroi*, *F. proliferatum*, *F. verticillioides*, *F. graminearum*, *F.*
154 *oxysporum* and rice tissue from diseased rice tissue. Moreover, real time PCR was also
155 used to evaluate the sensitivity with serial dilutions of genomic DNA (from 10 ng to 0.1 pg
156 DNA). Reactions were performed in an iCycler (BioRad) apparatus and the results were analyzed
157 using the manufacturer's software (Optical System Software, v. 3.0; BioRad). Each reaction
158 mixture (15 µl) contained 1 µl of DNA templates, 2× Quanti Fast PCR kit (Qiagen), and 10 µM of
159 the 2 primers pairs. The Real time PCR conditions were the same used in the conventional PCR
160 reaction.

161

162 **Results**

163

164 **Sequence analyses and primer design**

165 In order to identify regions of variability able to discriminate *F. fujikuroi* and *F. proliferatum*
166 among the other species of the GFSC and the other *Fusarium* spp., a portion of *TEF* gene was
167 sequenced for the 32 strains detailed in Table 1. Multiple sequence alignment showed a deletion of
168 six nucleotides in all the strains of *F. fujikuroi* and a difference in two nucleotides in the same
169 region of all the *F. proliferatum* strains (Figure 1) compared to the other species. These
170 observations were confirmed by comparison against existing sequences of *Fusarium* spp. *TEF* gene
171 in NCBI database (www.ncbi.nlm.nih.gov) and Fustablearium ID
172 (<http://isolate.fusariumdb.org/index.php>). Through Blastx analyses we located the deletion at the
173 position 688, in the second intron of *TEF* gene, just before the beginning of the third exon.
174 Specific forward primers Fuji1F and Prol1F were designed using this nucleotide variability. A
175 common reverse primer was designed downstream in order to generate two short PCR fragments
176 (smaller than 200 bp) suitable for real time PCR. Blast analyses on GeneBank showed 100%
177 homology of forward primers with sequences of *F. fujikuroi* and *F. proliferatum* for which the
178 primers were designed.

179

180 **Primer specificity with conventional PCR**

181 PCR reaction was set up at high annealing temperature (63°C) and by using a High Fidelity Taq
182 Polymerase to prevent amplification of non-specific DNA. Primer efficiency was tested on the
183 DNA of a collection of 256 *Fusarium* spp. isolated from rice seed and tissue in Northern Italy. The
184 collection includes 198 *F. fujikuroi* strains, 20 *F. proliferatum* strains and 38 strains of other species
185 of *Fusarium*. The expected amplicons of 179 bp and 188 bp were generated respectively only from
186 strains of *F. fujikuroi* and *F. proliferatum*. Primer specificity was confirmed by testing different
187 *Fusarium* spp. (Table 1). No amplicon was obtained from any of the 20 other species of *Fusarium*
188 spp. tested (Supplementary Figure 1), including other species of the GFSC from different
189 geographic origins.

190

191 **Primer specificity and sensitivity with real time PCR**

192 Primer specificity was confirmed through qPCR with the same conditions on *F. fujikuroi*, *F.*
193 *proliferatum*, *F. verticillioides*, *F. graminearum* and *F. oxysporum*, the five major species
194 encountered on rice seeds in Italy (Amatulli et al., 2010). The primer set Fuji1F/TEF1R
195 amplified selectively the DNA of *F. fujikuroi*, such as the other set Proli1F/TEF1R was
196 specific for *F. proliferatum*.

197 Sensitivity was evaluated by serial dilutions (1:10) of genomic DNA (from 10 ng μL^{-1} to 0.1
198 pg μL^{-1}) of *F. fujikuroi* (M-1149) and *F. proliferatum* (M-6580) in order to determine the
199 minimum amount of DNA required to generate a detectable product with each primer sets. Using
200 three replicates per dilution, amplification products were detected down to 10 pg μL^{-1} of DNA
201 (Supplementary Figure 2) for both primer pairs. An average squared regression (R^2) of 0.999 and a
202 PCR efficiency of 97.5% were obtained for the primer pair specific for *F. fujikuroi*, while the same
203 values were 0.999 and 97.9% for the primer pair for *F. proliferatum*. These results confirmed a
204 good correlation between the amount of template and Ct values.

205

206 **Detection from plant tissues and seeds**

207 In order to apply this technique to the detection of these pathogens in rice tissue of seedlings and
208 seeds, the primers Fuji1F/TEF1R and Proli1F/TEF1R were used on the total genomic DNA
209 extracted from diseased plants, and artificially inoculated or naturally contaminated rice seeds. No
210 amplification with the primers Fuji1F/TEF1R used in conventional and real time PCR was observed
211 on DNA extracted from healthy plants or plants inoculated with *F. proliferatum*, whereas a
212 fragment of the expected size was found on DNA extracted from 20-day-old rice seedlings
213 inoculated with *F. fujikuroi*.

214 On seeds artificially inoculated with *F. fujikuroi*, the primers Fuji1F/TEF1R gave a product of 179
215 bp, while the primer set Proli1F/TEF1R did not amplify in conventional PCR. On the contrary, on

216 seeds artificially inoculated with *F. proliferatum*, the primers Fuji1F/TEF1R did not amplify, while
217 the primer set Proli1F/TEF1R produced an amplicon of 188 bp. On seeds naturally contaminated,
218 the primers Fuji1F/TEF1R gave a positive result, while the primer Proli1F/TEF1R did not amplify,
219 as expected, due to the rare occurrence in seeds of this fungal species. On natural seeds, mixed
220 populations of *Fusarium* spp. can occur, but the amplification with Fuji1F/TEF1R was specific and
221 not influenced by the other species. Real time PCR could not be applied to seed, as the extraction procedure
222 adopted provided low quality DNA. The results were confirmed by plating on Komada.

223

224 **Discussion**

225 In recent decades, *F. fujikuroi* has emerged as an important rice pathogen in all the Italian rice
226 production areas mostly due to the reduction in pesticide availability, especially in Europe
227 (Regulation 1107/2009/EEC) and the United States (Food Quality Protection Act) for seed dressing
228 (Spadaro and Gullino, 2005; Tinivella et al., 2009). The early and correct identification of this
229 species is therefore very important for the rice seed industry to set up a good management strategy,
230 such as disease-free certified seeds. Among the other species of *Fusarium* that can be isolated from
231 rice, the discrimination between *F. fujikuroi* and *F. proliferatum* is particularly difficult because
232 they are morphologically identical, they both belong to the Asian clade of the GFSC and share a
233 high degree of genome similarity (Leslie and Zeller 1997). Therefore, reliable, sensitive and
234 specific molecular diagnostic markers for both species could make their identification easier and
235 could help to understand their ecological behaviour in order to develop a good management strategy
236 for disease control. PCR-based methods offer a rapid and cost-effective means of molecular
237 diagnosis. Sequencing of conserved genes can be a tool for species identification. However, to
238 reduce the time taken in sequencing, species specific primers can be designed on the differences
239 found after sequence analyses (SCAR primers). Due to its worldwide distribution and its broad
240 host range (maize, asparagus, fig, onion, palm, pine, and rice) in the last years, specific primers for
241 identifying *F. proliferatum* have been designed, based on the IGS region (Jurado et al. 2006) and on

242 the calmodulin gene (Mulé et al. 2004a,b) but species-specific primer able to identify *F. fujikuroi*
243 were not previously available. Moreover, until now no specific primers have been designed on the
244 *TEF* gene sequence. Our gene sequence analyses showed the deletion of six nucleotides in the
245 second intron of the *TEF* gene of *F. fujikuroi* is conserved. In fact, the deletion was found not only
246 in all our Italian isolates (198) but also in strains coming from other countries (Fusarium Research
247 Center collection) and in all the NCBI sequences analyzed. Moreover, in the same region where *F.*
248 *fujikuroi* deletion occurs, two nucleotides were conserved in all the *F. proliferatum* sequences
249 analysed but they differed from the other *Fusarium* spp. sequences. Although introns exhibit a very
250 poor level of sequence conservation and mutational changes might lead to false negatives, this study
251 is another important piece of evidence that the intron variation between species is greater than the
252 exon variation (Donnelly et al. 1999; Mulé et al. 2004b), especially for species so closely related as
253 *F. fujikuroi* and *F. proliferatum*. The primers proposed in the present study were tested for
254 specificity and sensitivity. Specificity was verified by the absence of cross-reaction with DNA from
255 different *Fusarium* spp. while sensitivity was established by Real-time PCR using a 10-fold
256 dilution series of DNA. Results showed that the described primers are able to discriminate the two
257 species from a large number of other *Fusarium* spp. and that the sensitivity level was 10 pg of
258 genomic DNA. Therefore this assay could be used to identify unknown strains belonging to these
259 pathogens, without the need of further analyses. The possibility to use these primers in Real-time
260 PCR, due to the small size of amplified fragment, allowed the quantification of fungal DNA in rice
261 tissues inoculated with *F. fujikuroi*. Moreover the primers were successfully used to detect *F.*
262 *fujikuroi* directly on artificially and naturally inoculated rice seeds. *F. proliferatum* was detected on
263 artificially infected seeds, but it was not detected on naturally infected seeds, due to the natural low
264 occurrence in rice seeds and diseased plants. In fact, *F. proliferatum* is accidentally present on rice
265 seeds and it is not able to cause bakane disease on rice (Amatulli et al., 2010; Desjardins et al.,
266 1997). The last application could be particularly useful for early detection of *F. fujikuroi* in rice
267 seed. Seed is a primary source of infection (Webster and Gunnell 1992), and using a molecular tool

268 which could confirm zero or very low level of infection with *F. fujikuroi* would be of value to seed
269 companies. The commercialization of heavily infected seed lots could be prevented, or appropriate
270 management strategies, such as tanning (Tinivella et al., 2009) or thermotherapy (Forsberg et al.,
271 2003), could be applied to prevent the spread of bakanae disease in rice fields.

272

273 **Acknowledgements**

274 Work was carried out with grants from the projects ITACA “Technological innovation, automation
275 and new analytical controls to improve the quality and safety of the Piedmontese food products”
276 funded by the Piedmont Region (Italy) and RISINNOVA “Integrated genetic and genomic
277 approaches for new Italian rice breeding strategies”, funded by AGER Foundation.

278

279 **References**

- 280 Amatulli, M.T., Spadaro, D., Gullino, M.L. & Garibaldi, A. (2010). Molecular identification of
281 *Fusarium* spp. associated with bakanae disease of rice in Italy and assessment of their
282 pathogenicity. *Plant Pathology* 59, 839-844
- 283 Bakan, B., Giraud-Delville, C., Pinson, L., Richard-Molard, D., Fournier, E. & Brygoo, Y. (2002).
284 Identification by PCR of *Fusarium culmorum* strains producing large and small amounts of
285 deoxynivalenol. *Applied and Environmental Microbiology* 11, 5472-5479
- 286 Carter, L.L.A., Leslie, L.F. & Webster, R.K. (2008). Population structure of *Fusarium fujikuroi*
287 from California rice and Water Grass. *Phytopathology* 9, 992-998
- 288 Desjardins, A.E., Plattner, R.D. & Nelson, P. (1997). Production of fumonisin B1 and moniliformin
289 by *Gibberella fujikuroi* from rice from various geographic areas. *Applied and Environmental*
290 *Microbiology* 63, 1838–42
- 291 Donnelly, S.M., Sullivan, D.J., Shanley, D.B. & Coleman, D.C. (1999). Phylogenetic analysis and
292 rapid identification of *Candida dubliniensis* based on analysis of ACT1 intron and exon sequences.
293 *Microbiology* 145, 1871-1882

294 Edwards, S., O' Callaghan, J. & Dobson, A.D.W. (2002). PCR based detection and quantification
295 of mycotoxigenic fungi. *Mycological Research* 106, 1005-1025

296 Forsberg, G., Kristensen, L., Eibel, P., Titone, P. & Hart, W. (2003) Sensitivity of cereal seeds to
297 short duration treatment with hot, humid air. *Journl of Plant Diseases and Protection* 110, 1-16

298 Geiser, D.M., Gasco, M.J., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N.,
299 Kuldau, G.A. & O'Donnell, K. (2004). FUSARIUM-ID v. 1.0: A DNA sequence database for
300 identifying *Fusarium*. *European Journal of Plant Pathology* 110, 473–479

301 Jurado, M., Vázquez, C., Marin, S., Sanchis, V. & González-Jaén, M.T. (2006). PCR-based strategy
302 to detect contamination with mycotoxigenic *Fusarium* species in maize. *Systematic and*
303 *Applied Microbiology* 29, 681–689

304 Komada, H. (1975). Development of a selective medium for quantitative isolation of *Fusarium*
305 *oxysporum* from natural soils. *Review of Plant Protection Research* 8, 114–24

306 Leslie, J.F. & Summerell, B.A. (2006). *The Fusarium Laboratory Manual*. (Blackwell Publishing
307 1st Ed., Ames, IA)

308 Leslie, J.F. & Zeller, K.A. (1996). Heterokaryon compatibility in fungi – More than just another
309 way to die. *Journal of Genetics* 75, 415-424

310 Moeller, E.M., Chelkowski, J. & Geiger, H.H. (1999). Species-specific PCR assays for the fungal
311 pathogens *Fusarium moniliforme* and *Fusarium subglutinans* and their application to diagnose
312 maize ear rot. *Journal of Phytopathology* 147, 497–508

313 Moukhamedov, R., Hu, X., Nazar, R. & Robb, J. (1994). Use of polymerase chain reaction-
314 amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*.
315 *Phytopathology* 84, 256-259

316 Mule', G., Susca, A., Stea, G. & Moretti, A. (2004a). A species-specific PCR assay based on the
317 calmodulin partial gene for identification of *Fusarium verticillioides*, *F. proliferatum* and *F.*
318 *subglutinans*. *European Journal of Plant Pathology* 110, 495–502

319 Mule', G., Susca, A., Stea, G. & Moretti, A. (2004b). Specific detection of the toxigenic species
320 *Fusarium proliferatum* and *F. oxysporum* from asparagus plants using primers based on calmodulin
321 gene sequences. FEMS Microbiology Letters 230, 235-240

322 Obanor, F., Erginbas-Orakci, G., Tunali, B., Nicol, J.M. & Chakraborty, S. (2010). *Fusarium*
323 *culmorum* is a single phylogenetic species based on multilocus sequence analysis. Fungal biology 4,
324 753-765

325 O'Donnell, K., Sutton, D.A., Rinaldi, M.G., Gueidan, C., Crous, P.W. & Geiser, D.M. (2009).
326 Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic
327 members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes
328 within the United States. Journal of Clinical Microbiology 47, 3851-3861

329 O'Donnell, K., Cigelnik, E. & Nirenberg, H.I. (1998). Molecular systematics and phylogeography
330 of the *Gibberella fujikuroi* species complex. Mycologia 90, 465-493

331 Ritieni, A., Fogliano, V., Randazzo, G., Scarallo, A., Logrieco, A., Moretti, A., Manndina, L. &
332 Bottalico, A. (1995). Isolation and characterization of fusaproliferin, a new toxic metabolite from
333 *Fusarium proliferatum*. Natural toxins 3, 17-20

334 Simpson, D.R., Rezanoor, H.N., Parry, D.W. & Nicholson, P. (2000). Evidence for differential host
335 preference in *Microdochium nivale* var. *majus* and *Microdochium nivale* var. *nivale*. Plant
336 Pathology 49, 261-268

337 Spadaro, D. & Gullino, M.L. (2005) Improving the efficacy of biocontrol agents against soilborne
338 pathogens. Crop Protection 24, 601-613

339 Sreenivasa, M.Y., González Jaen, M.T., Sharmila Dass, R., Charith Ray, A.P. & Janardhana, G.R.
340 (2008). A PCR-Based Assay for the Detection and Differentiation of Potential Fumonisin-
341 Producing *Fusarium verticillioides* Isolated from Indian Maize Kernels. Food Biotechnology, 22,
342 160-170

343 Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). *MEGA4*: Molecular evolutionary genetics
344 analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596-1599

345 Tinivella, F., Hirata, L.M., Celan, M.A., Wright, S.A.I., Amein, T., Schmitt, A., Koch, E., Van Der
346 Wolf, J.M., Groot, S.P.C., Stephan, D., Garibaldi, A. & Gullino, M.L. (2009). Control of seed-
347 borne pathogens on legumes by microbial and other alternative seed treatments. *European Journal*
348 *of Plant Pathology* 123, 139-151

349 Voigt, K., Schleier, S. & Bruckner, B. (1995). Genetic variability in *Gibberella fujikuroi* and some
350 related species of the genus *Fusarium* based on random amplification of polymorphic DNA
351 (RAPD). *Current Genetics* 27, 528–35

352 Webster, R.K. & Gunnell, P.S. (1992). *Compendium of rice diseases*. (The American
353 *Phytopathological Society Press, St. Paul, Minn*)

354 Zhou, M.G., Ye, Z.Y. & Liu, J.F. (1994). Progress of fungicide resistance research. *Journal of the*
355 *Nanjing Agricultural University* 17, 33-41

356

357 **Figure caption**

358

359 **Figure 1.** Multiple sequence alignment of the elongation factor 1 α gene sequences from different
360 *Fusarium* spp. of the GFSC. The regions of variability in *F. fujikuroi* and *F. proliferatum* used for
361 primers design are highlighted.

362

363 **Supplementary materials**

364 **Supplementary Figure 1.** (a) PCR amplification with the specific primer set Fuji1F/TEF1R. (b)
365 PCR amplification with the specific primer set Proli1F/TEF1R. Numbers correspond to the
366 different strains of *Fusarium* spp. listed in Table 1: 1: M1149 (ATCC38939); 2: M1150
367 (ATCC38940); 3: M1250; 4: M3096; 5: M5331; 6: M6461; 7: M3127; 8: M6580; 9: S09-10; 10:
368 S1S; 11: 2-27; 12: 3150FS; 13: M5598; 14: M7491; 15: M8386; 16: M8785; 17: ITEM 2809; 18:
369 ITEM 7611; 19: ITEM 7638; 20: ITEM 7614; 21: S09-8; 22: 2-130; 23: FR2b; 24: G2; 25: Lis41;
370 26: CCP1; 27: GL3S; 28: GL4S; 29: 1706FC; 30: G5S; 31: E87; 32: 11-17.

371

372 **Supplementary Figure 2.** Real-time quantification (a) Kinetic of fluorescence signal at different
373 concentrations of target genomic DNA (10 ng–1 pg) and (b) Standard curve obtained by plotting the
374 log of DNA concentrations (10 ng–1 pg) values and unknown DNA concentration of rice tissues
375 inoculated with *F. fujikuroi* (strain M1149).

376

Table 1. List of strains used for sequence alignment of *TEF* gene and species-specific primers amplification.

| Isolate | Species | Original host | Geographic origin | Accession number |
|--------------------|-----------------------------|---------------------------------|--------------------|------------------|
| M1149* (ATCC38939) | <i>F. fujikuroi</i> | <i>Oryza sativa</i> | Taiwan | HM243234 |
| M1150* (ATCC38940) | <i>F. fujikuroi</i> | <i>O. sativa</i> | Taiwan | JN092354 |
| M1250* | <i>F. fujikuroi</i> | <i>O. sativa</i> | Taiwan | JN092355 |
| M3096* | <i>F. fujikuroi</i> | <i>O. sativa</i> | USA, GA, Athens | JN092356 |
| M5331* | <i>F. verticillioides</i> | <i>O. sativa</i> (stem) | China, Beijing | AY337449 |
| M6461* | <i>F. verticillioides</i> | <i>Zea mays</i> | USA, KS, Rossville | JN092344 |
| M3127* | <i>F. sacchari</i> | Unknown | Unknown | JN092337 |
| M6580* | <i>F. proliferatum</i> | <i>O. sativa</i> | Thailand | JN092336 |
| S09-10 | <i>F. proliferatum</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | JN092352 |
| S1S | <i>F. proliferatum</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | JN092349 |
| 2-27 | <i>F. proliferatum</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | JN092351 |
| 3150FS | <i>F. subglutinans</i> | <i>Triticum</i> sp. | Poland | HM347131 |
| M5598* | <i>F. thapsinum</i> | <i>Sorghum vulgare</i> (stalks) | USA, KS | HM243235 |
| M7491* | <i>F. nygamai</i> | Unknown | Unknown | HM243236 |
| M8386* | <i>F. circinatum</i> | <i>Pinus radiata</i> | USA, CA | JN092335 |
| M8785* | <i>F. konzum</i> | <i>Sorghastrum nutans</i> | USA, KS | JN092341 |
| ITEM 2809** | <i>F. ramigerum</i> | Unknown | Unknown | JN092340 |
| ITEM 7611** | <i>F. guttiforme</i> | Unknown | Unknown | JN092343 |
| ITEM 7638** | <i>F. globosum</i> | Unknown | Unknown | JN092345 |
| ITEM 7614** | <i>F. phyllophilum</i> | Unknown | Unknown | JN092342 |
| S09-8 | <i>F. andiyazi</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | HM243238 |
| 2-130 | <i>F. napiforme</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | GQ848547 |
| FR2b | <i>F. oxysporum</i> complex | <i>Eruca sativa</i> | Lombardy, Italy | JN092346 |
| G2 | <i>F. oxysporum</i> complex | <i>Gerbera jamesonii</i> | Liguria, Italy | JN092347 |
| Lis41 | <i>F. oxysporum</i> complex | <i>Eustoma grandiflorum</i> | Liguria, Italy | JN092339 |
| CCP1 | <i>F. oxysporum</i> complex | <i>O. sativa</i> | Piedmont, Italy | JN092350 |
| GL3S | <i>F. sporotrichioides</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | GQ848546 |
| GL4S | <i>F. avenaceum</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | JN092348 |
| 1706FC | <i>F. culmorum</i> | <i>Z. mays</i> | Poland | JN092353 |
| G5S | <i>F. graminearum</i> | <i>O. sativa</i> | Piedmont, Italy | GQ848544 |
| E87 | <i>F. semitectum</i> | <i>O. sativa</i> | Italy | JN092338 |
| 11-17 | <i>F. equiseti</i> | <i>O. sativa</i> (seed) | Piedmont, Italy | GQ848541 |

* strains obtained from the Fusarium Research Center (FRC, The Pennsylvania State University, USA); ** strains obtained from the Culture Collection of

Institute of Sciences of Food Production (Bari, Italy).

Table 2. Primer sets designed and used in this study.

| Species | Primer name | Gene | Primer sequence | Amplicon size |
|------------------------|-------------|------------|------------------------|---------------|
| <i>F. fujikuroi</i> | Fuji1F | <i>TEF</i> | ACGTGTCAAACATAACATTCGA | 179 bp |
| | TEF1R | | GCGACAACATAACCAATGACG | |
| <i>F. proliferatum</i> | Proli1F | <i>TEF</i> | GTCACGTGTCAAGCAGCGA | 188 bp |
| | TEF1R | | GCGACAACATAACCAATGACG | |