Fungal Genetics and Biology 46 (2009) 936–948

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex

Kerry O'Donnell [a,*], Cécile Gueidan [b], Stacy Sink [a], Peter R. Johnston [c], Pedro W. Crous [b], Anthony Glenn [d], Ron Riley [d], Nicholas C. Zitomer [d], Patrick Colyer [e], Cees Waalwijk [f], Theo van der Lee [f], Antonio Moretti [g], Seogchan Kang [h], Hye-Seon Kim [h], David M. Geiser [h], Jean H. Juba [h], Robert P. Baayen [i,1], Matthew G. Cromey [j], Sean Bithell [j], Deanna A. Sutton [k], Kerstin Skovgaard [l], Randy Ploetz [m], H. Corby Kistler [n], Monica Elliott [o], Mike Davis [p], Brice A.J. Sarver [q]

[a] *Microbial Genomics Research Unit, Agricultural Research Service, US Department of Agriculture, Peoria, IL 61604, United States*
[b] *CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands*
[c] *Landcare Research, Auckland, New Zealand*
[d] *USDA-ARS, Athens, GA 30605, United States*
[e] *Louisiana State University Agricultural Center, Bossier City, LA 71040, United States*
[f] *Plant Research International, Wageningen University and Research, P.O. Box 16, 6700 Wageningen, The Netherlands*
[g] *CNR (Research National Council), ISPA Institute of Sciences of Food Production, Bari, Italy*
[h] *Department of Plant Pathology, The Pennsylvania State University, University Park, PA 16802, United States*
[i] *Ministry of Agriculture, Nature and Food Quality, P.O. Box 20401, 2500 EK The Hague, The Netherlands*
[j] *Crop and Food Research, Christchurch, New Zealand*
[k] *Department of Pathology, University of Texas Health Science Center, San Antonio, TX 78229, United States*
[l] *National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark*
[m] *University of Florida, Homestead, FL, United States*
[n] *Cereal Disease Laboratory, Agricultural Research Service, US Department of Agriculture, St. Paul, MN 55108, United States*
[o] *University of Florida, Ft. Lauderdale, FL 33301, United States*
[p] *Department of Plant Pathology, University of California, Davis, CA 95616, United States*
[q] *Department of Biological Sciences, University of Idaho, Moscow, ID 83844, United States*

ARTICLE INFO

Article history:
Received 8 June 2009
Accepted 18 August 2009
Available online 26 August 2009

Keywords:
Beauvericin
EF-1α
Enniatin
Fumonisin
Fusariosis
Genealogical discordance
IGS rDNA
Moniliformin
Mycotoxin
Phylogeny
Vascular wilt

ABSTRACT

We constructed a two-locus database, comprising partial translation elongation factor (*EF-1α*) gene sequences and nearly full-length sequences of the nuclear ribosomal intergenic spacer region (IGS rDNA) for 850 isolates spanning the phylogenetic breadth of the *Fusarium oxysporum* species complex (FOSC). Of the 850 isolates typed, 101 *EF-1α*, 203 IGS rDNA, and 256 two-locus sequence types (STs) were differentiated. Analysis of the combined dataset suggests that two-thirds of the STs might be associated with a single host plant. This analysis also revealed that the 26 STs associated with human mycoses were genetically diverse, including several which appear to be nosocomial in origin. A congruence analysis, comparing partial *EF-1α* and IGS rDNA bootstrap consensus, identified a significant number of conflicting relationships dispersed throughout the bipartitions, suggesting that some of the IGS rDNA sequences may be non-orthologous. We also evaluated enniatin, fumonisin and moniliformin mycotoxin production *in vitro* within a phylogenetic framework.

Published by Elsevier Inc.

1. Introduction

Members of the *Fusarium oxysporum* species complex (FOSC) are ubiquitous soil borne pathogens responsible for vascular wilts, rots, and damping-off diseases of a broad range of agronomically and horticulturally important crops (Baayen et al., 2000; Michielse

* Corresponding author. Fax: +1 309 681 6672.
E-mail address: kerry.odonnell@ars.usda.gov (K. O'Donnell).
URL: http://www.ars.usda.gov/sp2UserFiles/Place/36207000/MGB-ODonnell2.pdf (K. O'Donnell).
[1] Present address: European Commission, Directorate for Health and Consumers, Brussels, Belgium.

1087-1845/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.fgb.2009.08.006

and Rep, 2009). With more than 80 putatively plant host-specific formae speciales described (Kistler et al., 1998; Katan, 1999; Katan and Di Primo, 1999), members of the FOSC collectively represent the most commonly encountered and economically important species complex within *Fusarium*. A number of these fusaria are also clinically important, causing localized or deeply invasive life-threatening infections in humans and other animals (O'Donnell et al., 2004, 2007; Ortoneda et al., 2004). Mortality in patients who are persistently and severely neutropenic is typically 100% (Dignani and Anaissie, 2004).

Due to the staggering economic losses that members of the FOSC inflict on agriculture worldwide, an extensive body of literature exists on the genetic and pathogenic characterization of individual formae speciales into vegetative compatibility groups (VCGs). Formae speciales are defined based on pathogenicity to one or more plant hosts, whereas VCGs are defined based on their ability to anastomose and form heterokaryons (Gordon and Martyn, 1997). VCGs have proven to be excellent predictors of evolutionary origin (Elias et al., 1993), and they appear to be predominantly or exclusively clonal lineages. Some formae speciales are further divided into races based on virulence to a differential set of cultivars of one or more plant species. VCGs may harbor several races, which in such cases seem to differ only in cultivar-specific virulence genes (Baayen et al., 2000). Molecular phylogenetic analyses have shown that formae speciales with two or more VCGs may in some cases be polyphyletic (O'Donnell et al., 1998b; Baayen et al., 2000; Skovgaard et al., 2001; Mbofung et al., 2007; Fourie et al., 2009), calling into question the taxonomic value of the forma specialis naming system. Although putatively non-pathogenic strains have been described, and some have been employed successfully as biocontrol agents to suppress soil borne pathogens (Larkin et al., 1996; Fuchs et al., 1997; Olivain et al., 2006), the null hypothesis that some isolates may be non-pathogenic is virtually impossible to test given the huge number of potential vascular plant hosts. This problem is exacerbated because the phylogenetic history of the FOSC appears to be characterized by numerous host jumps based on geographic proximity rather than taxonomic relatedness (O'Donnell et al., 1998b; Baayen et al., 2000; Roy, 2001), and by the horizontal transfer of genes contributing to host specificity (van der Does et al., 2008).

Although diverse molecular markers have been developed to identify various formae speciales (Baayen et al., 2000; Groenewald et al., 2006; Lievens et al., 2007, 2008), time-consuming pathogenicity assays are currently the gold standard for identifying host-specific pathogens within the FOSC. Given the high level of phylogenetic diversity and large number of formae speciales, multilocus DNA sequence typing (MLST) currently represents the most robust approach for characterizing the genetic diversity of the FOSC. Moreover, DNA sequence data are ideally suited for sharing and developing web-accessible databases for the purpose of pathogen identification and strain typing via the Internet (Taylor and Fisher, 2003; Geiser et al., 2004; Park et al., 2008). Towards this end, the primary objective of the present study was focused on assessing the utility of partial translation elongation factor (*EF-1α*, 634 bp alignment) and nearly full-length nuclear ribosomal DNA intergenic spacer (IGS rDNA, 2220 bp alignment) region sequences for developing a two-locus database for the identification of formae speciales, opportunistic pathogens of humans and other animals, and environmental contaminants of hospital plumbing systems or food and beverage processing facilities. In addition, experiments were conducted to assess the potential of genetically diverse members of the FOSC to produce moniliformin, fumonisin, and enniatin mycotoxins *in vitro*. The FOSC database, including the electropherograms, will be incorporated into the next version of the web-accessible FUSARIUM-ID database (Geiser et al., 2004).

2. Materials and methods

2.1. Strains

To provide dense sampling of formae speciales genetic diversity, FOSC isolates were obtained from diverse sources (see Supplementary Table 1), with the majority obtained from the following three internationally accessible culture collections: CBS-KNAW Fungal Biodiversity Center (CBS, *N* = 157), Utrecht, The Netherlands; Fusarium Research Center (FRC, *N* = 119), Pennsylvania State University; and The International Collection of Microorganisms from Plants (ICMP, *N* = 246), Landcare Research, Auckland, New Zealand. We also included isolates characterized genetically in previous studies of the FOSC (O'Donnell et al., 1998b, 2004; Baayen et al., 2000; Skovgaard et al., 2001). In addition, some isolates included in this study were initially identified as members of the FOSC after conducting BLAST searches of the FUSARIUM-ID database (Geiser et al., 2004) (http://www.fusariumdb.org/), using partial translation elongation factor sequences as the query. Sequences of the sister taxon of the FOSC, *F. foetens*, were used to root the phylogeny (Schroers et al., 2004). We also included isolates associated with mycotic infections of humans and other animals, contaminants of food and beverage production facilities, putative non-pathogens, and biocontrol agents to assess their relationship to one another and to isolates of 68 described formae speciales, which we were able to obtain from internationally accessible culture collections (Table 1). All isolates are stored cryogenetically in liquid nitrogen vapors at ~175 °C in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL (http://nrrl.ncaur.usda.gov/).

2.2. DNA manipulations

Genomic DNA was extracted from freeze-dried mycelium using a CTAB protocol as described previously (O'Donnell and Cigelnik, 1997). The two-locus typing scheme consisted of sequences of the nuclear ribosomal DNA intergenic spacer region (IGS rDNA) and the 5′ intron-rich portion of the *EF-1α* gene. PCR and sequencing primers for the IGS rDNA are listed in Fig. 1. Of the seven internal sequencing primers designed in this study, only four were typically needed (i.e., iNL11, NLa, CNSa and iCNS1) to span the region analyzed (2220 bp alignment). PCR primers EF-1 and EF-2 for the *EF-1α* gene are reported in O'Donnell et al. (1998b). A partial sequence of the *EF-1α* gene was obtained using EF-3 (GTAA GGAGGASAAGACTCACC) and EF-22 (AGGAACCCTTACCGAGCTC) as sequencing primers. PCR amplifications employed Platinum® *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) in an Applied Biosystems (ABI) 9700 thermocycler (Emeryville, CA) using the following program: 1 cycle of 90 s at 94 °C; 40 cycles of 30 s at 94 °C, 90 s at 55 °C, and 3 min at 68 °C; followed by 1 cycle of 5 min at 68 °C and a 4 °C soak. All PCR products were sized via electrophoresis in 1.5% agarose gels run in 1× TAE buffer, stained with ethidium bromide, and then photographed over a UV trans-illuminator. Prior to sequencing on an ABI 3730 capillary sequencer, amplicons were purified using Montage PCR_{96} filter plates (Millipore Corp. Billerica, MA). DNA sequencing reactions were conducted in a 10 μl volume with 0.5–2 μl of ABI BigDye version 3.1 terminator reaction mix, 2–4 pmol of each sequencing primer and approximately 50 ng of cleaned amplicon. Sequence data was edited and then aligned with Sequencher version 4.1.2 (Gene Codes, Ann Arbor, MI), after which they were exported as nexus files. The *EF-1α* and IGS rDNA sequence files were imported separately into DnaSP (Rozas et al., 2007) so that they could be exported as FASTA files which were then aligned using CLUSTAL-X (Larkin et al., 2007). Due to the number of length-variable indels,