Molecular identification of Fusarium spp. associated with bakanae disease of rice in Italy and assessment of their pathogenicity

Molecular identification of *Fusarium* spp. associated with bakanae disease of rice in Italy and assessment of their pathogenicity

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Abstract

In the last 10 years the Fusarium disease of rice has spread through Italian rice fields becoming a serious problem for seed production and for seed companies. Between 2006 and 2008, 146 isolates of *Fusarium* spp. were obtained from diseased rice plants and seeds coming from the major rice-growing regions of Italy. These isolates were identified, based on the translation elongation factor sequence and pathogenicity tests to assess their aggressiveness against the susceptible rice cultivar Galileo. Use of the EF-1α sequence gave reliable identification and showed that *Fusarium fujikuroi*, the causal agent of bakanae disease, is the most abundant *Fusarium* spp. isolated. These data were confirmed by inoculation of the isolates in the greenhouse, showing that only *F. fujikuroi* strains are able to cause bakanae disease. We identified pathogenic strains with different levels of virulence. Phylogenetic analysis based on EF-1α sequences generated a tree which separated the various *Fusarium* species into different

clusters with high bootstrap values.

Key words: *Fusarium fujikuroi*, *Oryza sativa*, translation elongation factor, aggressiveness.

Introduction

Northern Italy has the largest production area (232,500 ha in 2007) of rice in Europe (FAO data). Several fungal diseases affect this crop; among these, *Fusarium* species are the agents of many different plant and seed diseases, such as bakanae. In the last decades, bakanae has emerged as a problem in Italian rice production (Desjardins *et al.*, 1997). The most evident symptom of this disease is yellowing and abnormal elongation of infected seedlings, due to gibberellic acid production, which led to the Japanese name *bakanae*, meaning "foolish seedling." In older plants, the roots, crowns, stems, leaf sheaths, and panicles can be infected. The disease is seed-borne and primarily seed transmitted (Desjardins *et al*., 1997).

Although bakanae disease was first described over 100 years ago in Japan, it is still not clear which *Fusarium* species are associated with the different symptoms. Early work in Japan identified the pathogen as "*Fusarium moniliforme*" in a broad sense (Ou, 1985); however, this taxon comprises a number of distinct species, now collectively termed the *Gibberella fujikuroi* species complex. The presence of the *Gibberella* sexual stage can distinguish mating populations, or biological species, within this group (Leslie, 1995). Three mating populations of the *G. fujikuroi* complex have been associated with bakanae diseased rice. Mating population C (MP-C) (anamorph *Fusarium fujikuroi*, Nirenberg) was first identified in 1977 among strains isolates from Taiwan rice (Hsieh, 1977). MP-A (anamorph *Fusarium verticillioides* (Sacc.) Nirenberg) and MP-D (anamorph *Fusarium proliferatum* (Matsushima) Nirenberg) have been isolated from

65 rice in Asia, and MP-D has also been isolated from rice from Africa, Australia, and the United
66 States (Desjardins *et al*., 1997; Voigt *et al*., 1995). The names for the sexual stage of these
67 pathogens are *G. fujikuroi*, *G. intermedia* and *G. moniliforme*. *Fusarium* species within this
68 complex are also able to produce mycotoxins that can affect human and animal health (Leslie *et*
69 *al*., 1992). In particular *F. fujikuroi* could produce moniliformin, beauvericin, and fumonisins; *F.*
70 *verticillioides* can produce fumonisins, and *F. proliferatum* are able to synthesize fumonisins,
71 beauvericin, and fusaproliferin. Recently molecular techniques to identify and group the species
72 are becoming important. Recent work has revealed great diversity among *Fusarium* species,
73 underestimated by earlier morphological criteria (O'Donnell *et al*., 1998a, b; Aoki & O'Donnell,
74 1999; O'Donnell *et al*., 2000; Ward *et a*l., 2002). Different techniques such as AFLP (amplified
75 fragment length polymorphism), RAPD (random amplification of polymorphic DNA), and RFLP
76 (restriction fragment length polymorphism) analysis have been used to characterize and identify
77 the different mating populations in the *G. fujikuroi* complex (Voigt *et al*., 1995; Patiño *et al*.,
78 2006; Moretti *et al*., 2004) as well as multiple gene phylogeny (O'Donnell *et a*l., 2000). Geiser
79 (2003) reported that intron-rich parts of protein-coding genes are excellent markers for
80 species-level phylogenetics in fungi. Gene introns seem better than the internal transcribed spacer
81 (ITS) regions of the nuclear ribosomal RNA gene repeat because they tend to evolve at a higher
82 rate (O'Donnell *et al*., 2000; O'Donnell *et al*., 1998a). Non-orthologous copies of the ITS2 may
83 also lead to incorrect phylogenetic inferences (O'Donnell *et al*., 1997). In these circumstances, it
84 is better to identify regions with high phylogenetic utility. The translation elongation factor 1-α
85 gene (EF-1α gene), which encodes an essential part of the protein translation machinery, is a
86 good single-locus identification tool in *Fusarium* because it shows high sequence polymorphism
87 among closely related species, even compared to the intron-rich portions of protein-coding genes
88 such as calmodulin, beta-tubulin and histone H3, and non-orthologous copies of the gene have

not been detected in the genus.

Since the identity of the bakanae causal agent seems to be still unclear, the objectives of this study were: i) to assess the variability of *Fusarium* from bakanae-infected rice in Italy, ii) to identify species by amplification and sequencing of the EF-1α gene, and iii) to evaluate the pathogenicity of the Italian isolates on the susceptible rice cultivar Galileo.

Materials and methods

Collection and isolation of Fusarium *strains*

Bakanae-infected rice samples were collected during the whole growing season for 3 years (2006-2008) in the major rice production areas of north-western Italy (Novara, Vercelli and Pavia provinces). Fungi were isolated from the stem just above the crown of diseased plants of different varieties. Tissues were washed under running tap water, sterilized with 1% NaOCl for 30 s, rinsed twice in sterile distillate water (SDW), placed in Petri plates containing Komada medium (Komada, 1975), and kept at room temperature. Fungi were isolated from the seeds after washing whole seeds under running tap water. The seed surface was treated with NaOCl for 45 s, rinsed twice in SDW, and placed on PDA. After 4 days, mycelia grown from tissues and seeds were transferred to fresh Petri dishes containing potato dextrose agar (PDA, Difco, USA) plus 25 mg/l streptomycin (AppliChem, Germany) in order to induce microconidia. In addition four representative strains (ITEM504 *F. verticillioides*, ITEM1720 *F. proliferatum*, ITEM231 *F. verticillioides*, and ITEM1746 *F. verticillioides*) from the of Institute of Food Production Sciences Culture Collection (Bari, Italy), and one (M-1149=ATCC 38939, *F. fujikuroi*) from the Fusarium Research Center (FRC, Pennsylvania State University, USA) were used. The strains were stored both at 4°C in Spezieller Nahrstoffarmer agar (SNA: 1.0 g/L KH_2PO_4, 1.0 g/L KNO_3,

113 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L glucose, 0.2 g/L sucrose) and at -80°C in 50% glycerol.

114

115 *DNA extraction and amplification*

116 About 100 mg of mycelium was scraped from a Petri dish containing PDA, and DNA extracted
117 from it using the NucleoMag Kit (Macherey Nagel Gmbh and Co., Duren, USA) according to the
118 manifacturer's instructions, adding 10 µL of proteinase K (10 mg/l) and 10 µL of RNAse A (12
119 mg/l) to MC1 buffer in each tube. DNA was extracted using Kingfisher (Thermo, Walthman,
120 USA) according to the manufacturer's protocols. The PCR amplified the EF-1α gene using
121 specific primers (O'Donnell *et al.*, 1998b): ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3')
122 and ef2 (5'- GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'). The reaction mixture of 20 µL
123 contained 1 µl of buffer 10X, 1 U of Taq DNA polymerase (QIAGEN, Chatsworth, USA), 0.5
124 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 mM of each primer, and 1.5 mM of $MgCl_2$.
125 Amplification was carried out in a T-Gradient thermal cycler (Biometra, Göttingen, Germany)
126 using the following steps: 95°C for 3 min, 35 cycles with denaturation at 95°C for 1 min, 60°C
127 for 1 min, 72°C for 3 min and a final extension at 72ºC for 5 min. Five µl of the PCR products
128 were separated by electrophoresis in 1.5 % agarose gel (Agarose D-1 LOW EEO Eppendorf,
129 Milan, Italy) containing 1 µl/100 ml of SYBR Safe DNA gel stain (Invitrogen, Eugene, USA) for
130 30 min at 3.3 V in 1 X TAE running buffer.

131

132 *Sequencing and phylogenetic analysis*

133 The PCR products were purified using QIAquick PCR purification kit (QIAGEN, Chatsworth,
134 USA) according to the manufacturer's instructions and were sequenced in both directions by
135 BMR Genomics Center (Padova, Italy). Sequencing data were aligned with the programme DNA
136 baser V2.71.0 (Heracle Software Lilienthal, Germany) and then blasted using both NCBI

137 (HYPERLINK "http://www.ncbi.nih.gov/" National Center for Biotechnology Information,
138 Rockville Pike, Bethesda, MS, USA) and FUSARIUM-ID (Geiser *et al*., 2004) to confirm the
139 results. Sequence alignments and analysis were performed using MEGA version 4 (Tamura *et al*.,
140 2007). The dendrogram was obtained using bootstrap analyses (1000 replicates, removing gaps)
141 with UPGMA. Sequences from strains M1148, M1150, M3096, M1250 (*F.fujikuroi*), M5598 (*F.*
142 *thapsinum*), M7491 (*F. nygamai*), M5331 (*F. verticillioides*) from the Fusarium Research Center
143 (FRC, The Pennsylvania State University, USA) were included in the analysis.
144
145 *Pathogenicity tests*
146 The susceptible cultivar Galileo was used to assess the pathogenicity of 121 strains of *Fusarium*
147 spp. Fungal cultures were sub-cultivated in PDA at room temperature. After 15 days plates were
148 flooded with sterile water and scraped with a sterile spatula. The resulting suspensions were
149 filtered through 2 layers of sterile cotton lint and brought to a final concentration of 10^6 spores/ml
150 in SDW. Before inoculation, rice seeds were treated with hot water for 15 minutes at 60°C, to
151 remove any surface contaminants. A hundred and fifty rice seeds were soaked in the spore
152 suspension and shaken for 10 minutes while control seeds were soaked in SDW for 10 minutes
153 then were air dried. The inoculated seeds were sown in plastic pots (50 seeds per pot) containing
154 sterile soil (60% peat, 40% sand). The plants were watered three times a day. The greenhouse
155 temperature was maintained at 24°-26°C during the day and 16-18°C during the night.
156 Fertilization with macro and micro nutrients was done twice during the 40-day period. Disease
157 symptoms were scored weekly starting from 15 days after inoculation using a 0-5 scale where 0
158 corresponded to asymptomatic plants, 1 to stunted plants with narrow and yellow leaves, 2 to thin
159 plants with narrow, pale yellow leaves, 3 to plants with narrow, pale yellow leaves, thin and long
160 internodes, short root system, 4 to plants with heavy bakanae symptoms and a starting crown

necrosis, and 5 corresponded to plant death due to bakanae. The score obtained was standardized onto a 0–100 scale.

Statistical analysis

For each strain, the final disease index was calculated as the means of the 3 pots in the 2 repetitions. Data analysis was performed by using the SPSS software (SPSS Inc., version 17.0, Chicago, USA). Statistical significance was judged at the level of /p/-value < 0.05. When the analysis of variance was statistically significant, Tukey's test was used to compare the means of pathogenicity level.

Results and Discussion

Collection of *Fusarium* spp. from rice and molecular identification of the species

Fusarium species were isolated from 98% of plant samples, including tissues and seeds of different rice varieties, collected in different fields. One hundred and forty-six isolates were confirmed as *Fusarium* spp. by their growth and morphological characteristics on PDA. Sequencing was used for further identification. Amplification of the translation elongation factor 1α gave a 660 bp product and its sequence was used for classification based on a BLAST analysis. All the 79 strains obtained from diseased tissues belonged to the *G. fujikuroi* complex, in particular 73 were *F. fujikuroi*. Of the 67 strains isolated from rice seeds, 26 proved to be *F. fujikuroi*, 12 *F. proliferatum*, and 7 *F. verticillioides*. Other strains belonging to different species (*F. equiseti*, *F. oxysporum*, *F. graminearum*, *F. brevicatenulatum*, *F. napiforme*, *F. avenaceum* and *F. sporotrichioides*) were identified (Table 1). Molecular identification showed that 85% of our isolates belonged to the *G. fujikuroi* species complex, confirming an earlier report from Italy

(Moretti *et al*.,2007). In particular, 72 out of 79 strains isolated from rice tissue showing bakanae symptoms were *F. fujikuroi* confirming the species as the most abundant in rice fields (Anderson, 2005; Zainudin *et al*., 2008). The other species were detected at very low levels and mainly from seeds.

Phylogenetic analysis

The EF-1α sequences obtained were used to create a dendrogram (Figure 1). The three clusters of *F. fujikuroi*, *F. proliferatum* and *F. verticillioides*, although sharing a high level of homology, grouped together with *F. oxysporum* strains and the other species of the *G. fujiku*roi complex in a larger cluster. In another cluster were grouped the other species: *F. equiseti, F. graminearum, F. avenaceum* and *F. sporotrichioides*. The isolates of *F. brevicatenulatum* and *F. napiforme* diverged from both groups. Phylogenetic trees obtained from analyses of EF-1α sequences clearly grouped the different species of *Fusarium* with high bootstrap values confirming that EF-1α gene is a good marker for species identification inside *Fusarium* spp..

Pathogenicity

One hundred and twenty one isolates of *Fusarium* spp., obtained from naturally infected rice plants, were tested for pathogenicity. The typical bakanae symptoms appeared 14 days after seed sowing only from seedlings inoculated with *F. fujikuroi* strains. The most severely infected plants died within 34 days of inoculation. Bakanae symptoms were not seen on healthy control plants. The *F. fujikuroi* strains were ranked based on their pathogenicity. Sixty one were pathogenic (rated between 52 and 97), 5 were slightly pathogenic (between 24 and 43), and 9 were not pathogenic (between 6 and 0) (Table 2). No bakanae symptoms were induced by other species of the *G. fujikuroi* complex or other *Fusarium* species. Reduction in germination were observed in

209 the plants inoculated with *F. graminearum* strains (15%). Pathogenicity tests showed that only *F.*
210 *fujikuroi* strains can cause bakanae symptoms. All the 61 pathogenic isolates showed abnormal
211 elongation of the seedling, thinning and yellowing, and most plants started to die from 25 dpi.
212 Some isolates did not show the classical elongation symptom, but only tanning and yellowing,
213 confirming the observations of Ou (1985) and Zainudin *et al*. (2008). Different disease indices
214 were observed also among isolates obtained from the same area or the same cultivar. Further
215 studies need to clarify if the variation in aggressiveness among the isolates is due to physiological
216 features of the strain or to environmental factors, such as temperature and humidity during the
217 experiment itself (Pasco *et al*., 2005). All the pathogenic and not pathogenic *F. fujikuroi* strains
218 and all the strains of *F. verticillioides* and *F. proliferatum* were reisolated from diseased
219 seedlings. This shows that other species besides *F. fujikuroi* can colonise rice tissues but they are
220 not involved in bakanae disease. *F. graminearum* was never re-isolated from inoculated rice
221 plants, though in literature it is reported as a rice pathogen (Lee *et al*., 2009). The other species,
222 all isolated from seeds, were not re-isolated, and they could probably be considered as epiphytic
223 or saprophytic species. *F. equiseti*, for instance, is known as a saprophytic coloniser that can
224 regularly be recovered as secondary invader of diseased tissues (Summerel *et al*., 2003).
225
226 **Conclusions**
227
228 The introduction of *F. fujikuroi* in Italy seems to have followed the use of infected material
229 originating from endemic areas. Infected rice seed is the primary source of inoculum, so the best
230 management strategy is to use healthy seed (Dodan *et al*., 1994). Tanning and thermotherapy (in
231 water at 60-61°C for 15 minutes or aerated steam at 74 ° C for 2 minutes) of seed can reduce the
232 disease by more than 90% (Titone *et al*., 2003). Others have studied the use of bacteria and fungi

as agents of biological control (Adhikari *et al*., 2001). Good management is also important to avoid contamination with mycotoxins. In the last decade, the organization of *Fusarium* spp. into well defined lineages and their mapping to specific geographic locations have led to the employment of genes involved in mycotoxin biosynthesis or other metabolic processes to study the pathogen populations prevalent in those regions (Brown *et al.*, 2007; Teichert *et al*., 2006). For this reason, knowledge of the distribution and aggressiveness pattern of *Fusarium* spp. in northern Italian rice fields is very useful for understanding and developing strategies to control the occurrence of bakanae disease.

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326

Table 1. *Fusarium* spp. isolates from diseased rice tissues and seeds identified by sequences in the EF-1α gene.

***Fusarium* species**	**N° of isolates identified for each species**				**Total**
	From seeds		From rice tissues		
	2006-2007	2008	2006-2007	2008	
*F. fujikuroi**	11	15	19	53	98
*F. proliferatum**	10	2	6	0	18
*F. verticillioides**	6	1	1	0	8
*F. equiseti**	6	2	0	0	8
F. graminearum	3	1	0	0	4
F. oxysporum	4	0	0	0	4
F. avenaceum	0	1	0	0	1
F. brevicatenulatum	1	0	0	0	1
F. napiforme	1	0	0	0	1
F. sporotrichioides	0	1	0	0	1
Fusarium spp.	2	0	0	0	2
Total	67		79		**146**

* The number of samples isolated from seed and tissue cannot be considered identical, based on the χ^2 test (p-value < 0.01).

336 **Table 2.** List of *F. fujikuroi* isolates tested for pathogenicity on rice susceptible cv. Galileo, their
337 degree of pathogenicity and Disease Severity Index (DSI) on inoculated rice seedling at 34 dpi.

Isolate Code	1st R	2nd R	M*	Isolate Code	1st R	2nd R	M*	Isolate Code	1st R	2nd R	M*
pathogenic				CSA 44	98	81	90 h	SG12	91	85	88 gh
11-3	96	88	92 h	D4	98	79	89 h	VAE2	78	95	87 fgh
11-80	79	87	83 gh	D8	85	56	71 e-h	VAE5	91	84	88 fgh
11-93	90	86	88 h	Da3	55	63	59 d-h	VAE7	65	62	64 d-h
14-22	96	98	97 h	Da5	71	67	69 e-h	VAL1	74	78	76 e-h
15-66	70	68	69 d-h	G1S	87	83	85 gh	Ve13	79	77	78 e-h
19-77	79	70	75 e-h	G3S	65	73	69 e-h	Ve9	81	83	82 gh
2-179	78	98	88 h	G6S	47	64	56 d-h	VN4	95	81	88 fgh
2-42	77	95	86 gh	Gl7S	81	79	80 gh	VPG2	90	77	84 gh
2-76	89	99	94 h	I 1,1	47	60	54 d-h	**slightly pathogenic**			
2-82	62	66	64 d-h	I 1,3	93	90	92 h	2-10	27	20	24 abc
A 2-44	91	77	84 gh	I 1,5	89	74	82 gh	2-147	24	35	30 a-d
A10	69	81	75 fgh	I 2,1	54	50	52 d-h	11-85	27	12	20 abc
A4-27	45	67	56 d-h	I 2,4	49	59	54 d-h	I 1,6	39	46	43 b-e
A5	97	91	94 h	I 2,6	75	83	79 fgh	S7	39	47	43 b-e
A6	69	79	74 e-h	I 3,1	64	62	63 d-h	**not pathogenic**			
A7	60	64	62 d-h	L5	98	80	89 h	11-23	6	5	6 ab
AC6	71	59	65 e-h	LS3	45	64	55 d-h	11-50	1	4	3 a
AG8	71	79	75 e-h	M1149a	73	66	70 e-h	19-53	1	8	5 ab
C1S	84	84	84 fgh	S10	85	71	78 fgh	AG2	8	0	4 a
C3S	73	82	78 fgh	S2	68	55	62 d-h	C2S	2	2	2a
C4S	73	76	75 e-h	S2S	96	92	94 h	LS6	5	1	3 a
C5S	68	65	67 e-h	S3	77	89	83 gh	SG4	1	0	1 a
C6S	98	79	89 fgh	S4	69	65	67 e-h	VAG6	1	0	1 a
Ca1	79	81	80 fgh	S5S	85	91	88 h	VPG6	0	2	1 a
Ca2	67	79	73 e-h	S6	63	75	69 e-h				
CFG	67	54	61 d-h	S6S	94	84	89 h	**Control**	0	0	0 a

338

339 [a] strains obtained from the Fusarium Research Center (FRC, The Pennsylvania State University, USA).

340 [b]Disease severity index . Statistical analysis were performed using Tukey test. Values with different letters are significantly different at

341 $p \leq 0.05$.

Figure caption

Figure 1. UPGMA analysis inferred from sequences of the elongation factor gene. Bootstrap values are indicated as percentage above the nodes for maximum parsimony analysis (1,000 replications). Because of the high number of samples, 5 Italian isolates were used as representative of *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* (GenBank accession number is given next to the strain code).