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# Isozyme Variation among Biological Species in the *Gibberella fujikuroi* Species Complex (*Fusarium* Section *Liseola*)†

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**Isozyme phenotypes were determined for 101 strains of *Gibberella fujikuroi* and 2 strains of *Gibberella nygamai* that represent seven biological species (mating populations) isolated from a variety of plant hosts in dispersed geographic locations. Fourteen enzymes were resolved in one or more of three buffer systems. Two of the enzymes, arylesterase and acid phosphatase, were polymorphic within two or more biological species and are suitable for intraspecific studies of population variation. Six enzymes, alcohol dehydrogenase, aspartate aminotransferase, glucose-6-phosphate dehydrogenase, mannitol dehydrogenase, phosphoglucomutase, and phosphogluconate dehydrogenase, were monomorphic in all of the isolates examined. The remaining six enzymes, fumarase, glucose phosphate isomerase, glutamate dehydrogenase (NADP), isocitrate dehydrogenase (NADP), malate dehydrogenase, and triose-phosphate isomerase, could potentially be used to distinguish the different biological species. Mating populations C and D are the most similar, since the mating population C isolates examined had the same isozyme phenotype as did a subset of the isolates in mating population D. Mating population E is the least similar to the other taxa examined. Unique isozyme phenotypes are present but are composed of banding patterns shared among the biological species. This finding supports the hypothesis that these biological species, with the possible exception of mating populations C and D, are reproductively isolated from one another and that no significant gene flow is occurring between them. Isozyme analysis is a useful method to distinguish these closely related biological species. Examination of isozyme phenotypes is more rapid than the present technique, which is based on sexual crosses; can be applied to strains that are not sexually fertile; and is more sensitive than traditional morphological characters, which cannot distinguish more than three or four morphological groups among the seven biological species. While emphasizing the discreteness of the mating populations as biological entities, our isozyme data also reaffirm the close genetic relationship among these groups.**

The *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura species complex contains at least six different biological species that have anamorphs in *Fusarium moniliforme* Sheldon, *Fusarium proliferatum* (Matsushima) Nirenberg, and *Fusarium subglutinans* (Wollenweber and Reinking) Nelson, Toussoun & Marasas with additional anamorphic groups in or related to *Fusarium* section *Liseola*, e.g., *Fusarium anthophilum* (A. Braun) Wollenweber and *Fusarium beomiforme* Nelson, Toussoun & Burgess, for which no sexual stage is yet known (20). The most reliable method for distinguishing the *G. fujikuroi* biological species (also termed mating populations) is in crosses with standard fertile tester isolates (18). These crosses are time-consuming, normally requiring 4 to 6 weeks from inoculation until final analysis, but usually provide clear results that are essential for population genetic analyses. Sexual crosses provide information only on strains that are sexually fertile. Infertile or poorly fertile strains can also play a role in fungal population dynamics (21) and may be important components of asexually reproducing populations. Thus, identifying strains that are associated with a mating population but that have greatly reduced fertility can be an important component in the analysis of field populations of these fungi.

Identifying the mating population with which a strain is associated can also be important for mycotoxicological and plant pathological purposes. Members of the same anamorph in different biological species may differ in the spectrum of mycotoxins that they produce. For example, both the A and the F mating populations have anamorphs in *F. moniliforme* (17), but the members of mating population A produce high levels of fumonisins and little if any moniliformin whereas the spectrum is reversed for mating population F (22, 24). Different mating populations are also more common on some host plants than on others. For example, mating population A commonly dominates on maize (6, 20, 23) whereas mating population F commonly dominates on sorghum (20, 23, 25). These differences and others (see reference 20 for a more complete list and additional references) suggest that mating populations are meaningful biological entities under field conditions and that techniques that can rapidly associate a strain with a mating population would be useful.

Isozymes have been used to distinguish fungal taxa and to assess the relatedness of many different fungal species (see, e.g., references 29 and 34). In the present study, we screened a standard set of strains described by Yan et al. (40) by using 14 resolvable isozymes on one or more of three different buffer systems. Our objectives in this study were (i) to identify isozymes that could be used for distinguishing mating populations and for intraspecific population genetic studies and (ii) to use the observed differences in isozymes to assess the relatedness of the different mating populations within *G. fujikuroi.*

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TABLE 1. Hosts from which strains of *G. fujikuroi* used in this study were recovered

| Mating population | No. of strains from host: | | | | | Total |
|---|---|---|---|---|---|---|
| | Maize | Sorghum | Rice | Sugarcane | Other[a] | |
| A | 22 | 3 | 1 | | 2 | 28 |
| B | | 2 | | 7[b] | 2 | 11 |
| C | | | 4 | | | 4 |
| D | 11[c] | 8 | | | 2 | 21 |
| E | 16 | | | | 1 | 17 |
| F | | 18[b] | | | 2 | 20 |
| Total | 49 | 31 | 5 | 7 | 9 | 101 |

[a] Includes banana, orchid, peanut, shattercane, and tobacco.
[b] Includes two $F_1$ progeny from crosses between field isolates.
[c] One strain, 2875, had been erroneously classified as a member of the B mating population (40) and has been reclassified as a member of the D mating population.

(A preliminary report of some of these results has been published [11].)

## MATERIALS AND METHODS

**Strains.** The strains we examined included 101 strains representative of the six mating populations (A to F) within *G. fujikuroi* that had been examined for sensitivity to benomyl and hygromycin by Yan et al. (40). These strains were isolated from diverse plant hosts and geographic locations and were purified through subcultures of uninucleate microconidia. Of these strains, 28 belonged to the A mating population, 11 belonged to the B mating population, 4 belonged to the C mating population, 21 belonged to the D mating population, 17 belonged to the E mating population, and 20 belonged to the F mating population (Table 1). All strains were classified on the basis of sexual cross-fertility with standard female-fertile testers (17, 18). We also included two strains, M-7491 and M-7492 from the *Fusarium* Research Center, The Pennsylvania State University, as representatives of the closely related and recently described *Gibberella nygamai* (15); these strains carry the KSU strain numbers G-05112 and G-05111, respectively.

Isolates were maintained on slants of complete medium (5) incubated at 25°C and preserved for the long term as spore suspensions in 15% glycerol frozen at −70°C.

**Sexual crosses.** Sexual crosses were made on carrot agar by the method of Klittich and Leslie (16). Crosses were scored as fertile if ascospores could be seen oozing from the body of the perithecium.

For the analysis of segregation ratios, single random ascospores from these crosses were separated from one another on 3% water agar slabs with a Cailloux stage-mounted micromanipulator as previously described (16). No more than 20 ascospores were taken from any single perithecium (19).

**Isozyme isolation and resolution.** Cultures were grown for 5 to 7 days in 100- by 15-mm petri dishes containing minimal medium (5) with a cellophane membrane overlay. The membrane with attached mycelium was flash frozen in liquid $N_2$ and then pulverized with a mortar and pestle. The powdered sample was mixed with 0.5 to 1.0 ml of extraction buffer (100 mM Tris-HCl [pH 7.5], 1 mM disodium EDTA, 10 mM KCl, 10 mM $MgCl_2 \cdot 6H_2O$, 350 mM polyvinylpyrrolidone, 0.1% β-mercaptoethanol, 10% dimethyl sulfoxide) and then frozen in microcentrifuge tubes. Protein extraction and starch gel electrophoresis were performed as previously described by Huss (10) and Murphy et al. (30). We found that the starch lot significantly affected resolution in the gel. Except for malate dehydrogenase (MDH) and NADP-dependent isocitrate dehydrogenase (IDH), protein extracts could be stored for up to 6 months at −80°C, but for less than 1 month at −20°C, without significant loss of detectable enzyme activity. MDH and IDH could be stored for up to 2 months at −80°C but for only a few days at −20°C. The enzymes, their abbreviations and EC numbers, the number of bands observed, and references for electrophoresis buffers and enzyme staining recipes are summarized in Table 2. Isozyme bands were scored and designated alphabetically with Greek letters in order of decreasing relative mobility within the starch gel (α is fastest, and ε is slowest).

**Analyses of isozyme data.** Numerical analysis of the isozyme data partitioned by mating population was performed with the computer software package NTSYS-pc, version 1.60 (31). The data were organized into a binary matrix, and a similiarity matrix was generated with the DICE coefficient. An unweighted pair grouping by mathematical averaging (UPGMA) cluster analysis was produced from this similarity matrix. By using this hierarchical system of clusters, a symmetrical matrix of cophenetic similarity values was generated. The goodness of fit of clustering to the data set was tested to compare the original similarity matrix with the cophenetic value matrix. From this comparison of these two matrices, the product-moment correlation, *r* (the cophenetic correlation coefficient), was calculated and used as a measure of the goodness of fit.

## RESULTS

**Monomorphic enzymes.** Six enzymes, some electrophoresed in more than one buffer system, were monomorphic across all

TABLE 2. Resolvable enzymes from *G. fujikuroi* by starch gel electrophoresis

| Enzyme | Abbreviation | EC no. | No. of bands | Buffer system[a] | Reference for stain recipe[b] |
|---|---|---|---|---|---|
| Acid phosphatase | ACP | 3.1.3.2 | 1/2 | All | 33 |
| Alcohol dehydrogenase | ADH | 1.1.1.1 | 1 | S6 | 30[c,d] |
| Arylesterase | EST | 3.1.1.2 | 1/2 | All | 30[e,f] |
| Aspartate aminotransferase | AAT | 2.6.1.1 | 1 | TM | 33 |
| Fumarase | FUM | 4.2.1.2 | 2 | M, S6 | 30[c,d,g] |
| Glucose-6-phosphate dehydrogenase | G6PDH | 1.1.1.49 | 1 | All | 37[c] |
| Glucose-4-phosphate isomerase | GPI | 5.3.1.9 | 1 | TM | 30[c,d] |
| Glutamate dehydrogenase (NADP) | GDH | 1.4.1.4 | 1 | TM | 29[c,h] |
| Isocitrate dehydrogenase (NADP) | IDH | 1.1.1.42 | 2 | M | 30[c,d] |
| Malate dehydrogenase | MDH | 1.1.1.37 | 1/2[i] | M, TM | 37[c] |
| Mannitol dehydrogenase | MADH | 1.1.1.67 | 1 | M, S6 | 27[c] |
| Phosphoglucomutase | PGM | 2.7.5.1 | 1 | TM | 33[j] |
| Phosphogluconate dehydrogenase | PGDH | 1.1.1.44 | 1 | M, S6 | 37[c] |
| Triose-phosphate isomerase | TPI | 5.3.1.1 | 1 | S6 | 30[c,d,k] |

[a] M buffer (4); TM buffer (32); S6 buffer (27, 33).
[b] The reference for basic recipe is given, with modifications from published references given in the footnotes.
[c] 200 mM Tris-HCl (pH 8.0) replaced with 100 mM Tris-HCl (pH 8.0).
[d] 5 mg of nitroblue tetrazolium per ml was replaced with 10 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide per ml.
[e] 300 mg of β-napthyl acetate was added to the 300 mg of α-napthyl acetate in a solution of 15 ml of acetone and 15 ml of $H_2O$.
[f] 200 mM Tris-HCl (pH 7.0) was replaced with 100 mM Tris-HCl (pH 7.0).
[g] Fumaric acid was increased to 1,200 from 50 mg, malic dehydrogenase was increased to 600 from 150 U, 1% MAD solution was increased to 6 from 1 ml, and 1% phenazine methosulfate solution was increased to 1 from 0.5 ml.
[h] NADPH is required for this enzyme activity to be detected (14).
[i] One band on Tris-maleate-EDTA buffer; two bands on morpholine buffer.
[j] 2 ml of 1.0 M $MgCl_2$ deleted, and only 50 ml of 100 mM Tris-HCl (pH 8.0) used instead of 100 ml.
[k] 24 mg of EDTA and an additional 40 ml of Tris-HCl (pH 8.0) added to increase the total volume of the staining solution.

TABLE 3. Distribution of polymorphic isozymes in *G. fujikuroi* and *G. nygamai*

| Group[a] | Isozyme distribution for[b]: | | | | | | | | | | | | No. of types |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ACP | | EST | | | FUM (M) | GDH (TM) | GPI (TM) | IDH (M) | MDH | | TPI (S6) | |
| | TM | S6 | M | TM | S6 | | | | | M | TM | | |
| A | α | α,β,γ | β | γ,δ | β,γ,δ | α | α | α,β | β | α | β | β | 2 |
| B | α | α | α,β,γ | β,γ | α | α,β | α | β | β | α | β | α | 2 |
| C | α | α | α | β | α | α | α | α | β | β | β | α | 1 |
| D | α,β | α,β,γ | α,β | α,β | α,β | α,β | α | α | β | β | β | α | 2 |
| E | α,β,γ | α,β | γ | ε | ε | δ | α,β,γ,δ | α | γ | γ | α,β,γ | α | 4 |
| F | α | α,β,γ | α,β | β | β | γ | α | α | α,β | α | β | α | 2 |
| *G. nygamai* | α | α | α | β | α | α | α | α | ω | α | β | α | 1 |

[a] Mating population or biological species.
[b] For each isozyme α is the fastest moving pattern followed by β, γ, δ, and ε. The ω pattern for IDH for *G. nygamai* contains one band that moves faster and one band that moves more slowly relative to the other patterns for this isozyme.

six of the mating populations. These enzymes were aspartate aminotransferase (AAT), alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PDH), mannitol dehydrogenase (MADH), phosphogluconate dehydrogenase (PGDH), and phosphoglucomutase (PGM). None of these enzymes was polymorphic when they were electrophoresed in another buffer system.

**Polymorphic enzymes.** Eight enzymes were polymorphic in one or more buffer systems (Table 3). Except for triose-phosphate isomerase (TPI), these enzymes were all polymorphic within at least one of the mating populations. The two groups represented by the fewest strains (mating population C and *G. nygamai*) are the only groups within which no isozyme polymorphism was detected. MDH was not variable within a group when the gels were run in morpholine or amine-citrate buffer (M buffer) but was polymorphic within mating population E when the MDH was resolved on a gel run in Tris-maleate-EDTA (TM buffer). Isozyme patterns for enzymes that can be used to identify the different mating populations are shown in Fig. 1.

In some cases, unique bands that were limited to a single group were found (Table 3). These bands can potentially be used in diagnostic analyses to distinguish the different groups from each other. With fumarase (FUM), the γ pattern is unique to the F mating population and the δ pattern is unique to the E mating population. For glutamate dehydrogenase (NADP) (GDH), the α allele is found in all of the groups but the β, γ, and δ patterns are all limited to mating population E. IDH has one pattern, γ, that is unique to mating population E, a second pattern (α) that is unique to mating population F, and a third pattern (ω) that is unique to *G. nygamai*. The ω pattern is unique in that the bands in this pattern have not both shifted in the same direction. Instead, one band moves faster and the other moves more slowly relative to their counterparts in the other groups (Fig. 1C). For MDH, the only unique patterns are found in the E mating population. Pattern uniqueness depends on the buffer system used for resolution. If the M buffer system is used, the γ pattern is unique, whereas if the TM buffer system is used, the α and the γ patterns are unique. For diagnostic patterns, M buffer is preferred because the γ pattern is found in all of the members of this mating population, whereas if TM buffer is used, some members of the E mating population may have the β pattern that is found in the other six groups. With TPI, the A mating population has a characteristically slow β pattern.

**(i) Acid phosphatase.** The amount of variability observed for acid phosphatase (ACP) depends on the buffer system used (Table 3). There were no observable differences in the banding pattern in M buffer; mating populations D and E were polymorphic in TM buffer; and mating populations A, D, E, and F were polymorphic in Soltis 6 buffer (S6 buffer). We observed no mating-population-specific banding patterns for ACP. On the basis of our limited sample size, we expect ACP to be a

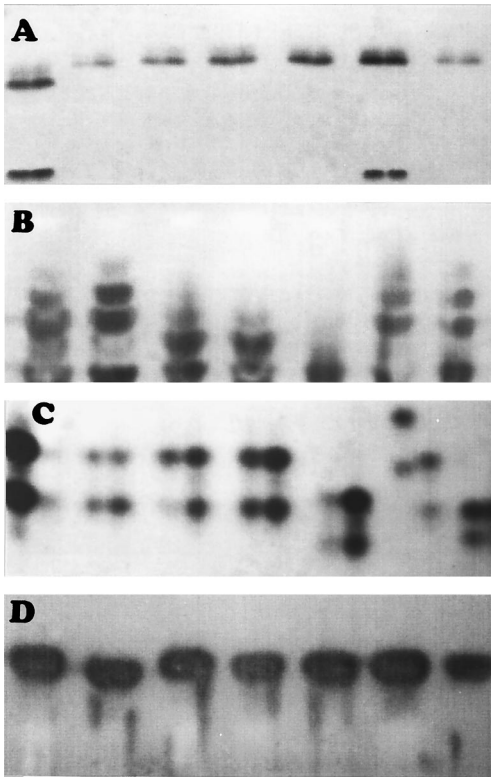


FIG. 1. Isozyme patterns for TPI (A), MDH (buffer M) (B), IDH (C), and GPI (D). In each case, the bands from left to right represent mating populations A (A-00149), B (B-03852), C (C-01993), D (D-04853), E (E-00990), and F (F-04093 and F-01183) and *G. nygamai* (G-05111). All bands are two lanes of the same strain except for the lanes containing strains from mating population F. For TPI, all of the mating populations have the α pattern except for the A mating population, which has the β pattern. For MDH(M), *G. nygamai* and mating populations A, B, and F have the α pattern; mating populations C and D have the β pattern; and mating population E has the γ pattern. For IDH, mating population F has both the α and β patterns, mating populations A to D have the β pattern, mating population E has the γ banding pattern, and *G. nygamai* has the ω banding pattern. For GPI, *G. nygamai* and all of the mating populations except B have the α banding pattern and mating population B has the β pattern.

TABLE 4. Heritability of GPI polymorphism in *G. fujikuroi* mating population A[a]

| Parent | | No. of progeny | | | |
|---|---|---|---|---|---|
| Female | Male | *matA*$^+$ *gpil*$^F$ | *matA*$^+$ *gpi1*$^S$ | *matA*$^-$ *gpi1*$^F$ | *matA*$^-$ *gpi1*$^S$ |
| A-00102 *matA*$^+$ *gpi1*$^F$ | A-00488 *matA*$^-$ *gpi1*$^S$ | 4 | 8 | 4 | 4 |
| A-04426 *matA*$^+$ *gpi1*$^F$ | A-00488 *matA*$^-$ *gpi1*$^S$ | 7 | 7 | 3 | 3 |

[a] Genotypes: *matA*$^+$/*matA*$^-$, mating-type alleles; *gpi1*$^F$/*gpi1*$^S$, GP1 fast-moving (α) or slow-moving (β) band (allele).

suitable marker for the analysis of genetic variability within a mating population.

**(ii) Arylesterase.** Arylesterase (EST) was the most variable of the enzymes that we surveyed. Intraspecific variation was observed in at least one buffer in all of the groups except for mating populations C and E and *G. nygamai*. Although we identified no more than four patterns per buffer system, many of the strains that are grouped together do not have identical patterns but may vary somewhat within a pattern. Therefore, the number of patterns we have described probably underestimates the amount of variability present with respect to this enzyme.

**Genetic basis for GPI polymorphism in mating population A.** One isolate in the A mating population (A-00488) had the β pattern that was typical of the B mating population (Table 3). We tested the heritability of the two glucose-6-phosphate isomerase (GPI) banding patterns through crosses between A-00488 and A-00102 and between A-00488 and A-04426 (Table 4). In addition to the GPI pattern, the progeny of these crosses were scored for mating type. The results of these crosses indicated that a single gene, unlinked to mating type, was responsible for the GPI phenotype. We did not test heritability patterns of other intraspecific polymorphisms.

**Isozyme phenotypes.** The patterns in the six polymorphic enzymes other than ACP and EST were used to group the strains into isozyme phenotypes; the resolution in M buffer was used for the MDH isozyme. This grouping results in 14 isozyme phenotypes (Table 3). If the more numerous polymorphisms in ACP and EST are included, the number of isozyme phenotypes can be significantly increased, with the exact number depending on which buffer is used for determining the number of banding patterns for ACP and EST.

*G. nygamai* and mating population C each contained only a single isozyme phenotype. Mating population E contains four isozyme phenotypes as a result of polymorphism in GDH; three of the four GDH forms are found only in this mating population. The most common of these four isozyme phenotypes accounts for nearly half (8 of 17) of the mating population E isolates examined. The other four mating populations contain two isozyme phenotypes each. In the B and D mating populations, the two different isozyme phenotypes result from polymorphism in FUM. In the D mating population, only 1 of 21 isolates examined had the α FUM pattern. This isozyme phenotype was the same as the single isozyme phenotype observed in mating population C. In mating population B, 4 of 11 isolates had the α FUM pattern and the remaining 7 had the β pattern. In mating population F, the frequency of the two banding patterns found for IDH were nearly identical; 9 were α, and 11 were β. The α pattern was unique to mating population F. In mating population A, GPI was polymorphic since 1 of 28 strains had the β banding pattern rather than the more common α banding pattern.

**Similarity among different isozyme phenotypes.** We constructed a phenogram (Fig. 2) indicating the similarity of different mating populations on the basis of the presence or absence of isozyme bands for all the enzymes resolved in this study except ACP and EST. The cophenetic correlation coefficient was high ($r = 0.96$), indicating that the UPGMA phenogram accurately portrays the original data matrix. The presence of monomorphic loci (AAT, ADH, G6PDH, MADH, PGM, and PGDH) shared by the different mating populations supports the previous conclusion based on morphology that these species are closely related.

Mating populations C and D are the most similar to one another. Mating populations A and B cluster with one another but are less similar to one another than are mating populations C and D. *G. nygamai* and mating populations A, B, C, and D are more similar to one another than to mating population F. Mating population E is least similar in its isozyme profile to the other biological species in this group.

## DISCUSSION

We analyzed 14 resolvable enzymes, 6 of which were monomorphic across the entire set of strains, 6 of which were useful for distinguishing the different biological species from one another, and 2 of which were variable enough to be used for the analysis of populations within a mating population. Our results can be used to estimate the genetic relatedness of the seven different biological species and in the development of a method using unique bands to quickly and reliably distinguish the different biological species without resorting to time-consuming and laborious sexual crosses for an initial diagnosis. Isozyme phenotypes also can be used to associate isolates that are poorly or totally infertile with a known mating population or to design crossing schemes for the detection of additional mating populations.

Isozymes have been used extensively in some fungi for both taxonomic and population analyses (see, e.g., references 2, 8, and 29). The use of isozymes in studies of *Fusarium* spp. has

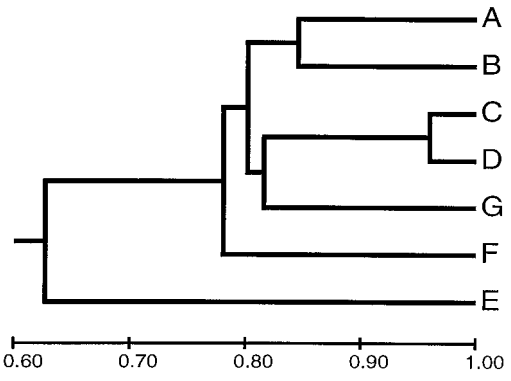


FIG. 2. UPGMA cluster analysis phenogram based on isozyme data for seven biological species of *Gibberella*. Mating populations of the *G. fujikuroi* species complex (*Fusarium* section *Liseola*) are designated A to F, and *G. nygamai* is designated G. This phenogram is based on the banding patterns for ADH, AAT, FUM, G6PDH, GPI, GDH, IDH, MDH, MADH, PGM, PGDH, and TPI, using M, TM, or S6 buffer for resolution.

been much more limited, although several recent population studies of *F. oxysporum* have been made (3, 7, 12, 26). These investigators usually tested for isozyme polymorphism(s) within one or a few pathogenic formae speciales by using a battery of different isozymes. In some cases, isozymes were used as independent markers to assess the relatedness of isolates within the same vegetative compatibility group. The general findings of these studies are consistent with our results (Table 3), namely, that some isozymes are polymorphic within one or more of these taxa. Enzymes such as EST and ACP were sufficiently variable within most of the mating populations to be generally useful for intraspecific population analyses. Other enzymes were polymorphic within one or a few of the biological species and might be of use in population analyses of those particular groups. These isozymes include GPI in mating population A, GDH and MDH in mating population E, IDH in mating population F, and FUM in mating populations B and D.

Many investigators commonly designate bands as alleles at one or more genetic loci bearing the name of the isozyme. We have not made such assignments because of the lack of genetic data to support the assignments. The only exception is GPI in mating population A, for which we have evidence (Table 4) to indicate that the two isozymic forms are both alleles at the *gpi1* locus. We attempted to place this locus on the existing genetic map of mating population A (38) but were unable to do so since both of the parents in the mapping cross carry the $gpi1^{F}$ allele (α banding pattern) at this locus. Determining the genetic bases of the remaining polymorphisms should be a relatively straightforward process since all the strains used in this study are known to be cross-fertile with standard testers (15, 18, 40). The identification of the genetic basis for the observed polymorphisms probably should be completed before population-level conclusions that assume allelic polymorphism are drawn.

**Relatedness of strains as assessed by isozyme polymorphisms.** Isozyme polymorphisms can be used to determine the relatedness of different species. The most extensive study of *Fusarium* spp. (34) was performed with single representatives of 39 different species and varieties. Relatedness was determined following isoelectric focusing of esterase isozymes that were separated in polyacrylamide gels. This study included one strain of *F. moniliforme* from onion, which could be in either the A or the F mating population, and one strain of *F. moniliforme* var. *subglutinans*, which could belong to either the B or the E mating populations. These strains were more closely related to one another than they were to anything else on the tree; the next nearest strains were from *F. oxysporum* and *F. lateritium*. Given the level of intraspecific population variation known in *F. oxysporum* for over 25 years (see, e.g., references 9 and 28), results such as these require increased numbers for substantiation.

Some more limited studies have also been done with larger numbers of strains from the *Liseola* section. Vágújfalvi and Szécsi (35) examined 70 strains from maize by using EST isozyme polymorphism. They resolved their strains into three groups: *F. moniliforme* (probably mating population A), *F. proliferatum* (probably mating population D), and *F. subglutinans* (probably mating population E). Polymorphism existed within each group, but the differences between groups were generally much larger than were the differences within the groups.

Kathariou (13) used isozymes to evaluate variation in strains from section *Liseola* that were collected from California and Italy. EST, ACP, peroxidase, G6PDH, and AAT (which she termed glutamate oxaloacetate transaminase) were polymorphic in her studies. She divided her strains into two allozyme types, AT-1 and AT-101, on the basis of polymorphisms in EST, AAT, and ACP. AT-1 is mating population A, and AT-101 is mating population D. She identified a third group of strains from China that were isozymically distinct from both AT-1 and AT-101 but included no further information on this group. Of the five polymorphic enzymes tested by Kathariou (13), peroxidase was not included in our screen and so we cannot compare our work with hers in this area. The polymorphisms in EST and ACP between and within mating populations A and D as observed by Kathariou are not the same as the polymorphisms that we observed. This difference may be due to differences in the electrophoresis buffers that were used to make the assessments. She also found polymorphism for AAT and G6PDH, while all of the strains that we examined from all seven biological species were monomorphic for this enzyme.

In our study, we found that *G. nygamai* and five of the six mating populations in *G. fujikuroi* could be distinguished from one another by using isozyme polymorphisms. The absence of variation for many loci between some mating populations of *G. fujikuroi* and the lack of variation for 6 of the 14 isozymes examined suggest that these seven entities are closely related species that have recently become reproductively isolated and diverged. Some bands for some of the loci were unique to a particular biological species. This result is somewhat surprising, considering that strains originated from a diversity of plant hosts (banana, maize, orchids, peanut, rice, sorghum, sugarcane, and tobacco) and dispersed geographic locations in Africa, Asia, Europe, and North America (40). These groups are also known to differ in other traits such as sensitivity to hygromycin and benomyl (40), electrophoretic karyotype (39), female fertility (20), genome organization (38), mycotoxin production (22, 24), and random amplification of polymorphic DNA (RAPD) banding patterns (1, 36). Our isozyme data both emphasize the discreteness of the mating populations as biological entities and reaffirm the close genetic relationship among these groups.

**Suggested protocol for distinguishing biological species.** The mating-population-specific bands that we have observed may be useful as diagnostic characteristics for preliminary assignments of unidentified strains to known mating populations. Only a few enzymes need to be resolved to make such assignments. As the amount of information on the reliability of these assignments increases, the need for determining mating type and/or male-female fertility could be limited to the cases for which that specific information is needed, e.g., studies of effective population size (21), and not used as the sole basis for assigning strains to a mating population.

Bands that are diagnostic for a particular mating population make the identification of strains belonging to that population relatively easy. For example, the β band for TPI is found only among members of the A mating population whereas all other isolates have the faster α band. Similarly, the γ pattern for IDH or the δ pattern for FUM is unique for mating population E, the α pattern for IDH is unique to mating population F, and the ω pattern at IDH is unique to *G. nygamai*. Mating populations C and D cannot be reliably distinguished from one another but can be distinguished from the other mating populations by the presence of the β banding pattern for MDH. Thus, *G. nygamai* and mating populations A, C/D, E, and F can each be identified on the basis of a unique banding pattern. Distinguishing the B mating population from the other six entities is somewhat more complicated. The β form of GPI has been found only in the A and B mating populations and so far is rare in the A mating population. Thus, isolates with the β

allele at GPI and the α allele at TPI can be assigned to the B mating population.

A practical scheme for differentiating the different mating populations depends on the sample being screened. If one particular biological species is thought to be dominant, the isozyme diagnostic for that group should be run first. For example, isolates from maize are most likely to be members of the A mating population, and the TPI assay should be run first. If nothing is known about the composition of the population, however, the first gel should be run in M buffer and slices should be stained for FUM, IDH, and MDH. These stains distinguish all of the groups except for mating populations A and B from one another.

Isozymes are preferable to many of the techniques that can be used to distinguish the *G. fujikuroi* mating populations. Isozyme analysis is faster than studies of cross-fertility (15–18, 20) and more accurate than studies of sensitivity to benomyl and hygromycin (40), two techniques that can be used to differentiate mating populations that require only a routine microbiology laboratory. Studies of electrophoretic karyotypes (39) and mycotoxin profiles (see, e.g., references 22 and 24) require sophisticated equipment and trained personnel; even when these are available, the methods are not necessarily faster than the isozyme analysis method. RAPD banding patterns also have been proposed as a means of distinguishing the mating populations (1, 36). These studies did not identify diagnostic protocols but, instead, focused on constructing phylogenies from relatively small sets of strains. Although a PCR-based protocol, perhaps based on a fragment(s) from a RAPD amplification, may ultimately be used to identify these fungi, no such protocol is presently available. Thus, isozyme analysis is presently the fastest molecular technique, with a defined protocol, that is available for distinguishing the *G. fujikuroi* mating populations, and should enable more rapid and more accurate surveys of commercial fields.

In addition to the resulting diagnostic protocol, our isozyme study sheds some light on the genetic relationships among the *G. fujikuroi* species complex. These results suggest that all of the groups observed, with the possible exceptions of mating populations C and D, need to be formally recognized as being distinct at the species level. Although isozymes provide a tool for distinguishing the mating populations, the development of additional techniques that can make these distinctions also should be encouraged. Only through the development of molecular traits that are correlated with biologically meaningful units can studies of genetic relatedness within this economically important group of fungi be successfully conducted.

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