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BIOMOLECULAR CHARACTERIZATION OF *FUSARIUM POAE* STRAINS ISOLATED FROM DURUM WHEAT IN CENTRAL ITALY

ABSTRACT

Fusarium Head Blight (FHB) is a worldwide disease affecting wheat, barley and other grains, reducing kernel weight and grain yield; infected seeds may contain a large number of mycotoxins, including trichothecenes of type A and B. These compounds have already been associated with human and animal toxicoses.

Most common species causing the disease are *F. graminearum*, *F. culmorum* and *F. avenaceum*, but in the last few years a gradual increase in incidence of another species, *F. poae*, has been reported. In general terms, *F. poae* is a relatively weak pathogen, but its contribute to the increase of mycotoxins level still has to be clarified.

Durum wheat is widely cultivated in the central part of Italy, however the effective incidence of *F. poae* in this area still has to be investigated.

In order to monitor *Fusarium* risk, we collected dozens of *F. poae* strains on seeds and glumes of durum wheat coming from some of the most important cultivated areas of Central Italy. Every isolate was identified both by microscope observation and by PCR assay with the primer pair Fp82 F/R.

Strains were therefore subjected to a more accurate molecular characterization by Translation Elongation Factor 1-alpha (TEF-1α) gene sequencing.

Key words: durum wheat, FHB, *F. poae*, gene sequencing, mycotoxins, trichothecenes

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INTRODUCTION

Fusarium Head Blight (FHB) is a wheat disease caused by different fungal species belonging to *Fusarium* and *Microdochium* genera. More than 17 different *Fusarium* species have been associated with FHB worldwide (Parry *et al.*, 1995), although *F. graminearum* (Schwabe), *F. culmorum* (Smith) Sacc., *F. avenaceum* (Corda ex Fr.) Sacc., and *M. nivale* (varieties *majus* and *nivale*) are the species that more often have been isolated from naturally infected wheat spikes and kernels.

F. graminearum is usually associated with warmer and humid conditions, *F. avenaceum* and *F. culmorum* are isolated in cooler and wet environments, while *Microdochium* species are common in regions with frequent short rainfalls (Xu *et al*., 2008).

FHB is one of the most important wheat diseases and it is spread worldwide: it reduces grain yield and affects germination and vigour of infected seeds. Moreover *F. graminearum* is able to digest proteins and starch thus reducing kernels quality and their suitability for bread and pasta production.

In the last few years another species, *F. poae* (Peck) Wr., has been studied and monitored because of its high frequency of isolation in drier warmer environments (Pancaldi *et al.*, 1995; Xu *et al.*, 2005; Bourdages *et al*., 2006 and Pancaldi *et al*., 2010).

F. poae is a weak pathogen if compared to *F. graminearum* and *F. culmorum*, since it only induces small spots on wheat glumes. Spots consist in distinct lesions or bleaching, often with a dark margin, on individual kernels or glumes (Vogelgsang *et al.,* 2008). Nevertheless it has to be considered as a potentially dangerous organism, as it can produce group A and B trichothecenes. This species was first described in 1902 as *Sporotrichum poae* and only ten year after was collocated in the *Fusarium* genus by Wollenweber.

On Potato Dextrose Agar (PDA) it produces a dense aerial mycelium that can range from pink to reddish-brown (in aged cultures); the colony undersurface has colours that can range from white to carmine red.

The mycelium is characterized by short branched and unbranched monophialides, that produce globose, oval or piriform microconidia, while macroconidia are usually not produced. The mycelium is able to produce a typical fruity aroma (Stenglein, 2009). *F. poae,* together with *F. sporotrichioides, F. tricinctum* and *F. chlamydosporum,* is classified in the section *Sporotrichiella* by Nelson *et al*. (1983), Another species, *F. langsethiae*, firstly reported as "powdery" variant of *F. poae* was recently described on the basis of morphological and toxicological observations, (Torp and Niremberg, 2004).

Phylogenetic relationships among *F. sporotrichioides, F. langsethiae* and *F. poae* were studied using Translation Elongation Factor-1 alpha (TEF 1-α) gene: *F. poae* showed an intraspecific population structure (Knutsen *et al.*, 2004).

Stenglein *et al.* (2009) studied a broad population of *F. poae* collected from different geographic areas, deducing that *F. poae* form a monophyletic group with 10 haplotypes.

The aims of this study were:

(i) to evaluate the presence of *F. poae* on durum wheat in one of the most important cultivation area of Italy,

(ii) to analyze differences between some isolates that we collected by TEF-1α sequencing.

MATERIALS AND METHODS

Fifty-two seed samples were collected during year 2009 from Tuscany, Emilia Romagna and Marche. These regions are the most economically important durum wheat growing areas of Central Italy (Fig. 1).

Fig. 1. Geografic location of *F. poae* isolates

In order to isolate the seedborne fungi, 400 seeds per sample were surface sterilised 5 min. with sodium hypochlorite 1% and then washed twice with sterile water for 5 min. Dried seeds were disposed in 90 mm Petri dishes containing Potato Dextrose

Agar (PDA) medium supplemented with 100µg/ml Streptomycin, 50µg/ml Neomicyn and 50µg/ml Chloramphenicol. Dishes were then incubated 7 days at 21 ± 1°C under 12 hrs cycles of light and darkness.

Monoconidial cultures of every fungal colony belonging to the *Fusarium* genus were prepared. *F. poae* strains were identified by microscope observation, on the basis of macroconidia, microconidia and conidiougenous cells morphology.

In order to confirm the identification and carry on molecular studies, genomic DNA was extracted accordingly with the protocol developed by Orsini and Romano-Spica (2001).

PCRs were performed with two specific primers pairs (Table 1): the first pair, Fp 82 F/R, is designed on a specific region of *F. poae* genomic DNA (Parry & Nicholson, 1996), while the second pair, EF1/EF2, is designed on a portion of the gene Translation Elongation Factor (TEF-1α) (O'Donnell *et al.*, 1998). Reactions were carried out in an Eppendorf Thermal Cycler (Mastercycler ep Gradient S) and performed in a 50 µl mixture containing 20 ng of genomic DNA, 100 µM each of dATP, dCTP, dGTP and dTTP, 5 µl of the 10X PCR Buffer (Applied Biosystem), 10 µM each of forward and reverse primers and 1,0 U AmpliTaq gold DNA polymerase.

Table 1

Primers used in this study for DNA amplification and sequencing

Locus	Primer name	Primer sequence (5'-3')	Reference
F. poae -220 bp	Fp 82 F	CAAGCAAACAGGCTCTTCACC	Parry and Nicholson (1996)
	Fp 82 R	ACCTGTTCCACCTCAGTGACAGGTT	
EF - 1α	EF1	ATGGGTAAGGA(A/G)GACAAGAC	O'Donnell *et al.* (1998)
	EF2	GGA(A/G)GTACCAGT(G/C)ATCATGTT	

DNA amplification with primers pair Fp 82 F/R, was conducted in accordance with Parry & Nicholson protocol (1996).

TEF-1α gene was amplified with primers EF1/EF2 accordingly with the protocol developed by Geiser *et al.* (2004) and then cloned in the pGEM-T easy vector and sequenced.

Data obtained were used to query the NCBI and FUSARIUM-ID v. 1.0 (http://fusarium.cbio.psu.edu) databases using BLAST program.

DNA sequences were edited, after primer region deletion, analysed and aligned using CLUSTAL W Method with MEGA v. 4 (Tamura *et al*., 2007).

Haplotype sequences were estimated by software Collapse 1.2© (David Posada 1998-2006) treating Gaps as a 5th state to increase pairwise distance with the default options.

Maximum parsimony trees were obtained with PAUP version 4.0b10 (Swofford, 1998) using the heuristic search option, with 1000 random addition sequences replicate, with MULPARS on and tree bisection-reconnection branch swapping. Gap are treated as "new state" and "missing" (option in Pset menu).

Table 2

***Fusarium poae* isolates used in this study and associated haplotypes**

Isolate	Geographic origin	District	Primer Fp 82	Haplotype TEF-1α (Collapse 1.2)
Ense 469	Tuscany	Arezzo	+	2
Ense 473	Tuscany	Arezzo	+	1
Ense 480	Tuscany	Arezzo	+	2
Ense 487	Tuscany	Arezzo	+	1
Ense 493	Tuscany	Arezzo	+	3
Ense 494	Tuscany	Arezzo	+	1
Ense 495	Tuscany	Firenze	+	11*
Ense 471	Tuscany	Grosseto	+	1
Ense 474	Tuscany	Grosseto	+	2
Ense 475	Tuscany	Grosseto	+	1
Ense 476	Tuscany	Grosseto	+	1
Ense 477	Tuscany	Grosseto	+	3
Ense 479	Tuscany	Grosseto	+	1
Ense 481	Tuscany	Grosseto	+	1
Ense 488	Tuscany	Grosseto	+	12*
Ense 489	Tuscany	Grosseto	+	1
Ense 490	Tuscany	Grosseto	+	1
Ense 491	Tuscany	Grosseto	+	2
Ense 468	Tuscany	Grosseto	+	1
Ense 470	Tuscany	Grosseto	+	1
Ense 472	Tuscany	Pisa	+	14*
Ense 478	Tuscany	Pisa	+	1
Ense 492	Tuscany	Siena	+	1
Ense 483	Tuscany	Siena	+	1

Table 2

***Fusarium poae* isolates used in this study and associated haplotypes— continued**

Isolate	Geographic origin	District	Primer Fp 82	Haplotype TEF-1α (Collapse 1.2)
Ense 486	Tuscany	Siena	+	2
Ense 482	Tuscany	Siena	+	1
Ense 485	Tuscany	Siena	+	1
Ense 484	Tuscany	Siena	+	13*
Ense 496	Tuscany	Siena	+	1
Ense 582	Emilia Romagna	Ferrara	+	1
Ense 580	Emilia Romagna	Ferrara	+	1
Ense 584	Emilia Romagna	Ferrara	+	1
Ense 585	Emilia Romagna	Ferrara	+	11*
Ense 578	Emilia Romagna	Modena	+	1
Ense 587	Emilia Romagna	Ravenna	+	1
Ense 581	Emilia Romagna	Ravenna	+	1
Ense 583	Emilia Romagna	Ravenna	+	16*
Ense 579	Emilia Romagna	Reggio Emilia	+	17*
Ense 575	Marche	Ancona	+	18*
Ense 577	Marche	Ancona	+	1
Ense 586	Marche	Ancona	+	15*
Ense 588	Marche	Macerata	+	1
Ense 589	Marche	Macerata	+	2

To assess confidence in phylogenetic analysis, a bootstrap test was conducted on 1000 pseudoreplicates.

Fusarium langsethiae CC321 (EU744847 Chandler E. and Nicholson P.) was used as out-group in order to root the tree, and the already known haplotype sequences, are jointed in the analysis as in-group (Table 2):

RESULTS AND DISCUSSION

The percentage of seed samples infected by *F. poae* was 46%; the colonies were identified on the basis of morphological characteristics. This frequency of

isolation is very high if compared with other Italian epidemiological studies (Pancaldi *et al*., 2010)

Forty-three strains were collected in total. All isolates analyzed produced a DNA fragment of 220 bp when genomic DNA was amplified with the primers pair Fp 82 F/R, as observed by Parry & Nicholson (1996).

Studies previously conducted by Stenglein *et al.* (2009) on a total of 98 *F. poae* strains, allowed to identify10 haplotypes, with sequences 626, 633, 638, 618 bp long respectively for the 5, 7, 8, 9 haplotypes and 641 bp long for the other haplotypes 1, 2, 3, 4, 6, 10. In agreement with these findings, all sequences analysed in the present work were 641 bp long and the total proportions of nucleotides were 26.7% T, 29.4% C, 22.4% A, 21.5% G.

The alignment conducted over 54 taxa (43 sequences corresponding to the strains collected, 10 reference sequences and 1 sequence considered as out group) originated a matrix with 669 characters, 619 constant, 22 variable, and 17 singletons; the parsimony informative characters were 6 when gaps were treated as missing and 11 when gaps were treated as 5th state.

Software Collapse 1.2 outlined 18 haplotypes, 8 never described before and 10 corresponding to those found by Steinglein *et al.* (2009). New haplotypes were named with numbers from 11 to 18.

The majority of our strains (≈ 60%) belongs to haplotype 1, followed by haplotype 2 (14%) and haplotype 3 (≈ 5 %); two strains (≈ 5%) belong to the haplotype 11. Only one strain was recovered for haplotypes from 12 to 18. Haplotypes from 4 to 10 were not represented (Table 1).

Maximum parsimony analysis produced only two trees 90 in length, with a CI=0.9889 RI=0.9697 with gaps treated as missing (tree not shown), and only one tree 166 in length, with a CI=0.9880 RI=0.9487 with gaps treated a new state (Fig. 2) but the topology are the same for the tree obtained with two parameters.

When a sequence from *F. langsethiae* was used as outgroup, the tree appeared to be composed of three main branches: the first comprised the reference sequence for haplotype 2 and eight of the examined sequences, the second comprised the reference sequence for haplotype 4 and the third comprised all other examined and reference sequences (Fig. 2).

Haplotype 1 resulted to be the haplotype most frequently isolated in the central part of Italy. This finding is in good accordance with studies previously conducted by Stenglein *et al*. (2009) where the only Italian strain analyzed resulted to belong to haplotype 1. These authors studied isolates of *F. poae* collected from two different areas South America (Argentina) and Europe (mainly England). Strains were compared on the basis of EF-1a and mtSSU sequences.

Data did not reveal any correlation between the haplotype and geographic origin of wheat samples.

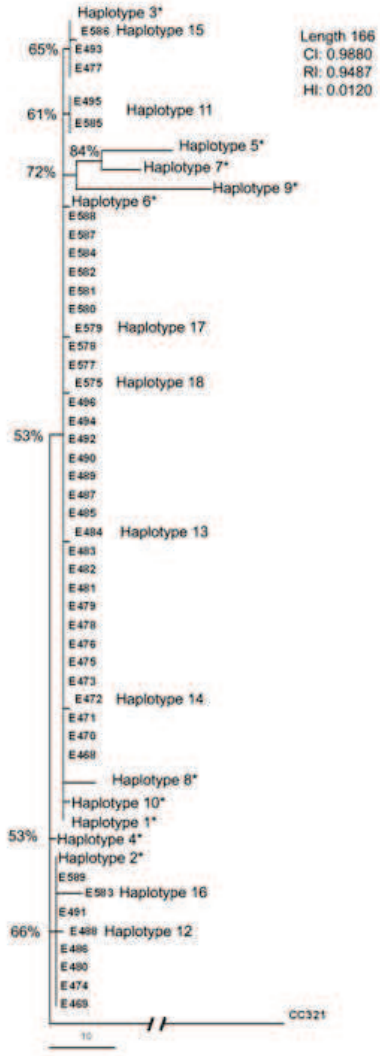

Fig. 2. Maximum parsimony tree obtained with hereustic search using gap as 5th state: 166 total length (CI: consistency index, HI: homoplasy index, RI: retention index) for all 43 isolates, 10 Haplotype references marked with (*) (Stenglein et al. 2009), and F. Langsethiae outgroup (close to node a bootstrap values [%])

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