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Structure of an *Aspergillus flavus* population from maize kernels in northern Italy

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ABSTRACT

In order to gain insight into the causal agents of aflatoxin contamination of maize in Italy, populations of *Aspergillus flavus* on maize produced in the most affected area were characterized. Forty-six percent of *A. flavus*, isolated from maize kernels collected in 5 districts of northern Italy between 2003 and 2010, were unable to produce detectable levels of aflatoxins. The genetic diversity of the population was assessed by analysis of vegetative compatibility groups (VCGs) and presence or absence of several aflatoxin biosynthesis genes. Forty-eight VCGs were identified through complementation between nitrate non-utilizing mutants. Twenty-five VCGs contained only atoxigenic isolates, and the remaining 23 only aflatoxin producers. Members of the largest atoxigenic VCG (IT6) were found in 4 of the 5 districts sampled. Six deletion patterns of genes in the aflatoxin biosynthesis gene cluster were detected. No deletions in the cluster were detected for twelve atoxigenic isolates and 10 had the entire cluster deleted. One isolate had a deletion pattern only seen once before in Nigeria. The basis for initial selection of endemic atoxigenic strains of *A. flavus* for biological control of aflatoxin contamination of maize in Italy is provided.

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1. Introduction

Maize (*Zea mays*) is a very important crop in Italy, cultivated on about 1 million ha; production amounts to about 8.5 million tons. The growing area is mainly located in 5 districts that supply 89% of the national production (Istat, 2011). These districts (Emilia Romagna, Friuli Venezia Giulia, Lombardy, Piedmont and Veneto) are situated in the north of the peninsula. Fumonisins, mycotoxins produced by *Fusarium verticillioides* in maize, represent a very frequent problem in this area (Pietri et al., 2004; Battilani et al., 2008). Unfortunately, in 2003 for the first time, high levels of aflatoxins were also detected (Giorni et al., 2007; Piva et al., 2006).

Aflatoxins are secondary metabolites produced by several members of *Aspergillus* section *Flavi* (Sweeney and Dobson, 1998). Giorni et al. (2007) reported that *Aspergillus flavus*, producers of aflatoxin B_1 and B_2, caused aflatoxin contamination in northern Italy. The International Agency for Research on Cancer (IARC, 2002) classified aflatoxin B_1 as a class 1 toxin, due to its demonstrated carcinogenic and teratogenic activity in humans (Wang and Tang, 2004).

A. flavus is a filamentous fungus with a vegetative incompatibility system (Papa, 1986), regulated by *vic* loci (Leslie, 1993), that limits hyphal fusion and gene flow between individuals belonging to different vegetative compatibility groups (VCGs; Leslie, 1993). Isolates are assigned to VCGs with functional Vegetative Compatibility Analyses (VCAs) typically utilizing nitrate nonutilizing auxotrophs (*nit*$^-$ mutants). In VCAs, VCG membership is defined by complementation of an isolate *nit*$^-$ by one or both members of a specific pair of tester mutants composed of complementary auxotrophs, usually one *cnx*$^-$ (deficient in the cofactor required by both nitrate reductase and xanthine dehydrogenase) and one *niaD*$^-$ (nitrate reductase deficient) (Cove, 1976; Papa, 1986; Bayman and Cotty, 1991a).

VCA is a useful tool to investigate diversity within *A. flavus* populations, and several VCGs are commonly found in each geographic area studied. Sweany et al. (2011) identified 16 VCGs from 669 isolates of *A. flavus* from ears and soil in 11 Louisiana corn fields. Habibi and Banihashemi (2008) identified 16 VCGs from 44 sesame seed isolates collected in Iran. Barros et al. (2006) identified 56 VCGs from 100 *A. flavus* isolates collected from soil in an Argentinean peanut-growing region. In these studies VCG diversity ranged from 0.02 in Louisiana corn to 0.56 in soil from Argentina.

Aflatoxin production is another character that is highly diverse within *A. flavus* populations. Isolates may produce anywhere from over 100 ppm aflatoxins (Mehl and Cotty, 2010) to no aflatoxins (i.e. are atoxigenic). The more than 25 genes involved in aflatoxin biosynthesis are contained within a 65 to 70 kb cluster (Yu et al., 2004), and several distinct deletions within this cluster have been described that may each be responsible for atoxigenicity in various isolates (Ehrlich et al., 2004; Chang et al., 2005; Yin et al., 2009; Donner et al., 2010). In recent years, specific primers have been designed

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for several aflatoxin biosynthesis genes, and PCR has been applied to study expression of aflatoxin biosynthetic genes by *A. flavus* in different matrices, including food commodities (Schmidt-Heydt and Geisen, 2007).

Since 2003, special attention has been dedicated to *A. flavus* populations in northern Italy; although only recently the distributions of deletions in the aflatoxin biosynthesis gene cluster have been partially examined (Gallo et al., 2012), the diversity of *A. flavus* VCGs has never been evaluated. The current study examined characteristics of Italian populations of *A. flavus* using VCA in order to gain insight into the characteristics and potential management of the causal agents of aflatoxin contamination of maize in Italy.

2. Materials and methods

2.1. Fungal isolates and culture conditions

One hundred and thirty-eight *A. flavus* isolates from maize kernels grown in 5 districts of northern Italy were used in the current study. The geographic area studied lies between longitude 7.49° and 13.33° E and latitude 43.85° and 46.16° N (Battilani et al., 2008) (Fig. 1). Fungi were isolated from maize collected in Emilia Romagna (54), Friuli Venezia Giulia (14), Lombardy (53), Piedmont (7) and Veneto (10) during the 2003–2010 growing seasons. Isolation and identification to species by morphological criteria followed Giorni et al. (2007). Isolates were also characterized by sclerotial morphology on 5/2 agar allowing for identification of S strain and L strain morphologies (Cotty, 1989; Probst et al., 2012). Sclerotial morphology combined with aflatoxin-producing potential (i.e. production of only B aflatoxins or production of both B and G aflatoxins) was used to determine potential occurrence of several recently described aflatoxin-producing taxa (Probst et al., 2012; Soares et al., 2012). All isolates have been incorporated into the culture collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore, Piacenza, Italy.

Isolates were transferred by single spore twice serially on malt agar (1% malt, 2% agar, 1000 ml of water). After 2 days on malt, colonies were transferred to 5/2 agar (5% V8 juice, 2% agar, pH 5.2, 1000 ml of water) and incubated at 31 °C for 5–6 days. Conidial suspensions from plugs of mature cultures were maintained in vials (4 ml) containing sterile distilled water and used as working cultures throughout the study. Long-term storage was on silica gel.

2.2. Aflatoxin quantification

All the isolates were tested for ability to produce aflatoxin. To this end, 20 g of undamaged kernels in 250-ml Erlenmeyer flasks sealed with gas-permeable plugs (Bugstoppers; Whatman, Piscataway, NJ) were autoclaved at 121 °C for 60 min (Probst et al., 2011). Kernels were inoculated with 100 μl of conidial suspension (10^5 to 10^6 conidia) and incubated for 7 days at 31 °C in the dark. After incubation, kernels were blended (30 s high) in 50 ml 80% methanol in a laboratory blender. The homogenized maize was filtered through Whatman No. 4 paper and the filtrate was spotted directly onto thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) together with aflatoxin standards (Aflatoxin Mix kit-M; Supelco Bellefonte, PA) containing a mixture of aflatoxin B_1, B_2, G_1 and G_2. Plates were developed in ethyl ether–methanol–water (96:3:1), air-dried, and aflatoxins were visualized under 365-nm UV light. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc., Wilmington, NC, USA) (Probst et al., 2011). Filtrates initially negative for aflatoxins were partitioned twice with methylene chloride (25 ml) and concentrated prior to quantification (Cotty, 1997). One replicate was made for each isolate. To confirm inability to produce aflatoxins, putative atoxigenic isolates were retested with 4 replicates. Isolates that were invariably negative for aflatoxins were considered atoxigenic for the purpose of this study. The limit of detection was 0.5 μg/kg.

2.3. Vegetative compatibility groups

Czapek–Dox (CZ) medium supplemented with 25 g/l of potassium chlorate and rose Bengal (50 mg/l) (Cotty, 1994), pH 7.0, was used to select nitrate-non-utilizing mutants of each isolate. Plates were center-point inoculated with 15 μl of conidial suspension and incubated at 31 °C until chlorate-resistant sectors arose. At least 4 mutants were recovered for each isolate. Mutants were phenotyped on CZ (30 g sucrose, 3 g $NaNO_3$, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$.$7H_2O$, 0.5 g KCl, 20 g Bacto agar, pH 6.0), hypoxanthine (HYP) agar (50 g sucrose, 10 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, 0.2 g hypoxanthine, 20 g Bacto agar, pH 5.5) and nitrite (NIT) medium (50 g sucrose, 10 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, 0.5 g $NaNO_2$, 20 g Bacto agar pH 5.5). CZ, HYP and NIT media allowed identification of *niaD*, *cnx* and *nirA* mutants, respectively, as previously described (Bayman and Cotty, 1991b).

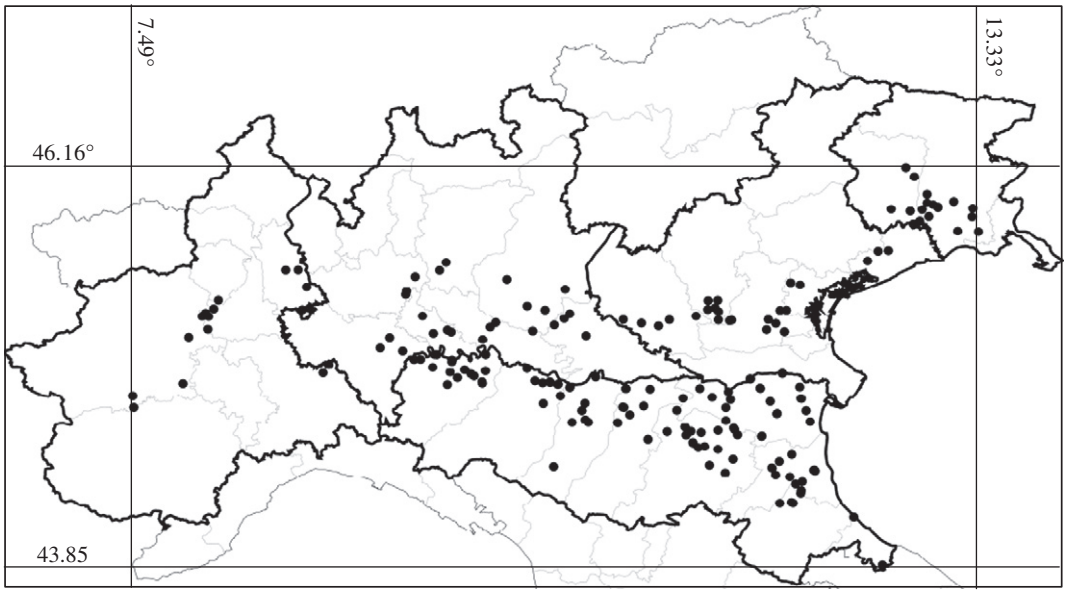

Fig. 1. Locations from which maize samples were collected from 2003 to 2010. *Aspergillus flavus* used in the current study were isolated from these samples.