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Fusarium subglutinans f. sp. *pini* Represents a Distinct Mating Population in the *Gibberella fujikuroi* Species Complex†

H. BRITZ,[1]* T. A. COUTINHO,[1] M. J. WINGFIELD,[1] W. F. O. MARASAS,[2] T. R. GORDON,[3] AND J. F. LESLIE[4]

Forestry and Agricultural Biotechnology Institute, Faculty of Biological and Agricultural Sciences, University of Pretoria, Pretoria, 0002,[1] and PROMEC, Medical Research Council, Tygerberg 7505,[2] South Africa; Department of Plant Pathology, University of California, Davis, California 95616[3]; and Department of Plant Pathology, Kansas State University, Manhattan, Kansas 66506-5502[4]

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***Fusarium* strains in the *Gibberella fujikuroi* species complex cause diseases on a variety of economically important plants. One of these diseases, pitch canker of *Pinus* spp., is caused by strains identified as *Fusarium subglutinans* f. sp. *pini*. Fertile crosses were detected between *F. subglutinans* f. sp. *pini* strains from South Africa, California, and Florida. *F. subglutinans* f. sp. *pini* strains were not cross-fertile with the standard tester strains of six of the seven other mating populations of *G. fujikuroi*. Sporadic perithecia with ascospores were obtained in two crosses with the mating population B tester strains. These perithecia were homothallic, and the ascospores derived from these perithecia were vegetatively compatible with the mating population B tester strain parent. We concluded that fertile *F. subglutinans* f. sp. *pini* isolates represent a new mating population (mating population H) of *G. fujikuroi* and that they belong to a unique biological species in a distinct taxon.**

There are a variety of reproductive systems in ascomycetous fungi, including both homothallism and heterothallism (8). In heterothallic ascomycetes, individuals belonging to the same biological species may be sexually fertile; i.e., they may belong to the same mating population and cross to produce viable offspring. Fertility has been used to distinguish seven mating populations in the *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura species complex (11, 12, 17, 19). The *G. fujikuroi* mating populations (designated mating populations A to G) encompass several heterothallic *Fusarium* species in the section *Liseola*, including *Fusarium moniliforme* Sheldon (synonym, *Fusarium verticillioides* (Sacc.) Nirenberg), *Fusarium proliferatum* (Matsushima) Nirenberg, *Fusarium subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas, *Fusarium nygamai* Burgess & Trimboli, and *Fusarium thapsinum* Klittich, Leslie, Nelson & Marasas (12, 14, 17, 19, 24). So far, two teleomorphs have been associated with strains of *F. subglutinans*, mating populations B and E of *G. fujikuroi* (17, 19). Isolates that are morphologically consistent with the *F. subglutinans* description but are not fertile in crosses with standard tester strains of these two mating populations also are known. Thus, identification of additional mating populations associated with the *F. subglutinans* anamorph should be expected. In practice, the mating populations are closely related biological species that can be distinguished on the basis of the infertility of isolates belonging to different mating populations (28).

Recently, O'Donnell et al. (27) recognized 36 *Fusarium* taxa in the *G. fujikuroi* species complex on the basis of molecular and morphological characteristics and described 12 of these taxa as new species (25, 26). For one of these species, *Fusarium circinatum* Nirenberg & O'Donnell, the teleomorph, *Gibberella circinata* Nirenberg & O'Donnell, was also described (25). However, the authors included no data which indicated that strains of *G. circinata* were reproductively isolated from other *G. fujikuroi* strains and thus constitute a distinct mating population, as has been the case with other recently described species in the *G. fujikuroi* complex (12, 14).

The morphological species *F. subglutinans* occurs on a variety of host plants, including maize (*Zea mays*), mango (*Mangifera indica*), pine (*Pinus* spp.), pineapple (*Ananas comosus*), and sugarcane (*Saccharum officinarum*) (1, 10, 16, 31, 32). Nirenberg and O'Donnell (25) assigned the name *Fusarium sacchari* (Butler) W. Gams to *F. subglutinans* strains from sugarcane, the name *Fusarium guttiforme* Nirenberg & O'Donnell to *F. subglutinans* f. sp. *ananas* Ventyra, Zambolim & Gilb. strains from pineapple, and the name *F. circinatum* to the causal agent of pitch canker of pines, *F. subglutinans* (Wollenweber & Reinking) Nelson et al. f. sp. *pini* Correll et al.

The pitch canker fungus is found in the United States, Haiti, Japan, and Mexico (4, 7, 10) and is a serious pathogen of pine seedlings in South Africa (33–37). One isolate of *F. subglutinans* f. sp. *pini* (ATCC 38479) has been reported to be sexually fertile with mating tester strains of the mating population B (15), but Correll et al. (3) and Viljoen et al. (33) were not able to repeat this cross and questioned the validity of this observation. Sexual cross-fertility was not observed between *F. subglutinans* f. sp. *pini* isolates from California and Florida (3). However, fertile crosses were reported among *F. subglutinans* f. sp. *pini* isolates from South Africa, and appropriate tester strains have been selected (6, 33). Isolates from this collection were among the isolates used by Nirenberg and O'Donnell (25) and O'Donnell et al. (27) to describe *F. circinatum* and *G. circinata*.

Our objectives in this study were (i) to determine whether field isolates of *F. subglutinans* f. sp. *pini* belong to a new or previously described mating population of *G. fujikuroi* and (ii) to determine whether *F. subglutinans* f. sp. *pini* isolates from California and Florida are cross-fertile with the South African tester strains.

* Corresponding author. Mailing address: Forestry and Agricultural Biotechnology Institute, Faculty of Biological and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa. Phone: 27-12-420-4111. Fax: 27-12-420-3960. E-mail: Henriette.Britz@fabi.up.ac.za.

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TABLE 1. Mating types and references for previously described strains used in this study

Strain[a]	Other designation(s)[b]	Mating population[c]	Reference(s)
MRC 6213		H^+	2, 33, 36
MRC 7488		H^-	2
MRC 6209	BBA 69854	H^+	25, 27, 34, 36
MRC 7454	BBA 69722	H^+	25, 27
MRC 6191	KSU 00999, FGSC 7603, M-3703, ATCC 201261	A^+	13, 17, 19, 22, 27
MRC 6155	KSU 0149, M-3125, FGSC 7600	A^-	3, 13, 17, 19, 22, 27
MRC 6524	KSU 03852, FGSC 7610, ATCC 201264, M-6865	B^+	3, 19, 22, 27, 33
MRC 6525	KSU 03853, FGSC 7611, ATCC 210265, M-6866	B^-	3, 19, 22, 27, 33
MRC 6570	KSU 04921	C^+	19, 22, 27
MRC 6571	KSU 04922	C^-	19, 22, 27
MRC 6568	KSU 04853, FGSC 7614, ATCC 201268	D^+	19, 22, 27
MRC 6569	KSU 04854, FGSC 7615, ATCC 201269	D^-	19, 22, 27
MRC 6512	KSU 02192, M-3693, FGSC 7617	E^+	3, 17, 19, 22, 27, 33
MRC 6483	KSU 00990, M-3696, BBA 65921, FGSC 7616	E^-	3, 17, 19, 22, 25, 27, 33
MRC 6536	KSU 04092, FGSC 7055, ATCC 200520	F^+	14, 19, 22, 25, 27
MRC 6537	KSU 04093, FGSC 7056, ATCC 200521	F^-	14, 19, 22, 25, 27

[a] MRC, Medical Research Council Culture Collection at PROMEC, Tygerberg, South Africa.
[b] KSU, Department of Plant Pathology, Kansas State University, Manhattan; BBA, Biologische Bundesanstalt für Land- and Forstwirtschaft, Berlin, Germany; M, Fusarium Research Center, The Pennsylvania State University, University Park; FGSC, Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Kans.; ATCC, American Type Culture Collection, Rockville, Md.
[c] The mating population (letter) and the mating type (plus or minus) are indicated.

MATERIALS AND METHODS

Cultures. *F. subglutinans* f. sp. *pini* strains were isolated from *Pinus* spp. (Tables 1 and 2) and were pathogenic to *Pinus patula* seedlings (3, 37). South African *F. subglutinans* f. sp. *pini* isolates were obtained from a single nursery in 1990 (37). Representatives of the South African *F. subglutinans* f. sp. *pini* isolates have been deposited in the culture collection of the Medical Research Council (MRC), Tygerberg, South Africa, and duplicates are stored in glycerol-water (15:85) at −70°C in the *Fusarium* culture collection of the Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

Sexual fertility. Crosses were made on carrot agar as described by Klittich and Leslie (13), except that 300 g of fresh carrots was used per liter of medium rather than 400 g. All crosses that were considered positive were successful on at least two different occasions. Crosses were examined weekly and were considered positive when ascospores were observed exuding from perithecia. Reciprocal crosses, in which the male and female roles of the strains were reversed, were made whenever fertile crosses were obtained. Strains that were fertile only as males were designated female sterile, while strains that could serve as either the male parent or the female parent were designated hermaphrodites (Table 2).

Crosses were retained for up to 6 weeks after fertilization. Developing perithecia were usually evident 1 to 2 weeks after fertilization, and the ascospore cirrhus usually could be detected 1 to 3 weeks after perithecium formation was observed. Ascospore viability was determined by streaking a portion of an ascospore cirrhus onto 2% water agar (20 g of agar per liter of distilled water) and estimating the percentage of germination after 24 h. The level of ascospore viability was 85% or greater in crosses with the tester strains.

Mating types were designated H^+ and H^-, where the H indicates the mating population and the plus and minus signs indicate the mating type, similar to the terminology of Leslie (17, 19). The plus and minus signs were assigned arbitrarily and were not necessarily indicative of molecular similarity to other plus and minus alleles in the other mating populations of the *G. fujikuroi* species complex.

Vegetative compatibility. Vegetative compatibility tests were used to determine the thallism of putative crosses. Non-nitrate-utilizing (*nit*) mutants were generated from the parents and eight of the progeny of each putative cross by using standard protocols (5). Mutants belonging to different phenotypic classes (*nit1*, *nit3*, and NitM) were paired to determine the vegetative compatibility and the thallism of the putative crosses. When pairs of *nit* mutants formed a distinct line of prototrophic growth, they were considered vegetatively compatible and, therefore, members of the same vegetative compatibility group (VCG).

We used VCGs to determine if the ascospore progeny originated from a heterothallic cross or a homothallic cross. The parents of the crosses analyzed were members of different VCGs, which means that they must have had different alleles at least one *vic* locus (18). If at least one of the ascospore progeny was not in the same VCG as either of the parents, then we concluded that the progeny resulted from a heterothallic cross. If all of the progeny were in the same VCG as one of the parents, then we concluded that the cross was homothallic, since with at least eight progeny analyzed per cross the probability that all of the progeny would be in the same VCG as either parent was $(0.5)^7$ (i.e., 0.0078).

RESULTS

All isolates of *F. subglutinans* f. sp. *pini* were used as males in the crosses with the mating population B and E standard tester strains (MRC 6483, MRC 6512, MRC 6524, and MRC 6525). Additionally, the H^+ and H^- tester strains were crossed

TABLE 2. Characteristics of *F. subglutinans* f. sp. *pini* strains used in this study

Geographic origin, mating type, and female fertility (no. of strains)	MRC strain numbers[a]
South Africa, hermaphrodites, H^+ (13)	6213, 7448, 7462, 7464, 7469, 7471, 7483, 7487, 7490, 7494, 7501, 7502, 7503
South Africa, hermaphrodites, H^- (4)	7452, 7470, 7484, 7488
South Africa, female sterile, H^+ (21)	6208, 6209, 6217, 7449, 7454, 7456, 7457, 7459, 7461, 7466, 7467, 7476, 7477, 7478, 7482, 7489, 7493, 7495, 7497, 7498, 7500
South Africa, female sterile, H^- (25)	7445, 7446, 7447, 7450, 7451, 7453, 7455, 7458, 7460, 7463, 7465, 7468, 7472, 7473, 7474, 7475, 7479, 7480, 7481, 7485, 7486, 7491, 7492, 7496, 7499
California, hermaphrodites, H^+ (2)	7506 (FSP 52), 7507 (FSP 75)
California, hermaphrodites, H^- (1)	6229
California, female sterile, H^+ (1)	7505 (FSP 48)
California, female sterile, H^- (2)	7504 (FSP 14), 7508 (FSP 90)
Florida, hermaphrodites, H^- (2)	7439 (FL 15), 7510 (FL 17)
Florida, female sterile, H^+ (2)	7437 (FL 103), 7512 (FL 27)
Florida, female sterile, H^- (3)	7509 (FL 3), 7511 (FL 19), 7513 (FL 58)

[a] The FL and FSP designations are from T. R. Gordon, University of California-Davis.

with the standard tester strains of mating populations A, C, D, and F of *G. fujikuroi*. Most of these crosses did not produce perithecia; there was only one exception (see below).

All isolates of *F. subglutinans* f. sp. *pini* were crossed with each other in all possible pairwise combinations. Two South African hermaphrodites having opposite mating types, MRC 6213 (H^+) and MRC 7488 (H^-), were selected as standard tester strains based on the number of perithecia exuding ascospores. Of the 80 South African *F. subglutinans* f. sp. *pini* isolates, 64 were fertile in crosses with one of the two tester strains. Twenty *F. subglutinans* f. sp. *pini* isolates from Florida were crossed in all possible pairwise combinations, but only two fertile crosses occurred (MRC 7439 × MRC 7512 and MRC 7439 × MRC 7437). Six of these 20 strains (MRC 7439, MRC 7509, MRC 7510, MRC 7511, MRC 7512, and MRC 7513) could cross with one of the two South African tester strains. Thirty *F. subglutinans* f. sp. *pini* isolates from California were crossed in all possible pairwise combinations, but no fertile crosses were detected. Five of these 30 strains (MRC 7504, MRC 7505, MRC 7506, MRC 7507, and MRC 7508) could cross with one of the South African tester strains. At least one strain of each mating type was obtained from each geographical region.

We used vegetative compatibility to analyze ascospore progeny from two crosses, MRC 6213 × MRC 7488 and MRC 6213 × MRC 7460, to determine if *F. subglutinans* f. sp. *pini* reproduces heterothallically. We analyzed eight progeny from each of these two crosses. Fifteen of these 16 progeny were not vegetatively compatible with the parental strains; one of the progeny from the MRC 6213 × MRC 7488 cross was in the same VCG as the MRC 6213 parent. We concluded that both of these crosses were heterothallic and that heterothallic outcrossing is the common reproductive mode in this biological species.

We also observed two unexpected putative crosses. In 2 of 10 replicate plates of MRC 6525 × MRC 6512 and 3 of 10 replicate plates of MRC 6524 × MRC 7460, sporadic perithecia exuding viable ascospores were seen. Eight progeny were recovered from each cross, and in each case the progeny were vegetatively compatible with the mating population B tester strain that served as the parent in the putative cross. The four parental strains all belonged to different VCGs. We concluded that these perithecia were the result of homothallism in the mating population B tester strains and did not result from heterothallic crosses between strains belonging to two different mating populations.

DISCUSSION

Our results showed that *F. subglutinans* f. sp. *pini* represents a unique biological species in the *G. fujikuroi* species complex, which we designate mating population H. We identified heterothallic strains that were fertile with other members of this biological species but that were not cross-fertile with representatives of the other mating populations in the *G. fujikuroi* species complex. Isolates belonging to mating populations B, E, and H represent distinct biological species with anamorphs in *F. subglutinans* that can be distinguished on the basis of their sexual outcrossing abilities. Members of these mating populations have also been reported to occur primarily on different hosts (15, 17, 19, 35); mating population B strains occur on sugarcane, mating population E strains occur on maize, and mating population H strains occur on *Pinus* spp.

The status of *F. subglutinans* f. sp. *pini* as a distinct mating population has been open to question due to inconsistent reports of fertility by various investigators. Kuhlman (15) reported that strains of *F. subglutinans* f. sp. *pini* could cross with tester strains of *G. fujikuroi* var. *subglutinans*, which is also designated mating population B (11, 17, 19). Neither Correll et al. (3) nor Viljoen et al. (33), however, were able to repeat these experiments, and these authors questioned Kuhlman's results. Our results provide an explanation for the results of all three sets of researchers. We observed sporadic perithecia in putative crosses between strains belonging to mating population B and representatives of mating populations E and H. Such perithecia could explain the fertile crosses observed by Kuhlman (15) and could explain his conclusion that the isolates obtained from pine belonged to mating population B. Initially, we interpreted the putative crosses that we observed to mean that members of mating populations B, E, and H could occasionally intercross and produce viable perithecia in a manner similar to that seen for different *Neurospora* species (28, 29). We tested this hypothesis by analyzing random ascospores from the sporadic perithecia. We found that all of the progeny from both putative crosses were vegetatively compatible with the mating population B parent of the putative cross and concluded that the perithecia and ascospores resulted from homothallic reproduction, as previously described by Marasas (24) and Leslie et al. (20). Thus, mating population B has a mixed reproductive system that is both heterothallic and homothallic and resembles the reproductive system of *Cryphonectria parasitica* (23, 30). In cases in which crosses with mating population B tester strains occasionally produce sporadic perithecia, it is critical to confirm the recombinant nature of the progeny before concluding that a sexual cross has occurred.

Nirenberg and O'Donnell (25) described a new species, *F. circinatum* with the teleomorph *G. circinata*, by using four *Fusarium* strains isolated from pine. Two of the four strains which these authors used, MRC 7454 and MRC 6209, were included in our study, but the other two strains, including the ex-holotype strain of *F. circinatum* (BBA 69720), were not. The names *F. circinatum* and *G. circinata* may be synonymous with *F. subglutinans* f. sp. *pini* and the reproductively isolated biological species identified as *G. fujikuroi* mating population H. However, the distinguishing morphological characteristics identified thus far for *F. circinatum* (25) appear to be inadequate to distinguish this taxon from other strains of *F. subglutinans* sensu lato. These characteristics are critical because many strains of *F. subglutinans* f. sp. *pini* are not cross-fertile with our tester strains and, at present, can be identified reliably only on the basis of their pathogenicity. Also, from the description of the *G. circinata* material deposited as the holotype, it is not clear whether the perithecia described are the result of homothallic or heterothallic reproduction. Furthermore, there has been no evidence which shows that strains of the newly described species are reproductively isolated from other *Fusarium* biological species with teleomorphs in the *G. fujikuroi* species complex. We believe that a much more extensive effort will be required to determine if the species descriptions provided by Nirenberg and O'Donnell (25) are sufficient to encompass all of the strains in our extensive, biologically cohesive, global set of *F. subglutinans* f. sp. *pini* cultures.

The relative importance of sexual reproduction in the natural history of *F. subglutinans* f. sp. *pini* is unclear. Britz et al. (2) found that the effective population number (N_e) was 42 to 46% of the total number of strains. The N_e for the California and Florida populations is even lower since female hermaphroditic strains are rare (<7%) in these populations. Indeed, the N_e for the California and Florida populations may be lower than the N_e for any of the other populations of strains within the *G. fujikuroi* species complex studied thus far (2, 21, 22). These

results are consistent with the hypothesis that *F. subglutinans* f. sp. *pini* is a relatively recently introduced pathogen in both California and South Africa and that it reproduces primarily asexually (9). It is possible that the strains obtained from pine were derived from strains found in either a native or agricultural setting that have adapted to *Pinus* spp. Identifying the native population from which the *F. subglutinans* f. sp. *pini* isolates were derived remains critical to understanding pitch canker of *Pinus* spp. and its potential to spread beyond *Pinus* spp., upon which it is already known to be capable of inflicting significant damage.

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