Evidence for recombination and segregation of virulence to pine in a hybrid cross between Gibberella circinata and G. subglutinans

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\textbf{A R T I C L E   I N F O}

Article history:
Received 8 April 2006
Received in revised form 27 February 2007
Accepted 17 May 2007
Published online 27 May 2007
Corresponding Editor: Brenda Wingfield

Keywords:
Fusarium circinatum
Pinus radiata
Pitch canker
Species concepts

\textbf{A B S T R A C T}

Two species associated with the Gibberella fujikuroi species complex, G. circinata (the cause of pitch canker in pines) and G. subglutinans (avirulent on pine), were found to have limited interfertility in hybrid crosses. MAT idiomorphs, polymorphisms in the histone H3 gene, vegetative compatibility, and virulence phenotypes were used to verify recombination. The MAT idiomorphs appeared to be assorting independently, but the histone H3 haplotype was disproportionately represented by that of the G. subglutinans parent. Ninety-eight percent (45/46) of the progeny tested were vegetatively incompatible with both parents. All F1 progeny were avirulent to pine, but a wide range of virulence was restored through a backcross to the virulent parent (G. circinata). Attempts at hybrid crosses using other isolate combinations were rarely successful (1/26). This limited interfertility supports retention of G. circinata and G. subglutinans as separate species, but offers opportunities to characterize the inheritance of virulence to pine.

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\textbf{Introduction}

The ascomycete Gibberella fujikuroi was originally described as a pathogen of rice and was later shown to be associated with the anamorphic species Fusarium moniliforme (Nirenberg 1976). It subsequently became apparent that G. fujikuroi was comprised of several intersterility groups, also known as mating populations (Puhalla & Spieth 1985). Nine mating populations (A–I) within this complex were shown to be concordant with phylogenetic groupings based on DNA sequence comparisons and were accorded specific status (Nirenberg & O’Donnell 1998; Zeller et al. 2003). However, reproductive isolation between these species is apparently not complete in all cases. For example, Leslie et al. (2004) reported that viable progeny were obtained from crosses between G. fujikuroi sensu stricto (mating population C; anamorph F. fujikuroi) and G. intermedia (mating population D; anamorph F. proliferatum). In fact, these authors concluded that interfertility between the two taxa was high enough that they could be regarded as a single biological species (Leslie et al. 2004). They also noted that G. intermedia and G. fujikuroi are morphologically very similar and both may be isolated from the same host plant.

Another example of fertility between species in the G. fujikuroi complex, involved a cross between G. circinata (mating
population H; anamorph F. circinatum) and G. subglutinans (mat-
ing population E; anamorph F. subglutinans) (Desjardins et al. 2000). In this case, fertility was relatively weak and the two spe-
cies involved appear to be well separated based on multigene
genealogies (Steenkamp et al. 2001; Nirenberg & O’Donnell 1998). Their host relationships are divergent as well, with G. circinata and G. subglutinans known as pathogens of pines
and corn, respectively (Edwards 1935; Reid et al. 2002). Thus, in-
terfertility between G. circinata and G. subglutinans seems more
likely to represent a hybridization event rather than an indica-
tion the two species fully share a common gene pool. Further-
more, the very different ecological activities of the fungi
involves suggest that genetic analysis of progeny from a hybrid
cross might yield insights into the inheritance of traits that
influence virulence and host range. With this in mind, the pres-
cent study was undertaken to: (1) use both phenotypic and
molecular markers to confirm recombination in crosses be-
 tween G. circinata and G. subglutinans; (2) examine additional
isolate combinations in order to better characterize the extent
of interfertility between the two species; and (3) assess segrega-
tion of virulence to pine among progeny of a hybrid cross.

Methods and materials

Fungus cultures

All isolates referred to in this paper are stored in the Depart-
ment of Plant Pathology, University of California at Davis,
and are available to interested parties on request.

Mating experiments

Crosses were performed as described by Desjardins et al. (2000), and these procedures are briefly outlined here. Isolates
serving as female parents were cultured on carrot agar, and
candidate male isolates were grown on potato dextrose agar
(PDA) (Difco, Detroit, MI). Both cultures were allowed to incu-
bate in a growth chamber at 25 °C light/21 °C dark with a 12 h
photoperiod until carrot agar plates were fully colonized. At
that time, a spor suspension prepared from the PDA culture
was spread over the surface of a fully colonized carrot agar
plate. Cultures thus “fertilized” were returned to the growth
chamber. After approximately four weeks, crosses were rated
as fertile when ascospores were observed to ooze from osti-
oles of mature perithecia as described by Desjardins et al. (2000).
Single ascospores, collected by crushing intact perithe-
cia that had been rinsed in sterile water to ensure removal of
any contaminating conidia, were cultured on either V8 agar or
PDA and stored on dried filter paper at 4 °C.

Vegetative compatibility tests

A nitrate non-utilizing (nit) mutant was obtained from each of
the progeny to be tested by culturing on PDA amended with
4% potassium chloride. Each nit mutant thus obtained was
paired with a complementary nit mutant derived from each of
the two parents of the cross that produced the progeny
being tested, as described by Gordon et al. (2006). Reactions
were evaluated 7–10 d later. Two strains were judged to be
vegetatively compatible when abundant aerial mycelium (in-
dicative of wild-type growth) developed in the area of contact
between them; whereas the absence of wild-type growth was
taken to indicate the strains in question were incompatible
(Gordon et al. 1996).

Molecular markers

Fungi were grown in potato dextrose broth (Difco) in 500 ml
flasks on a rotatory shaker (ca 200 rev min⁻¹) at room temper-
ature (22 ± 2 °C). After one week, mycelia were filtered, rinsed
in sterile, deionized water, lyophilized, and powdering using
liquid nitrogen. Approximately 25 mg powdered mycelium
was added to 450 µl extraction buffer [0.25 M NaCl, 0.017 M
SDS, 0.2 M TRIS (pH 8), 0.025 M EDTA (pH 8.0)] and 4 µl RNase
A (100 mg ml⁻¹; Qiagen, Germantown, MD). These ingredients
were mixed and incubated at 65 °C for 10 min before adding
250 µl 3 M sodium acetate pH 5.2. This mixture was vortexed
for 5 s and centrifuged at 12,000 rev min⁻¹ (15,300 g) for 10 min.
The liquid phase was removed and DNA was precipi-
tated in 500 µl 2-propanol, centrifuged at 12,000 rev min⁻¹
(15,300 g) for 10 min and washed in 500 µl of 70 % ethanol.
The pellet was dissolved in 40 µl TE buffer and this served as
a source of template for DNA amplifications using PCR, as
described below.

A duplex reaction was used to assay for the MAT1 and MAT2
idiomorphs. The reaction mixture contained 2 µM MAT1 and
MAT2 primers (Steenkamp et al. 2000), 2.5 mM MgCl₂, 0.05 U µl⁻¹
Hotstar Taq DNA polymerase (Qiagen), 1 mM dNTP (0.25 mM of each), 1 mM 10× buffer (Qiagen) and 2 µl DNA tem-
plate in a total volume of 25 µl. Reactions were initiated with
denaturation at 95 °C for 15 min, followed by 25 cycles of 92
°C for 30 s, 56 °C for 30 s and 72 °C for 45 s. A final extension
was performed at 70 °C for 5 min. Fragments were resolved
on 0.9 % agarose gels in 1 % TAE buffer. MAT1 and MAT2 ampli-
cons were identified as 700 and 250 bp bands, respectively.

Polymorphisms in the histone H3 gene were determined as
described by Steenkamp et al. (1999) with minor modifications.
DNA was extracted as described above and 2 µl of the extract
served as the template for amplification using PCR, as de-
scribed by Steenkamp et al. (1999), except that total reaction
volume was 35 µl. A double restriction was performed using
Dde1 and Hha1, an isoschizomer of Cfo1 (New England Biolabs;
NEB, Ipswich, MA). Each reaction had a total volume of 20 µl
and contained 5 U of each restriction enzyme, 1 µl 100× bovine
serum albumen (BSA; NEB), 3.25 µl Buffer1 (NEB) and 15 µl PCR
product. The reaction was incubated at 37 °C for 4 h. The unique
PCR-RFLP profiles associated with G. circinata and G. subglutinans
were visualized as described by Steenkamp et al. (1999).

Virulence assays

Progeny were tested for virulence by inoculating three to four-
year-old Monterey pines (Pinus radiata). Trees were main-
tained in a growth chamber with a diurnal cycle of 12 h light
at 22 °C and 12 h dark at 18 °C. Each tree was inoculated
once, by making a shallow wound in the main stem with a 1.6 mm drill bit and depositing therein approximately 250
spores suspended in 5 µl of 0.5 % potassium chloride. Each of
the tested progeny was inoculated separately into three different trees. For comparison, three trees were also inoculated with the virulent parent (GL 343). After an incubation period of 28 d, the bark was removed and the lesion length at each inoculation site was measured.

**Results**

*Gibberella circinata* (isolate GL 343) was crossed twice as a male to a female fertile strain of *G. subglutinans* (isolate GL 52 = Fst 51, Desjardins et al. 2000). GL 343 carries the MAT1 allele at the mating type locus, whereas GL 52 has the MAT2 allele. The crosses produced a modest number of fertile perithecia. Two hundred and seventy-seven ascospores were collected, up to 23 from each of 19 perithecia (12 perithecia from the first cross, seven from the second); mean ascospore viability, averaged across the sampled perithecia, was 77 % (± 24 %). Thirteen of these F1 progeny, representing four different perithecia (GL 343 × GL 52), were confirmed to be the opposite mating type of GL 52 (as described below), to which they were backcrossed as males. None of these backcrosses produced fertile perithecia. Three F1 progeny of the appropriate mating type were backcrossed to GL 343 (female parent) and all produced a modest number of fertile perithecia. From each of the backcrosses to GL 343, 22 ascospores were collected from a single perithecium (66 ascospores, all of which were further characterized as described below.

Of 178 F1 progeny from the two GL 343 × GL 52 crosses described above, 50.6 % (90/178) were found to be MAT1 (Table 1). This result suggests the MAT idiomorphs were assorting independently and segregating in a 1:1 ratio through recombination (χ² = 3 × 10⁻⁴, P > 0.99). One hundred and thirty-three of these same 178 progeny (75 %) had a banding pattern corresponding to the histone H3 gene sequence of the *G. subglutinans* parent (Gs haplotype), suggesting a bias in the hybrid progeny as compared to the histone H3 gene sequence of the *G. circinata* parent (C1, C2) and thus were judged to be avirulent. However, these lesions were distinctly different in appearance from those induced by *G. circinata*. The affected tissue was only slightly darkened and was not resinous. This suggested that the discoloration might reflect damage to the host that was independent of the inoculation. Consequently, these five F1 progeny were re-tested, and all produced lesions, only 2–3 mm in length, that were similar in appearance to those produced by the avirulent parent. Fourteen progeny from the second successful cross (GL 318 × GL 52) were also tested and found to produce only short lesions: 2.9 ± 0.9 mm (n = 56 inoculations) and thus were judged to be avirulent.

A subset of the progeny collected from GL 343 × F1 backcrosses that were confirmed as recombinants, were shown

<table>
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<tr>
<th>Table 1 – Summary of crosses and characterization of progeny</th>
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<tr>
<td>Crossa (♀ × ♂)</td>
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<tr>
<td>GL 52 × GL 343 (178)</td>
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<td>GL 52 × GL 318 (14)</td>
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<td>GL 343 × GL 680 (F1) (22)</td>
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<td>GL 343 × GL 681 (F1) (22)</td>
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<td>GL 343 × GL 682 (F1) (22)</td>
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</table>

**a** The male and female parents of the cross, with the number of progeny evaluated given in parentheses.

**b** The pattern of restriction digest products associated with the histone H3 gene sequence of the *Gibberella* species indicated.

**c** Recombination is inferred from an assessment of two loci: histone H3 haplotype and MAT idiomorph.
were confirmed as recombinants. However, the complete
and vegetative compatibility) 63.5% (113/178) of the progeny
cating they constituted recombinant genotypes. Considering
addition, 50% of progeny were avirulent and were associated
with the MAT idiomorph of the virulent parent (MAT1), indi-
tions of MAT idiomorphs and histone H3 gene haplotypes. In
Pinus radiata
margin defined by callus tissue. Both were inoculated on
G. subglutinans
by inoculation with
G. circinata
695) induced lesions similar in length to those produced by
mean lesion lengths
Fig 2 – Mean lesion lengths induced in
Pinus radiata
by progeny of a backcross, and both parents of the cross
(GL 343 × GL 680). Error bars correspond to the standard
error of the mean.

Discussion

The initial report on the cross between Gibberella circinata and
G. subglutinans showed a subset of progeny to be vegetatively
incompatible with both parents (Desjardins et al. 2000). The present study confirmed this indication of recombination by
showing that 34% of F1 progeny had non-parental combina-
tions of MAT idiomorphs and histone H3 gene haplotypes. In
addition, 50% of progeny were avirulent and were associated
with the MAT idiomorph of the virulent parent (MAT1), indic-
cating they constituted recombinant genotypes. Considering
all four markers (MAT locus, histone H3 haplotype, virulence,
and vegetative compatibility) 63.5% (113/178) of the progeny
were confirmed as recombinants. However, the complete
absence of virulent progeny in the F1 generation implies
a bias towards the genome of the G. subglutinans (avirulent)
parent. Although a higher density of markers would be re-
quired to confirm the relative contributions of each parent,
our results may be indicative of unbalanced hybrid genomes
due to genetic divergence of the parents, as described by
Kile & Brasier (1990) for crosses between Ophiostoma ulmi and
O. novo-ulmi.

Interfertility within the G. fujikuroi species complex has
previously been documented by crosses between G. fujikuroi
and G. intermedia. Whereas that result was taken to indicate
the two taxa may in fact represent a single species (Leslie
et al. 2004), there remains abundant support for retaining
specific status for G. circinata and G. subglutinans. The crosses
between G. circinata and G. subglutinans that we report were
characterized by low fertility and moderate ascospore viabil-
ity, as would be expected for a hybrid cross. Furthermore, in-
terspecific fertility was the exception, with successful crosses
occurring in only two of 27 combinations tested and then only
with G. circinata as the male parent. One of these combina-
tions, GL 318 × GL 52 produced only a single fertile perithecium.

Hybrid crosses were limited, in part, by female fertility.
Although both parents were functionally hermaphroditic in
intraspecific matings (Desjardins et al. 2000), only G. subglu-
tinans was female fertile in hybrid crosses. F2 crosses were
generally successful with either parent serving as the female
data not shown), but backcrosses beyond the initial cross of
F1 progeny to G. circinata did not succeed and repeated at-
ttempts to cross progeny of the GL 343 × F1 backcross among
themselves also failed. Finally, 14 mating combinations of
backcross progeny and the avirulent parent (G. subglutinans)
proved to be infertile (data not shown).

There is no overlap in the host ranges reported for G. subglu-
tinans and G. circinata, with G. circinata being a pathogen of pine
and G. subglutinans known principally as a pathogen of corn. As
reported by Desjardins et al. (2000), G. subglutinans is also asso-
ciated with teosinte, although the nature of this relationship
has not been explored. Our inoculation tests showed that
G. subglutinans from teosinte had a very limited ability to colo-
nize pines when spores were placed in wounded tissue. The
fungus was recoverable from lesions, suggesting it could sur-
vive in pines at least for the 28 d incubation period, but survival
beyond that time was not tested. The toxicity of a constitutive
resin component to G. subglutinans also indicates that it is
poorly adapted for growth on pines (Friel & Gordon 2005).

All F1 progeny tested produced lesions averaging less than
5 mm, and most were similar in appearance to those induced
by the avirulent parent, in being confined by callus tissue. However,
all three backcrosses of F1 progeny to G. circinata
produced offspring that revealed a wide range of virulence
to pine. These results suggest that more than one gene con-
tributes to the virulent phenotype and that genomes of the
F1 progeny were likely unbalanced, with an incomplete repre-
sentation of the genes required for virulence. The complete
absence of virulent F1 progeny is somewhat surprising and
we can only speculate that this reflects a very low probability
of finding a sufficiently high proportion of the G. circinatum ge-
nome in viable progeny of this hybrid cross.

Interfertility between G. subglutinans and G. circinata could
be an indication of recent divergence from a common
ancestor. However, phylogenetic analyses suggest they are not sister taxa (O'Donnell et al. 1998; Steenkamp et al. 2001) and that G. subglutinans (anamorph Fusarium subglutinans) is more closely related to F. bactridioides than to G. circinata. Thus, the ability of G. subglutinans and G. circinata to cross may overstate their evolutionary proximity. Retention of fertility between phylogenetically distinct species in the G. fujikuroi complex is also indicated by the detection of distinct lineages within G. subglutinans s. str. by Steenkamp et al. (2002), which these authors regarded as a case of cryptic speciation.

The ability of G. circinata to colonize maize and other grasses that are host to G. subglutinans has not been characterized, but G. subglutinans appears ill-adapted to growth on pine. As such, it seems unlikely that either teosinte or maize serve as contemporary reservoirs of inoculum for pine. In principle, genes could be transferred between G. circinata and G. subglutinans by introgressive hybridization, but clear phylogenetic separations between the two species and low fertility of the hybrid crosses suggest this has been at most an infrequent occurrence.

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