ELSEVIER

British Mycological Society promoting fungal science

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

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

Christopher J. FRIEL[a], *Anne E. DESJARDINS*[b], *Sharon C. KIRKPATRICK*[a], *Thomas R. GORDON*[a,*]

[a]*Department of Plant Pathology, University of California, Davis 95616, USA*
[b]*USDA ARS 1815 North University Street, Peoria, Illinois 61604, USA*

ARTICLE INFO

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

ABSTRACT

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 F_1 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.

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

* Corresponding author.
E-mail address: trgordon@ucdavis.edu
0953-7562/$ – see front matter © 2007 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2007.05.004

population H; anamorph *F. circinatum*) and *G. subglutinans* (mating population E; anamorph *F. subglutinans*) (Desjardins *et al.* 2000). In this case, fertility was relatively weak and the two species 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, interfertility between *G. circinata* and *G. subglutinans* seems more likely to represent a hybridization event rather than an indication the two species fully share a common gene pool. Furthermore, the very different ecological activities of the fungi involved 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 present study was undertaken to: (1) use both phenotypic and molecular markers to confirm recombination in crosses between *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 segregation 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 Department 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 incubate 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 spore 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 ostioles of mature perithecia as described by Desjardins *et al.* (2000). Single ascospores, collected by crushing intact perithecia 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 chlorate. 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 (indicative 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 temperature (22 ± 2 °C). After one week, mycelia were filtered, rinsed in sterile, deionized water, lyophilized, and powdered 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 precipitated in 500 µl 2-propanol, centrifuged at 12,000 rev min^{-11} (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 $MgCl_2$, 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 template 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 amplicons 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 described by Steenkamp *et al.* (1999), except that total reaction volume was 35 µl. A double restriction was performed using *Dde*1 and *Hha*1, an isoschizimer of *Cfo*1 (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 Buffer3 (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 maintained 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 F_1 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 F_1 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 F_1 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 ($\chi^2 = 3 \times 10^{-4}$, $P > 0.99$). 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 toward this parent. Based on these two markers, 34 % (60/178) of the F_1 progeny represented recombinants (Table 1).

Forty-six randomly selected F_1 progeny were tested for vegetative compatibility with both parents. One gave a positive pairing reaction with the *G. subglutinans* parent and the remaining 45 were incompatible with both parents and therefore represented non-parental (recombinant) genotypes. Of these, 20 had not been confirmed as recombinants based on the two molecular markers described above.

To further asses the extent of fertility between the two species, 26 additional isolates of *G. circinata* (all collected in California) were tested for fertility with isolate GL 52 of *G. subglutinans*. All 26 *G. circinata* isolates were associated with the same vegetative compatibility group (VCG) as GL 343, C1, and all were determined to carry the MAT1 idiomorph. No mating combinations with *G. subglutinans* as the male parent produced fertile perithecia. With *G. circinata* as the male, 25 combinations failed to produce fertile perithecia, but a single perithecium was obtained from a cross between GL 318 (*G. circinata*) and GL 52. Fourteen ascospores were recovered, of which 63 % (9/14) were MAT2, and 14 (100 %) had the Gs haplotype; 36 % (5/14) of these represented recombinant genotypes (Table 1). Finally, 66 progeny collected from three backcrosses (GL 343 × F_1) were characterized, and recombinant progeny were identified in all cases (Table 1).

The average lesion length produced by the virulent parent (GL 343) on inoculated pines was 19.4 ± 6.3 mm ($n = 15$); lesions produced by *G. subglutinans* averaged 3.3 ± 0.4 mm ($n = 9$). The lesions induced by *G. subglutinans* typically had a distinct ring of callus tissue around the wound, suggesting they were effectively contained by a host response; they also lacked the dark discolouration and resin-soaking characteristic of lesions made by *G. circinata* (Fig 1). The average lesion length produced by 178 F_1 progeny of the GL 343 × GL 52 cross was 3.5 ± 1.9 mm ($n = 534$ inoculations); many were no bigger than the wound made for the inoculation (2 mm). Although most progeny were clearly avirulent, five induced lesions of 8 mm or longer on at least one inoculated tree. 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 discolouration might reflect damage to the host that was independent of the inoculation. Consequently, these five F_1 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 × F_1 backcrosses that were confirmed as recombinants, were shown

Table 1 – Summary of crosses and characterization of progeny

Cross[a] (♀ × ♂)	MAT 1 idiomorph (%)	MAT 2 idiomorph (%)	*Gibberella subglutinans* haplotype[b] (%)	*Gibberella circinata* haplotype[b] (%)	Recombinant progeny[c] (%)
GL 52 × GL 343 (178)	51	49	75	25	34
GL 52 × GL 318 (14)	36	64	100	0	36
GL 343 × GL 680 (F_1) (22)	86	14	18	82	18
GL 343 × GL 681 (F_1) (22)	55	45	27	73	14
GL 343 × GL 682 (F_1) (22)	82	18	91	9	77

MAT = mating type idiomorph.

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.

Fig 1 – The darkened lesion with irregular margins was produced by *Gibberella circinata* (top). The lesion produced by inoculation with *G. subglutinans* (bottom); has a distinct margin defined by callus tissue. Both were inoculated on *Pinus radiata*.

to induce a wide range of lesion lengths when inoculated into pine branches (Fig 2). Eighteen backcross progeny produced mean lesion lengths $\geq$ 6 mm and two of these (GL 687 and GL 695) induced lesions similar in length to those produced by *G. circinata*. The lesions had irregular margins, discolouration, and resin soaking typical of lesions made by *G. circinata* (Fig 1).

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 F_1 progeny had non-parental combinations 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), indicating 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

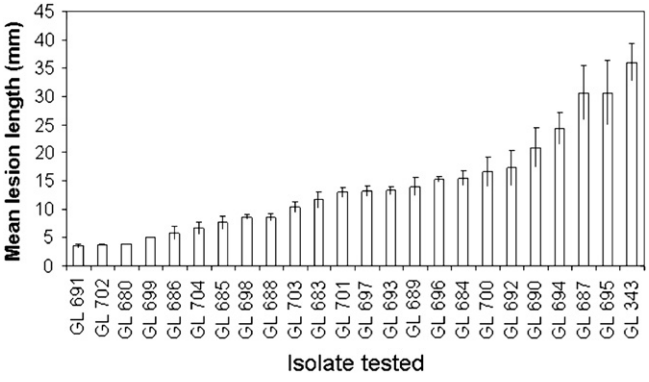

Fig 2 – Mean lesion lengths induced in *Pinus radiata* by progeny of a backcross, and both parents of the cross (GL 343 × GL 680). Error bars correspond to the standard error of the mean.

absence of virulent progeny in the F_1 generation implies a bias towards the genome of the *G. subglutinans* (avirulent) parent. Although a higher density of markers would be required 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 viability, as would be expected for a hybrid cross. Furthermore, interspecific 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 combinations, 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. subglutinans* was female fertile in hybrid crosses. F_2 crosses were generally successful with either parent serving as the female (data not shown), but backcrosses beyond the initial cross of F_1 progeny to *G. circinata* did not succeed and repeated attempts to cross progeny of the GL 343 × F_1 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. subglutinans* 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 associated 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 colonize pines when spores were placed in wounded tissue. The fungus was recoverable from lesions, suggesting it could survive 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 F_1 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 F_1 progeny to *G. circinata* produced offspring that revealed a wide range of virulence to pine. These results suggest that more than one gene contributes to the virulent phenotype and that genomes of the F_1 progeny were likely unbalanced, with an incomplete representation of the genes required for virulence. The complete absence of virulent F_1 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* genome 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.

REFERENCES

Desjardins A, Plattner RD, Gordon TR, 2000. *Gibberella fujikuroi* mating population A and *Fusarium subglutinans* from teosinte species and maize from Mexico and Central America. *Mycological Research* **104**: 865–872.

Edwards ET, 1935. Studies on *Gibberella fujikuroi* var. *subglutinans* the hitherto undescribed ascigerous stage of *Fusarium moniliforme* var. *subglutinans* and on its pathogenicity on maize in New South Wales. *Science Bulletin, Department of Agriculture, New South Wales* **49**: 1–68.

Friel CJ, Gordon TR, 2005. Inhibition of growth and spore germination of *Gibberella circinata* by volatile components of pine resin. *Phytopathology* **95**: S32.

Gordon TR, Storer AJ, Okamoto D, 1996. The population structure of the pitch canker pathogen, *Fusarium subglutinans* f. sp. *pini*, in California. *Mycological Research* **100**: 850–854.

Gordon TR, Kirkpatrick SC, Petersen JC, Friel CJ, 2006. Potential diversity in vegetative compatibility groupings in the California population of *Gibberella circinata*. *Mycological Research* **110**: 936–940.

Kile GA, Brasier CM, 1990. Inheritance and inter-relationship in fitness characters in progeny of an aggressive × non-aggressive cross of *Ophiostoma ulmi*. *Mycological Research* **94**: 514–522.

Leslie JF, Zeller K, Wohler M, Summerell BA, 2004. Interfertility of two mating populations in the *Gibberella fujikuroi* species mating complex. *European Journal of Plant Pathology* **110**: 611–618.

Nirenberg HI, 1976. Untersuchungen uber die morphologische and biologische Diferenzierung in der *Fusarium*— Sektion *Lesiola*. *Mitteilungen aus der Biologischen Bundesanstalt für Land-und Forstwirtschaft, Berlin-Dahlem* **169**: 1–117.

Nirenberg HI, O'Donnell K, 1998. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia* **90**: 434–458.

O'Donnell K, Cigelnik E, Nirenberg HI, 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**: 465–493.

Puhalla JE, Spieth PT, 1985. A comparison of heterokaryosis and vegetative incompatibility among varieties of *Gibberella fujikuroi* (*Fusarium moniliforme*). *Experimental Mycology* **9**: 39–47.

Reid LM, Woldermeriam XZ, Stewart DW, Schaafsma AW, 2002. Effect of inoculation and point of entry on disease severity in *Fusarium graminerearum*, *Fusarium verticilloides*, or *Fusarium subglutinans* inoculated maize ears. *Canadian Journal of Plant Pathology* **24**: 162–167.

Steenkamp ET, Coutinho TA, Desjardins AE, Wingfield BD, Marasas WFO, Wingfield MJ, 2001. *Gibberella fujikuroi* mating population E is associated with maize and teosinte. *Molecular Plant Pathology* **2**: 215–221.

Steenkamp ET, Wingfield BD, Coutinho TA, Wingfield MJ, Marasas WFO, 1999. Differentiation of *Fusarium subglutinans* f. sp. *pini* by histone gene sequence data. *Applied and Environmental Microbiology* **65**: 3401–3406.

Steenkamp ET, Wingfield BD, Coutinho TA, Zeller KA, Wingfield MJ, Marasas WFO, Leslie JF, 2000. PCR- based identification of *MAT-1* and *MAT-2* in the *Gibberella fujikuroi* species complex. *Applied and Environmental Microbiology* **66**: 4378–4382.

Steenkamp ET, Wingfield BD, Desjarsins AE, Marasas WFO, Wingfield MJ, 2002. Cryptic speciation in *Fusarium subglutinans*. *Mycologia* **94**: 1032–1043.

Zeller KA, Summerell BA, Bullock S, Leslie JF, 2003. *Gibberella konza* (*Fusarium konzum*) *sp. Nov.* from prairie grasses, a new species in the *Gibberella fujikuroi* species complex. *Mycologia* **95**: 943–954.