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Genome distribution and validation of novel microsatellite markers of *Fusarium verticillioides* and their transferability to other *Fusarium* species

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ABSTRACT

Improved population studies in the fungus *Fusarium verticillioides* require the development of reliable microsatellite markers. Here we report a set of ten microsatellite loci that can be used for genetic diversity analyses in *F. verticillioides*, and are equally applicable to other fungi, especially those belonging to the *Gibberella fujikuroi* clade.

1. Introduction

Fusarium verticillioides, the most common maize pathogen in the world, is the cause of stalk, root and ear rot (Munkvold, 2003; Morales-Rodríguez et al., 2007). In addition to this disease and its negative impacts on grain yield and quality, *F. verticillioides* produces a diversity of mycotoxins, some of which are potentially carcinogenic. Among these are the fusarins, fumonisins, and 8-bostrycoidin (Leslie and Summerell, 2006). *Fusarium* species show high levels of intraspecific genetic diversity, which could explain such traits as pathogenicity variation, mycotoxin production and host selectivity between isolates of the same species (Manicom et al., 1990; Miedaner et al., 2001; Saharan and Naef, 2008). This genetic diversity has been investigated by molecular techniques including Random Amplification of Polymorphic DNA (RAPDs; Gherbawy et al., 2001; Pamphile and Azevedo, 2002), Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLPs; Patiño et al., 2006) and microsatellites (Saharan and Naef, 2008; Xu et al., 2012). Microsatellites, simple sequence repeats (SSR), or short tandem repeats (STR) are small motifs of DNA that are repeated in tandem, which are typically 1–6 nucleotides in length. Microsatellites have been successfully used to elucidate genetic diversity and to examine species differentiation and population structure in several *Fusarium* species, including *Fusarium oxysporum* (Bogale et al., 2005), *Fusarium culmorum* (Giraud et al., 2002; Bayraktar et al., 2008), *Fusarium circinatum* (Santana et al., 2009) and *Fusarium graminearum* (Karaoglu et al., 2005; Naef and Dégafo, 2006; Saharan and Naef, 2008; Vogelgsang et al., 2009). Genetic diversity in *F. verticillioides* has also been assessed by microsatellites designed for related species, e.g. *F. graminearum* (Saharan and Naef, 2008), although cross-species transferability often results in the amplification of either only a small set of microsatellites or no amplification at all (Saharan and Naef, 2008; Vogelgsang et al., 2009). With the recent sequencing of the *F. verticillioides* genome (Ma et al., 2010), significant advances have been made in the mining and generation of microsatellites in this species. For instance, Santana et al. (2009) identified genome microsatellites and Xu et al. (2012) reported di-, tri- and tetranucleotide microsatellites that are useful for genetic diversity studies in Chinese populations of *F. verticillioides*. To date, microsatellites used in population genetic studies of *Fusarium* species have been mainly di- and trinucleotides that range from seven to twenty-eight repetitions. Almost all of these are polymorphic, and have been shown to be useful for differentiating diverse *formae specialis* of *F. oxysporum* (Bogale et al., 2006), isolate identification (Giraud et al., 2002; Vogelgsang et al., 2009), and genetic variability assessment (Naef and Dégafo, 2006; Bayraktar et al., 2008; Saharan and Naef, 2008). Tetra-, penta- and hexanucleotides are rarely used, since it is presumed

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that they are less likely to be polymorphic, due to their lower repetition numbers. However, short microsatellites (arranged in five to seven tandem repeats) are reported to be polymorphic in other *Fusarium* species (Giraud et al., 2002; Bogale et al., 2006; Naef and Dégafo, 2006; Bayraktar et al., 2008; Saharan and Naef, 2008; Vogelgsang et al., 2009). Thus, polymorphism can also occur in microsatellites that are longer than hexanucleotides, as well as in compound microsatellites (Bogale et al., 2005).

A successful examination of the high genetic diversity of *F. verticillioides* will require a greater number of microsatellites than currently available. To respond to this need, we provide detailed information on the distribution and patterns of microsatellite regions over eleven of the twelve chromosomes and validate a set of ten microsatellite loci that include tetra-, penta- and hexanucleotides.

2. Materials and methods

2.1. Genome survey and distribution of microsatellites

The nucleotide sequences of eleven of the twelve chromosomes in the *F. verticillioides* (strain 7600) genome (previously obtained by the Broad Institute (Ma et al., 2010)) were downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). The sequences of all chromosomes were scanned for microsatellite repeats with the Tandem Repeat Finder (TRF) software, version 4.04 (Benson, 1999). The following parameters, recommended by the software authors, were used: +2, −7, and −7 for match, mismatch and indels respectively; 0.80 and 0.10 for matching and indel probability respectively; a maximum period size of 500 for patterns and a minimum alignment score to report repeats of 50. Finally, only tandem repeats of exact copies of a particular motif (perfect repeats or perfect microsatellites) were selected for further analysis with the following selection criteria: seven or more repetitions for mononucleotides; five or more repetitions for di- and trinucleotides; and four or more repetitions for tetra-, penta- and hexanucleotides. The identified repeat motifs were grouped into classes that include all possible positions of nucleotides in the repeat and its complementary sequence. For example, $(AG)_n$ is equivalent to $(GA)_n$, $(CT)_n$, and $(TC)_n$. This yields two classes for mononucleotide repeats, four classes for dinucleotides, 10 classes for trinucleotides, 33 classes for tetranucleotides, 102 classes for pentanucleotides and 350 classes for hexanucleotides (Jurka and Pethiyagoda, 1994). To confirm the identified microsatellites using the TRF software, all chromosome sequences were also scanned by the SciRoKo 3.4 software (Kofler et al., 2007). This tool has been successfully used in previous studies to identify microsatellite repeats (Merkel and Gemmell, 2008; Kofler et al., 2008; Xiao et al., 2011). Both programs employ probabilistic methods, but use different approaches making SciRoKo a faster tool (Sharma et al., 2007; Grover et al., 2012). Aside from their differences in speed and approach, both programs are recommended for mining perfect, imperfect and compound microsatellite repeats (Sharma et al., 2007) and the characterization of genomic microsatellite distribution (Merkel and Gemmell, 2008; Grover et al., 2012). A compound microsatellite is an arrangement where two or more repeated motifs are next to each other without interruptions (e.g. $(CT)_{22}$-$(CA)_6$-), whereas an interrupted compound microsatellite is interrupted by a sequence (e.g. $(AC)_{14}$-AG-AA-$(AG)_{12}$-), and a complex microsatellite is an arrangement with three or more repeat units that may or may not be interrupted (e.g. $(TTTC)_{3-4}$-$(T)_6$-$(CT)_{0-1}$-$(CYKY)_n$-CTCC-$(TTCC)_{2-4}$) (Chambers and MacAvoy, 2000). The frequency of each repeat unit (mono-, di-, tri-, tetra-, penta- and hexanucleotide) in the genome was also determined, and the relative frequency was calculated by dividing the total amount of each type of microsatellite by the total number of microsatellites found in the *F. verticillioides* genome. Relative abundance (number of microsatellites of each type/Mb of analyzed sequence) and relative density (length of microsatellites/Mb of analyzed sequence) were calculated for each repeat unit. Normalizing the data per Mbp allows comparisons with similar studies.

Finally, microsatellites were mapped to their respective chromosomes with the software pDRAW32 version 1.0 (ACACLONE), using the chromosomal coordinates obtained by TRF.

2.2. Development of markers

Eleven microsatellite loci were selected on the basis that they showed a higher number of repetitions than average for each microsatellite repeat (mono-, di-, etc.). For instance, if the average number of repetitions observed for hexanucleotides was five, then a hexanucleotide with more than five repetitions would be selected for primer design. Subsequently, a set of eleven primer pairs was designed to target the surrounding sequence (500 bp) of the 11 microsatellites, using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). Primer design considerations included: an amplicon size from 100 to 300 bp; a Tm between 50 and 65 °C; GC content between 40 and 60 °C; and a primer size between 18 and 21 bp (Roux, 1995; Singh and Kumar, 2001). Four of the selected *loci* (Fv-98, Fv-114, Fv-120 and Fv-312) matched the microsatellites reported by Xu et al. (2012). However, new primer pairs were designed for each microsatellite, and therefore the Tm and expected size of the fragments were different (Table 3).

2.3. Microsatellite validation

Sixty-two mono-conidial strains of *F. verticillioides* isolated from seed and root from different locations in Northern Mexico were used for this study (Table S1). Cross-transferability was examined using two mono-conidial isolates each of *Fusarium thapsinum* (isolates F33 and F65), *Fusarium nygamai* (isolates P01 and DA31), *Fusarium andiyazi* (isolates F116 and F133), and one isolate of *F. oxysporum* (Isolate Fol 11). *Fusarium* strains were previously identified using partial DNA sequences of the calmodulin (Mulé et al., 2004) and elongation factor 1α (O'Donnell et al., 1998) genes, which were deposited in the GenBank database (accession numbers are reported in Table S1). Isolates were grown in 125 ml Erlenmeyer flasks containing 50 ml of PD broth (BD Difco) on a shaker (180 rpm) at 25 °C. After four days, mycelia were harvested by centrifugation (15,000 *g* for 20 min), dried with sterile filter paper (Faga-lab No. 1), frozen, and ground in liquid nitrogen with a mortar and pestle. Genomic DNA was extracted from 10 mg of frozen mycelium using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol adapted for yeast. Quality and purity of genomic DNA were estimated by measuring the 260/280 nm ratio in a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) and by electrophoresis in a 1% agarose gel. DNA extracted from all samples had a 260/280 ratio ranging from 1.7 to 1.9, indicating high quality. DNA was quantified using the Quant-iT ™ dsDNA HS kit (Invitrogen). PCR was performed in a total volume of 25 μl containing 1 ng template DNA, 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 μM of each primer (forward and reverse), and 0.05 U of Taq DNA polymerase (Invitrogen). The following amplification program was used: 5 min initial denaturation at 94 °C, followed by 35 cycles of 45 s denaturation at 94 °C, 30 s annealing at the optimal Tm for each primer pair (Table 3), and 30 s extension at 72 °C. PCR was completed with a 5 min final extension at 72 °C. The same PCR conditions were used for each primer pair to amplify microsatellite loci in all tested species. PCR yield was first measured in a NanoDrop 2000c spectrophotometer prior to QIAxcel analysis. Allele amplification and visualization were then determined by capillary electrophoresis in a QIAxcel system using the QIAxcel DNA High resolution kit (Qiagen). The DNA concentration used for all samples was between 100 and 135 ng/μL, and the OH800 method was therefore selected for microsatellite analysis in the QIAxcel system. This method provides a resolution of 3 bp for amplicons between 100 and 500 bp in size. Alleles obtained from the