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Interfertility of two mating populations in the *Gibberella fujikuroi* species complex

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Abstract

Gibberella fujikuroi and *Gibberella intermedia* (mating populations 'C' and 'D' of the *G. fujikuroi* species complex) can be distinguished by differences in the spectrum of mycotoxins produced, the lack of sexual cross-fertility and diagnostic differences in their DNA sequences. Some isolates from these two biological species, however, can interbreed and complete meiosis to produce viable progeny. Analysis of marker segregation amongst such hybrid progeny can be used to estimate the degree of genomic rearrangement and genetic incompatibility that has accumulated since these sibling species diverged. Recombinant progeny were isolated from crosses of the standard tester strains for these two species and from crosses between these standard testers and a field isolate (KSU X-10626) that was cross-fertile with tester strains of both species. Progeny in all of the crosses segregated for amplified fragment length polymorphisms (AFLPs). Segregation of AFLP loci deviated from 1:1 for two thirds of the loci amongst the progeny of the cross between the 'C' and 'D' mating population tester strains, but <20% of the polymorphic loci in the cross of either tester with KSU X-10626 showed such distortion. It was concluded that *G. intermedia* and *G. fujikuroi* are sufficiently interfertile to belong to the same biological species, but that changing the nomenclature to reflect this interfertility requires more evidence for the natural occurrence of a continuum in fertility than is presently available.

Introduction

Species in the genus *Fusarium* can be defined using morphological, biological or phylogenetic species concepts (Leslie et al., 2001; Summerell et al., 2003). In many cases, these different concepts result in the same grouping of strains. The 'C' and 'D' mating populations of the *Gibberella fujikuroi* species complex, also known as *Fusarium fujikuroi* (*Gibberella fujikuroi*) and *Fusarium proliferatum* (*Gibberella intermedia*), respectively, are difficult to distinguish morphologically, and are very closely related based on the similarity of sequenced loci (O'Donnell et al., 1998). Mating population 'C' was first defined by Hsieh et al. (1977), based on isolates from rice, which often are associated with bakane disease, and commonly are assigned to *F. fujikuroi*. Tester strains for determining cross-fertility for the 'C' and 'D' mating populations were identified by Kuhlman (1982), who characterized these groups as varieties of *G. fujikuroi*. Strains of mating population 'D' have been recovered from diverse agricultural (Leslie, 1995) and non-agricultural (Leslie et al., 2004) hosts. Kuhlman (1982) did not report any cross-fertility between the tester strains of these two mating populations, and thus the two entities were raised to species rank by Samuels et al. (2001).

The possibility of cross-fertility between isolates of these two mating populations was suggested by Desjardins et al. (1997) based on the cross-fertility of field isolates from rice with reference mating

type tester strains of *F. proliferatum*. Such cross-fertility, if it occurred at a sufficiently high level, could be used to argue that these two entities are not distinct species, but should instead be separated at some sub-specific level. Additional strains that would be classified as mating population 'C' on the basis of amplified fragment length polymorphism (AFLP) fingerprints, but which were cross-fertile with mating population 'D' tester strains, have recently been identified from native grasses in the tallgrass prairie of the Konza Prairie Biological Station in Kansas (Leslie et al., 2004). The available tester strains for *G. fujikuroi* are known to be relatively poorly fertile under laboratory conditions (Kuhlman, 1982; Puhalla and Spieth, 1985; Moretti et al., 1996; Desjardins et al., 1997), and <50% of the field isolates of *G. intermedia* are fertile as female parents (Leslie and Klein, 1996). Thus, crosses based solely on field isolates might not be optimal for determining whether *G. fujikuroi* and *G. intermedia* are cross-fertile to any significant degree.

The objectives of this study were: (i) to develop highly fertile tester strains for *G. intermedia*, (ii) to determine if the available tester strains of *G. fujikuroi* were cross-fertile with the *G. intermedia* tester strains, and (iii) to assess the cross-fertility of an unusual field isolate with standard testers of both *G. fujikuroi* and *G. intermedia*. The working hypothesis was that these species were not cross-fertile and were genetically isolated from one another. This work provides a test of the utility of the biological species concept for differentiating these two groups of strains.

Materials and methods

Strains and culture conditions

The mating type tester strains available from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas, USA) for *G. fujikuroi* (FGSC 8931 (*MATC-1*) and FGSC 8932 (*MATC-2*)) and *G. intermedia* (FGSC 7615 (*MATD-1*; KSU D-04854, ATCC 201269) and FGSC 7614 (*MATD-2*; KSU D-04853, ATCC 201268)) were used for the interspecific crosses. Random ascospore progeny were collected from crosses in which KSU strain X-10626 (*MAT-1*), from a surface-sterilized stem of an asymptomatic plant of *Andropogon scoparius* (common name 'little bluestem') from the Konza Prairie Biological Station (Riley County), Kansas, USA in October, 1997, was used as the male parent and either FGSC 7614 or FGSC 8932 as the female parent. The 'D' tester strains were synthesized from a cross in which FGSC strain 7612 (*MATD-2*; KSU D-00502, ATCC 201266) collected from diseased maize in St. George (Pottawottamie County), Kansas, USA in September 1985 was used as the female parent, and FGSC strain 7613 (*MATD-1*; KSU D-02945, ATCC 201267) collected from asymptomatic sorghum in Holcomb (Grenada County), Mississippi, USA in August, 1986 (Leslie et al., 1990) was used as the male parent.

Strains were propagated vegetatively on minimal medium (Correll et al., 1987) and stored for long-term preservation as spore suspensions in 15:85 (v:v) glycerol:H_2O. Sexual crosses were made on carrot agar as described by Klittich and Leslie (1988). Single ascospores were separated from one another on slabs of water agar with a micromanipulator, allowed to germinate overnight at 25 °C, and germlings transferred to individual slants of minimal medium.

Photography protocols

Perithecia were photographed *in situ*. Asci and ascospores were mounted in water for photography. Whole perithecia were fixed in 6.5% glutaraldehyde in 100 mM sodium cacodylate buffer at pH 7.6 for 4 h at room temperature (Bullock et al., 1980), dehydrated in a graded ethanol series, and finally infiltrated and embedded in LR White resin. Sections, 1.5 μm thick, were cut with a Reichert ultramicrotome, dried onto poly-L-lysine-coated glass slides, and stained with 0.5% Toluidine Blue *O* for 10 s (Feder and O'Brien, 1968). Sections were mounted in immersion oil.

DNA extractions

Strains were cultured for DNA isolation and DNA was extracted (Kerényi et al., 1999). DNA concentrations were quantified densiometrically with an ISO-1000 digital imaging system (Alpha Innotech, San Leandro, CA) by running samples and sample dilutions on agarose gels in comparison to *Hind*III-digested bacteriophage λDNA of known concentration.

AFLP procedures

AFLPs (Vos et al., 1995) were generated as described by Zeller et al. (2000). AFLP data for the parents and the progeny of the three crosses (FGSC 8932 × FGSC 7615, KSU 10626 × FGSC 7614, and KSU 10626 × FGSC 8932) were generated for the specific primer combinations EAA/MAA and ECC/MCG. AFLP markers were scored as present/absent for all of the polymorphic bands between 100 and 800 bp in the parents and progeny of each of the three crosses.

MAT *polymorphism*

MAT idiomorphs for the progeny screened as potential tester isolates were determined in crosses in which the progeny were used as the female strains and the parents of the cross (FGSC 7612 and FGSC 7613) were used as the male strains. For the cross between FGSC 7615 and FGSC 8932 the *MAT* idiomorph was first determined in a PCR-based assay (Kerényi et al., 1999; Steenkamp et al., 2000). These initial results were confirmed either by two or more additional PCR tests, or in crosses with the mating type tester isolates of *G. intermedia* (FGSC 7614 and FGSC 7615) with the standard *G. intermedia* tester isolates used as the female parent.

FUM1 *polymorphism*

Degenerate PCR primers were designed based on the *FUM1*, also called *FUM5*, sequence (Proctor et al., 1999) from *Gibberella moniliformis* (Genebank accession number AF155773). Primer Fum-Lft1 (5′-GCNGCATATGCAGCTGGGGCC-3′) matches a conserved amino acid sequence (AAYAAGA) in the acyl transferasae (AT) domain, while primer Fum-Rgt1 (5′-GCCATNGCTAGGTAGCCCGC-3′) is the reverse complement to a conserved amino acid sequence (AGYLAMA) in the dehydratase (DH) domain of the *G. moniliformis FUM1* sequence. The region spans 323 amino acids and an intron in *G. moniliformis*, with an expected amplification product size of between 1015-1040 nucleotides.

FUM1-specific PCR reactions contained from 2–10 ng genomic DNA, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 pM of each primer and 0.025 units/μl *Taq* DNA polymerase. PCR amplification of the *FUM1* DNA fragments was done in an MJ PTC-200 thermocycler (MJ Research, Watertown, Massachusetts, USA) with the following cycling parameters: 1 cycle of 1 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 45 s at 60 °C, and 90 s at 72 °C. Positive (DNA from *G. moniliformis* strain FGSC 7600) and negative (no target DNA) controls, and DNA from the parental strains were included in each set of reactions. PCR amplification products were resolved by running aliquots of each reaction on 1% agarose (Fisher Scientific, Fair Lawn, New Jersey, USA), 1 × TAE (40 mM Tris acetate (pH 8.5) 2 mM EDTA) gels stained with ethidium bromide. Product sizes were estimated relative to a 100-bp ladder (New England Biolabs, Beverly, Massachusetts, USA). Each reaction was scored on at least two separate occasions.

Results

Synthesis of mating type tester strains of G. intermedia

Field isolates of *G. intermedia* often are poorly or intermittently fertile as female parents. Two field isolates, FGSC 7612 and FGSC 7613, that are relatively female-fertile were crossed and 49 progeny were collected. These progeny were backcrossed to both parents, with the progeny serving as the female parent of the crosses. Of the 49 progeny, 24 were female-fertile. Four of these progeny were weakly cross-fertile with both of the parents and could represent strains with weak homothallic capabilities such as those already observed for the tester strains of *Fusarium sacchari* (Britz et al., 1999). Of the remaining 20 strains, 11 were *MATD-1* and nine were *MATD-2*. Six of the *MATD-2* and two of the *MATD-1* strains were consistently female-fertile at a relatively high frequency, and one strain of each mating type was selected and deposited at FGSC to serve as the tester strains (FGSC 7614 and FGSC 7615) for *G. intermedia*.

Morphology of crosses and progeny

All three crosses were relatively poorly fertile in terms of the number of perithecia and the number of ascospores produced. The crosses involving the

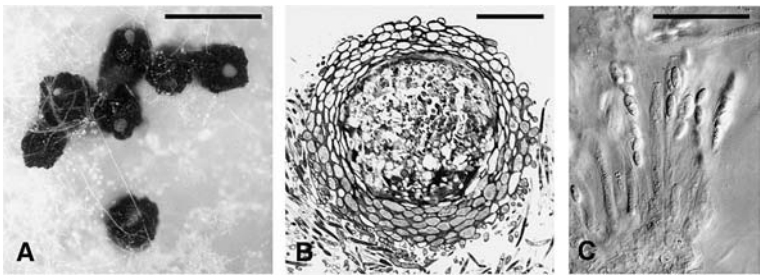

Figure 1. (A) Perithecia from the cross FGSC 8932 × KSU X-10626 three weeks after fertilization on carrot agar. Bar = 500 μm. (B) Cross-section of a perithecium from FGSC 8932 × KSU X-10626. Bar = 50 μm. (C) Asci from a cross between FGSC 8932 and KSU X-10626. Note that the asci do not contain eight mature ascospores. Bar = 50 μm.

G. fujikuroi tester strains were the least fertile, and were repeated numerous times as they often produced neither asci nor perithecia. The KSU X-10626 × FGSC 7615 cross was the most fertile. In general, the perithecia produced were typical in size and color for *Gibberella* (Figure 1A and B) and there was no observable difference in perithecial size for any of the crosses made. When asci and ascospores were produced there also were no significant differences in size or morphology, although the number of asci was reduced and these asci usually did not contain a full complement of eight viable spores (Figure 1C). Ascospore viability also was low (33–65%), but those that did germinate usually produced vegetative colonies that were morphologically similar to those produced by other strains of either *G. fujikuroi* or *G. intermedia*.

AFLP comparisons of progeny and parents

AFLPs have been used to assess genetic relationships between different species (Marasas et al., 2001; Zeller et al., 2003). Pairwise UPGMA similarity between the tester strains and KSU X-10626 ranged from 0.49 to 0.67 (Table 1). The genetic similarity of a tester strain to the complementary tester strain is relatively high (>89%), while the similarity of a tester strain from one species to that of the other species was only around 50%. KSU X-10626 was more similar to the *G. fujikuroi* tester strains (67–69% similarity) than it was to the *G. intermedia* testers (49–54% similarity). Based on these data, KSU X-10626 would be most accurately grouped with the *G. fujikuroi* testers, but this level of relatedness is near the lower limit of what has previously been reported for similarity between isolates that clearly belong to the same species.

Crosses between FGSC 7615 × FGSC 8932

Forty-seven progeny were collected from this interspecific cross and scored for 80 segregating AFLP markers. Approximately 66% (53/80) of the AFLP markers were not segregating in a 1:1 manner (Table 2). The mean frequency of the presence (AFLP band present) allele was 52%, with a range of 15–88%. The proportion of presence alleles derived from each parent was similar —*G. fujikuroi*, 54% and *G. intermedia*, 46%—and not significantly different from 1:1. The mean frequency of the allele derived from the *G. intermedia* parent amongst the progeny was 61%, with a range of 30–88%.

Table 1. Pairwise UPGMA similarity among KSU X-10626 and the *G. intermedia* and *G. fujikuroi* mating type tester strains estimated from polymorphism in banding patterns for two pairs of AFLP primers (EAA/MAA and ECC/MCG)

	FGSC 8932	FGSC 7614	FGSC 7615	KSU X-10626
FGSC 8931	0.94	0.49	0.49	0.67
FGSC 8932		0.50	0.51	0.69
FGSC 7614			0.89	0.54
FGSC 7615				0.49

Table 2. Probability that AFLP markers are segregating in a 1:1 ratio and the proportion of the segregating markers in each significance class

1:1 Segregation[a]	Percentage of segregating loci		
	FGSC 7615 × FGSC 8932	FGSC 7614 × KSU X-10626	FGSC 8932 × KSU X-10626
>5% (not significant)	34	81	83
5.0% ≥ *P* ≥ 1%	31	16	13
>1.0%	35	3	4
Total number of loci	80	70	24

[a]Probability from a χ^2 test with one degree of freedom that the segregation ratio observed for a locus in the class was 1:1 as described by Perkins (1994a).

The *FUM1* fragment amplified by PCR is found in FGSC 7615, but not in FGSC 8932. Among the progeny of this cross the presence/absence of this fragment segregates 18 *FUM1*$^+$:29 *FUM1*$^-$, which also is not significantly different from 1:1.

Based on PCR amplification of the *MAT* region from 44 of the 47 progeny, the *MAT-1*:*MAT-2* segregation ratio amongst the progeny was 23:21, which is not significantly different from 1:1. When these progeny were backcrossed to the *G. intermedia* testers, 77% (36/47) were fertile as males.

Crosses with KSU X-10626

Thirty-two progeny were collected from the cross between KSU X-10626 and FGSC 7614 and scored for 70 segregating AFLP markers. Approximately 19% (13/70) of the AFLP markers did not segregate in a 1:1 manner. The mean frequency of the presence (AFLP band present) allele was 53%, with a range of 26–79%. Fifty percent of the presence alleles were derived from each parent. The mean frequency of the allele derived from the *G. intermedia* parent amongst the progeny was 54% with a range of 26–79%.

Eighteen progeny were collected from the cross between KSU X-10626 and FGSC 8932 and scored for 24 segregating AFLP markers. Approximately 17% (4/24) of the AFLP markers were not segregating in a 1:1 manner. The mean frequency of the presence (AFLP band present) allele was 54%, with a range of 17–94%. The proportion of presence alleles derived from each parent was similar—KSU X-10626, 46% and *G. fujikuroi*, 54%—and not significantly different from 1:1. The mean frequency of the allele derived from the *G. fujikuroi* parent amongst the progeny was 56%, with a range of 29–94%.

Discussion

Gibberella fujikuroi and *G. intermedia* are difficult to distinguish morphologically, with the length of microconidial chains and the occasional presence of pyriform conidia in the aerial mycelium sometimes used as differentiating characters (Nirenberg and O'Donnell, 1998). None of the morphological characters associated with the production of perithecia, asci or ascospores suffice to differentiate these taxa. Strains assigned to these species generally differ in the ability to produce at least some secondary metabolites. For example, *G. fujikuroi* strains generally produce high levels of gibberellic acid (Tudzynski, 1999), while strains of *G. intermedia* generally produce high levels of fumonisins (Rheeder et al., 2002). Crosses between strains of these species under field conditions could result in progeny that produce unusual sets of secondary metabolites, and these progeny could make it more difficult to determine the risks these strains pose in terms of mycotoxin production. Isozymes are not adequate to separate these two groups as the patterns observed thus far for *G. fujikuroi* are a subset of the patterns observed for *G. intermedia* (Huss et al., 1996).

DNA sequences also are not a particularly powerful tool for separating *G. fujikuroi* and *G. intermedia*, whether a genome-wide measure (AFLPs or DNA reassociation kinetics) or specific DNA sequences are used. The genetic similarity based on AFLPs, approximately 50%, is intermediate between cutoffs at which the isolates

are clearly conspecific (>70%) or are clearly in different species (<40%) that have been suggested recently (Marasas et al., 2001; Zeller et al., 2003). The similarity between the tester strains of *G. fujikuroi* and *G. intermedia* is comparable to that between some of the phylogenetic lineages of *G. zeae*. Strains from the different *G. zeae* lineages are sufficiently cross-fertile for a genetic map to be made based on the segregation patterns of progeny from a cross between strains belonging to different lineages (Jurgenson et al., 2002). Ellis (1988a, b, 1989) found strains of *G. intermedia* and *G. fujikuroi* to have sufficiently high genetic similarities (>80%), based on reassociation kinetic values, to be termed conspecific. With respect to differences in sequences of cloned loci, there are differences between the species reported for β-tubulin (O'Donnell et al., 1998), *MAT-1* and *MAT-2* (Steenkamp et al., 2000), histone H3 (Steenkamp et al., 1999), and mitochondrial SSU rDNA (O'Donnell et al., 1998), but not for nuclear ribosomal DNA—ITS1, ITS2 or 28S rDNA (O'Donnell et al., 1998). The sequence differences that have been identified are usually based on a small number of strains. The utility of these sequence differences for identification of field samples remains untested.

Fertility is at the basis of biological species concepts, but is a continuum with varying degrees of fertility separating clearly fertile from clearly infertile. The crosses described in this study are sparingly fertile, as defined by Perkins (1994b), in that fewer than the normal number of ascospores is produced per perithecium. The *Gibberella* crosses are intermittently fertile in that sometimes the crosses are completely barren while at other times relatively numerous fertile perithecia are produced. Thus individual perithecia from the *Gibberella* crosses are more fertile than similar interspecific crosses within *Neurospora*, but the number of perithecia produced by the *Gibberella* crosses is lower than what is seen in the *Neurospora* crosses. Mating type appears to be segregating normally in the present crosses, but only three quarters of the progeny from the cross between the tester strains of *G. fujikuroi* and *G. intermedia* were cross-fertile with the *G. intermedia* tester strains when the progeny were used as the male parent. There may be genetic components of fertility that could be identified through more detailed studies of the progeny of this cross. Thus, the intermediate degree of fertility between the tester strains can be used to argue both for and against the hypothesis that these testers represent distinct species.

The recent identification of KSU X-10626 as an apparently naturally-occurring hybrid between *G. fujikuroi* and *G. intermedia* complicates the analysis. The existence of such a strain on a native prairie grass (Leslie et al., 2004) suggests that crosses between isolates identified as *G. fujikuroi* and *G. intermedia* have occurred under field conditions and are not simply a laboratory artifact. If the proportion of loci at which segregation is not 1:1 is taken as a measure of the genetic distance between the parental strains, then the testers for *G. fujikuroi* and *G. intermedia*, in whose intercross approximately two thirds of the loci do not segregate 1:1, are more distant from each other than either of these testers is from KSU X-10626, for which <20% of the loci are not segregating in a 1:1 manner. One interpretation of these results is that the standard testers for *G. fujikuroi* and *G. intermedia* represent distinct species, but that one, or more, hybrid swarms, perhaps separated geographically or specialized to a particular host, exist between these species. As *G. fujikuroi* normally is associated with rice and is easily confused with *G. intermedia* on the basis of morphology, the hybrid nature of many field strains might easily be missed unless relatively extensive crossing and/or molecular analyses were conducted. Indeed the entity currently identified as *G. intermedia*/*F. proliferatum*, which is known to have a broad host range and geographic distribution, could be a series of hybrid swarms that share taxonomically important morphological characters. The results of Desjardins et al. (1997) with strains of *G. intermedia* and *G. fujikuroi* from rice also are consistent with such a hybrid swarm hypothesis. The number and relatedness of such putative hybrid swarms remain important questions for further study and analysis.

In conclusion, the cross-fertility observed in the crosses analyzed and the natural occurrence of an apparent hybrid strain on a native tallgrass prairie suggests that the genetic isolation between *G. intermedia* and *G. fujikuroi* is not complete. The possible existence of hybrid swarms in either natural or agricultural ecosystems, however, prevents us from suggesting that these two species should be combined into a single entity until more data from

studies designed to detect the presence and relatedness of such hybrid swarms has been collected.

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