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Fusarium diversity in soil using a specific molecular approach and a cultural approach

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ABSTRACT

Fusarium species are ubiquitous in soil. They cause plant and human diseases and can produce mycotoxins. Surveys of *Fusarium* species diversity in environmental samples usually rely on laborious culture-based methods. In the present study, we have developed a molecular method to analyze *Fusarium* diversity directly from soil DNA. We designed primers targeting the translation elongation factor 1-alpha (EF-1α) gene and demonstrated their specificity toward *Fusarium* using a large collection of fungi. We used the specific primers to construct a clone library from three contrasting soils. Sequence analysis confirmed the specificity of the assay, with 750 clones identified as *Fusarium* and distributed among eight species or species complexes. The *Fusarium oxysporum* species complex (FOSC) was the most abundant one in the three soils, followed by the *Fusarium solani* species complex (FSSC). We then compared our molecular approach results with those obtained by isolating *Fusarium* colonies on two culture media and identifying species by sequencing part of the EF-1α gene. The 750 isolates were distributed into eight species or species complexes, with the same dominant species as with the cloning method. Sequence diversity was much higher in the clone library than in the isolate collection. The molecular approach proved to be a valuable tool to assess *Fusarium* diversity in environmental samples. Combined with high throughput sequencing, it will allow for in-depth analysis of large numbers of samples.

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1. Introduction

Fusarium is a large genus of filamentous fungi widely distributed around the world in all types of soils (Backhouse et al., 2001). It groups very diverse species with various morphological, physiological and ecological characteristics. There is no known relationship between species distribution and environment. Numerous species are pathogens responsible for severe diseases on a variety of plants of agronomic, horticultural or forestry importance. For example, the formae speciales of the *Fusarium oxysporum* cause wilt or root rot diseases in more than 100 plant species (Armstrong and Armstrong, 1981; Kistler et al., 1998). Several species cause destructive diseases on cereals, among which *Fusarium graminearum*, *Fusarium culmorum*, and *Fusarium avenaceum* are responsible for Fusarium head blight of wheat. Based on their economic and scientific importance, both *F. graminearum* and *F. oxysporum* were recently classified among the "Top 10" fungal pathogens in molecular fungal pathology (Dean et al., 2012). *Fusarium* species can also produce mycotoxins that result in crop and food contamination, especially on cereals. Common examples are the production of trichothecenes such as deoxynivalenol by *F. graminearum* on wheat and the production of fumonisin by *Fusarium verticillioides* on maize. These mycotoxins are hazardous to human and animal health. Conversely, some beneficial populations of *Fusarium* can reduce the infectious activity of pathogenic *Fusarium* and disease severity (Steinberg et al., 2007). These diverse fungi have various habitats, they are common in soil and on living plants and plant residues but they can also be found in the air and in water (Almaguer et al., 2012; Donat et al., 2012; Sautour et al., 2012). Besides their economic importance, *Fusarium* is also of clinical importance: in recent years they have been increasingly associated with human skin infections and invasive infections of immunocompromised patients (Nucci and Anaissie, 2007; Migheli et al., 2010).

Although abundant in soils, *Fusarium* communities can vary in terms of species diversity, depending on crops, soil physico-chemical characteristics, climatic conditions and human activities (Bateman and Murray, 2001; Steinkellner and Langer, 2004; Summerell et al., 2010). In addition, non-cultivated areas may represent conservation sites of a primary biodiversity where microbial communities have not been subjected to human activities. A few studies have investigated the impact of cultural practices and seasonal and climatic conditions on the incidence of *Fusarium* species in soil (Bateman and Coskun, 1995; Bateman and Murray, 2001; Steinkellner and Langer, 2004). The diversity of *Fusarium* isolates was also analyzed at different geographic scales (Backhouse and Burgess, 1995; Balmas et al., 2010; Summerell et al., 2010; Laurence et al., 2012). But very little is currently known about the relative importance of the different environmental factors that affect *Fusarium* communities.

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The majority of *Fusarium* isolates cannot be identified down to the species level using only morphological characters. Molecular analyses and multilocus phylogenetic studies have identified several informative loci that resolve relationships between and within *Fusarium* species (O'Donnell et al., 2009, 2010, 2012). They have revealed the high level of phylogenetic diversity within several morphological species such as *F. oxysporum* and *Fusarium solani*, which are now recognized as species complexes. Among useful loci, the translation elongation factor 1-alpha (EF-1α) gene allowed both to differentiate closely related species and to reveal intraspecific polymorphism (O'Donnell et al., 2009, 2012; Edel-Hermann et al., 2012) and can be used as a BLAST query against the FUSARIUM-ID sequence database (Geiser et al., 2004) or the Fusarium MLST database (O'Donnell et al., 2012).

Standard procedures to analyze fungal diversity are based on isolation on selective media and species identification using morphological and/or molecular characters. Because they are laborious and time-consuming, these methods generally restrict the number of isolates one can analyze. During the last two decades, the development of culture-independent molecular approaches has made it easier to monitor microbial communities in soils. They rely on direct extraction of DNA from soil and selective amplification by polymerase chain reaction (PCR) of a marker of the community of interest (Edel-Hermann et al., 2008; van Elsas and Boersma, 2011; Plassart et al., 2012; Suenaga, 2012). Such strategies have been developed to characterize whole bacterial or fungal communities but can also be set up to address a specific component of the microflora, the limiting factor being the availability of specific primers.

The objectives of the present study are (i) to set up a specific PCR assay to analyze the diversity of *Fusarium* communities in soils by direct amplification from soil DNA, and (ii) to compare the diversity of *Fusarium* communities yielded by this direct molecular approach with the diversity yielded by a cultural approach.

2. Materials and methods

2.1. Fungal isolates

Nineteen *Fusarium* species and 20 other fungal species commonly found in soil were obtained from the "Microorganisms of Interest for Agriculture and Environment" (MIAE, INRA Dijon, France) collection (Table 1).

Table 1
Fungal strains used in this study.

Species	MIAE accession[a]
Fusarium oxysporum	MIAE00047
F. acuminatum	MIAE00244
F. avenaceum	MIAE00286
F. chlamydosporum	MIAE00287
F. commune	MIAE00101
F. culmorum	MIAE00688
F. incarnatum–equiseti species complex	MIAE00050
F. graminearum	MIAE01623
F. langsethiae	MIAE00289
F. lateritium	MIAE00291
F. poae	MIAE00299
F. redolens	MIAE00129
F. sambucinum	MIAE00301
F. solani species complex	MIAE01624
F. sporotrichioides	MIAE01625
F. subglutinans	MIAE00068
F. tricinctum	MIAE00305
F. venenatum	MIAE00076
F. verticillioides	MIAE00306
Aspergillus sp.	MIAE01626
Cladosporium sp.	MIAE00388
Clonostachys rosea	MIAE00156
Colletotrichum coccodes	MIAE00168
Cylindrocarpon sp.	MIAE00384
Gliocladium roseum	MIAE00088
Mortierella elongata	MIAE00403
Mucor hiemalis	MIAE00009
Penicillium sp.	MIAE01627
Rhizoctonia solani	MIAE00082
Rhizopus oryzae	MIAE01628
Stereum rugosum	MIAE00005
Trichoderma tomentosum	MIAE00417
T. viride	MIAE01083
T. spirale	MIAE00977
T. velutinum	MIAE00411
Trichosporon porosum	MIAE00979
Umbelopsis autotrophica	MIAE01629
Ulocladium sp.	MIAE00408
Verticillium dahliae	MIAE01064

[a] MIAE collection, Microorganisms of Interest for Agriculture and Environment (INRA, Dijon, France).

2.2. Soils

Three soils from eastern France near Dijon were used to analyze the diversity of *Fusarium* communities by cloning PCR products and by colony isolation: an arable soil from an experimental farm in Epoisses (soil E, 47°14 N, 5°5 E); a meadow soil from Blaudes (soil B, 46°47 N, 5°11 E); and a forest soil from Morvan (soil M, 47°16 N, 4°13 E). At each location, 20 kg of soil was taken within an area of several m^2 at 2–15 cm depth, and then homogenized. The main characteristics of the soils were: 37.9% clay, 55.5% loam, 6.6% sand, 2.41% organic matter and pH 6.8 for soil E; 13.8% clay, 37.8% loam, 48.4% sand, 3.3% organic matter and pH 6.8 for soil B; and 19.2% clay, 21.2% loam, 59.6% sand, 4.81% organic matter and pH 5.0 for soil M.

2.3. DNA extraction

Nucleic acids were extracted from fungal strains using cultures on potato dextrose agar (PDA) (39 g l^{-1}; Sigma Chemical Co., St Louis, MO, USA) and the procedure described by Edel et al. (2001). Nucleic acids were extracted from soils using a procedure adapted from Edel-Hermann et al. (2004). Two grams of soil was added to 4 g of 100-μm-diameter silica beads, 5 g of 1.4-mm-diameter ceramic beads, eight 4-mm-diameter glass beads and 8 ml of lysis buffer containing 100 mM Tris–HCl (pH 8), 100 mM EDTA (pH 8), 100 mM NaCl and 2% (w/v) sodium dodecyl sulfate. The physical and chemical disruptions were performed as described in the ISOm procedure by Plassart et al. (2012). The crude nucleic acid extracts were purified twice using a polyvinylpolypyrrolidone spin column and once using a Geneclean® Turbo kit (MP Biomedicals, Illkirch, France). Purified DNA extracts were quantified by electrophoresis in agarose gels using dilutions of calf thymus DNA, and then stored at −20 °C.

2.4. Design of primers and PCR assay specific for Fusarium spp.

Yergeau et al. (2005) proposed specific primers Alfie1/Alfie2 targeting the EF-1α gene to assess *Fusarium* species diversity in asparagus. We first used this primer pair and the PCR conditions described by Yergeau et al. to assess *Fusarium* diversity from total DNA extracted from soil E. After cloning and sequencing of the PCR products, 47 out of 96 clones were identified as *Trichoderma tomentosum*. Consequently we designed new primers by comparing the EF-1α sequences of several *Fusarium* species with a range of other fungi including *Trichoderma* species. Primer specificity was assessed in silico using Primer-BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) and experimentally by PCR using the fungal isolates listed in Table 1.

Part of the EF-1α gene was amplified from fungal DNA using these specific primers Fa (5′-TCGTCATCGGCCACGTCGACTCT-3′) and Ra (5′-CAATGACGGTGACATAGTAGCG-3′) in a final volume of 25 μl containing 1 μl of DNA, 0.2 μM of each primer, 150 μM dNTP, 3 U of *Taq* DNA polymerase (MP Biomedicals) and PCR reaction buffer. Amplifications were