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

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

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Abstract

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

Introduction

Fusarium fujikuroi Niremberg [teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura] is an important rice pathogen and a member of the polyphyletic taxon, *Gibberella fujikuroi* species complex (GFSC). This fungus is seed-borne and the causal agent of bakanae diseases of rice in a number of rice growing areas (Carter et al. 2008). Chemical seed dressing has been the most common way to control rice bakanae for a long time. In some countries, such as Japan and China, seeds are soaked in water suspensions containing carbendazim, prochloraz, trifumezol, thiram or other fungicides (Zhou et al., 1994). Due to the reduction in pesticide availability, in recent years the disease incidence has increased also in Italy (Amatulli et al. 2010), becoming a serious problem especially for the seed companies which often have to sell certified rice seed, free of *F. fujikuroi*. The most evident symptom of the disease is yellowing and abnormal elongation of infected seedlings, which led to the Japanese name *bakanae*, meaning "foolish seedling." In older plants, the roots, crowns, stems, leaf sheaths and panicles can be infected (Desjardins et al. 1997). Although it is known that *F. fujikuroi* is the cause of bakanae disease of rice, other *Fusarium* spp. also occur on rice including two other species of the GFSC, *Fusarium verticillioides* (Sacc.) Nirenberg (tel. *G. moniliforme* Wineland) and *Fusarium proliferatum* (Matsushima) Nirenberg (*G. intermedia* (Kuhlman) Samuels, Nirenberg & Seifert) that have been isolated on diseased plants in different parts of the world (Desjardins et al. 1997; Voigt et al., 1995) and considered for many years to be involved in the development of the disease. In particular, *F. proliferatum* is morphologically indistinguishable from *F. fujikuroi* and can only be distinguished by making tests of sexual cross-fertility (Leslie and Zeller 1997) or through DNA sequencing. Phylogenetically, *F. proliferatum* and *F. fujikuroi* belong to the Asian clade of the GFSC (O'Donnell et al., 1998) and are very closely related, although their karyotypes are different (Leslie and Summerell 2006). Both species can produce a broad range of mycotoxins, such as fumonisin B_1, moniliformin, beauvericin, fusaric acid, fusarin (Leslie and Summerell 2006). Moreover *F. proliferatum* is also able to produce

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

Materials and Methods

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 manifacturer'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

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

***TEF* amplification**

Amplifications of the elongation factor 1α gene were done by using specific primers (O'Donnell et al. 1998): ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'). The reaction mixture of 20 µl contained 1 µl of buffer 10X, 1 U of Taq DNA polymerase (Qiagen, USA), 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 mM of each primer, and 1.5 mM of $MgCl_2$. Amplification was carried out in a T-Gradient thermal cycler (Biometra, Germany) using the following steps: 95°C for 3 min, 35 cycles with denaturation at 95°C for 1 min, 60°C for 1 min, 72°C for 3 min and a final extension at 72ºC for 5 min. Five µl of the PCR products were separated by electrophoresis in 1.5% agarose gel (Agarose D-1 LOW EEO Eppendorf, Italy) containing 0.001% of SYBR Safe DNA gel stain (Invitrogen, USA) for 30 min at 3.3 V in 1 X TAE buffer.

Sequence analyses and primer design

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

Primer specificity and sensitivity

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

Real-time PCR

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

Results

Sequence analyses and primer design

In order to identify regions of variability able to discriminate *F. fujikuroi* and *F. proliferatum* among the other species of the GFSC and the other *Fusarium* spp., a portion of *TEF* gene was sequenced for the 32 strains detailed in Table 1. Multiple sequence alignment showed a deletion of six nucleotides in all the strains of *F. fujikuroi* and a difference in two nucleotides in the same region of all the *F. proliferatum* strains (Figure 1) compared to the other species. These observations were confirmed by comparison against existing sequences of *Fusarium* spp. *TEF* gene in NCBI database (www.ncbi.nlm.nih.gov) and Fustablearium ID (http://isolate.fusariumdb.org/index.php). Through Blastx analyses we located the deletion at the position 688, in the second intron of *TEF* gene, just before the beginning of the third exon.

Specific forward primers Fuji1F and Proli1F were designed using this nucleotide variability. A common reverse primer was designed downstream in order to generate two short PCR fragments (smaller then 200 bp) suitable for real time PCR. Blast analyses on GeneBank showed 100% homology of forward primers with sequences of *F. fujikuroi* and *F. proliferatum* for which the primers were designed.

Primer specificity with conventional PCR

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

Primer specificity and sensitivity with real time PCR

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

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

Detection from plant tissues and seeds

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

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

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

Discussion

In recent decades, *F. fujikuroi* has emerged as an important rice pathogen in all the Italian rice production areas mostly due to the reduction in pesticide availability, especially in Europe (Regulation 1107/2009/EEC) and the United States (Food Quality Protection Act) for seed dressing (Spadaro and Gullino, 2005; Tinivella et al., 2009).The early and correct identification of this species is therefore very important for the rice seed industry to set up a good management strategy, such as disease-free certified seeds. Among the other species of *Fusarium* that can be isolated from rice, the discrimination between *F. fujukuroi* and *F. proliferatum* is particularly difficult because they are morphologically identical, they both belong to the Asian clade of the GFSC and share a high degree of genome similarity (Leslie and Zeller 1997). Therefore, reliable, sensitive and specific molecular diagnostic markers for both species could make their identification easier and could help to understand their ecological behaviour in order to develop a good management strategy for disease control. PCR-based methods offer a rapid and cost-effective means of molecular diagnosis. Sequencing of conserved genes can be a tool for species identification. However, to reduce the time taken in sequencing, species specific primers can be designed on the differences found after sequence analyses (SCAR primers). Due to its worldwide distribution and its broad host range (maize, asparagus, fig, onion, palm, pine, and rice) in the last years, specific primers for 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
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356

Figure caption

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

Supplementary materials

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

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

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)	*F. fujikuroi*	*Oryza sativa*	Taiwan	HM243234
M1150* (ATCC38940)	*F. fujikuroi*	*O. sativa*	Taiwan	JN092354
M1250*	*F. fujikuroi*	*O. sativa*	Taiwan	JN092355
M3096*	*F. fujikuroi*	*O. sativa*	USA, GA, Athens	JN092356
M5331*	*F. verticillioides*	*O. sativa* (stem)	China, Beijing	AY337449
M6461*	*F. verticillioides*	*Zea mays*	USA, KS, Rossville	JN092344
M3127*	*F. sacchari*	Unknown	Unknown	JN092337
M6580*	*F. proliferatum*	*O. sativa*	Thailand	JN092336
S09-10	*F. proliferatum*	*O. sativa* (seed)	Piedmont, Italy	JN092352
S1S	*F. proliferatum*	*O. sativa* (seed)	Piedmont, Italy	JN092349
2-27	*F. proliferatum*	*O. sativa* (seed)	Piedmont, Italy	JN092351
3150FS	*F. subglutinans*	*Triticum* sp.	Poland	HM347131
M5598*	*F. thapsinum*	*Sorghum vulgare* (stalks)	USA, KS	HM243235
M7491*	*F. nygamai*	Unknown	Unknown	HM243236
M8386*	*F. circinatum*	*Pinus radiata*	USA, CA	JN092335
M8785*	*F. konzum*	*Sorghastrum nutans*	USA, KS	JN092341
ITEM 2809**	*F. ramigerum*	Unknown	Unknown	JN092340
ITEM 7611**	*F. guttiforme*	Unknown	Unknown	JN092343
ITEM 7638**	*F. globosum*	Unknown	Unknown	JN092345
ITEM 7614**	*F. phyllophilum*	Unknown	Unknown	JN092342
S09-8	*F.andiyazi*	*O. sativa* (seed)	Piedmont, Italy	HM243238
2-130	*F. napiforme*	*O. sativa* (seed)	Piedmont, Italy	GQ848547
FR2b	*F. oxysporum* complex	*Eruca sativa*	Lombardy, Italy	JN092346
G2	*F. oxysporum* complex	*Gerbera jamesonii*	Liguria, Italy	JN092347
Lis41	*F. oxysporum* complex	*Eustoma grandiflorum*	Liguria, Italy	JN092339
CCP1	*F. oxysporum* complex	*O. sativa*	Piedmont, Italy	JN092350
GL3S	*F. sporotrichioides*	*O. sativa* (seed)	Piedmont, Italy	GQ848546
GL4S	*F. avenaceum*	*O. sativa* (seed)	Piedmont, Italy	JN092348
1706FC	*F. culmorum*	*Z. mays*	Poland	JN092353
G5S	*F. graminearum*	*O. sativa*	Piedmont, Italy	GQ848544
E87	*F. semitectum*	*O. sativa*	Italy	JN092338
11-17	*F. equiseti*	*O. sativa* (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
F. fujikuroi	Fuji1F	*TEF*	ACGTGTCAAACTAAACATTCGA	179 bp
	TEF1R		GCGACAACATACCAATGACG	
F. proliferatum	Proli1F	*TEF*	GTCACGTGTCAAGCAGCGA	188 bp
	TEF1R		GCGACAACATACCAATGACG	