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Biological Species in the *Gibberella fujikuroi* Species Complex Isolated from Maize Kernels in Argentina

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Abstract: The present study was carried out to evaluate Fusarium species within the $Gibberella\ fujikuroi$ species complex isolated from maize from a population level point of view. Three mating populations among the G. fujikuroi species complex were found, $Fusarium\ verticillioides\ (Sacc.)$ Nirenberg (= F. $moniliforme\ Sheldon = <math>G$. $fujikuroi\ mating\ population\ A$; teleomorph, G. $moniliformis\ Wineland$), F. $proliferatum\ (Matsushima)\ Nirenberg\ (= <math>G$. $fujikuroi\ mating\ population\ D$; teleomorph, G. $intermedia\ (Kuhlman)\ Samuels\ et\ al.$) and F. $subglutinans\ (Wollenweber\ and\ Reinking)\ Nelson,\ Toussoun\ and\ Marasas\ (= <math>G$. $fujikuroi\ mating\ population\ E$; teleomorph, G. $subglutinans\ (Edwards)\ Nelson,\ Toussoun\ and\ Marasas\)$. Both mating types were observed MAT-1/MAT-2 in the three populations; the allele frequencies were different according to the mating population evaluated. The effective population number (N_e) based on mating type $[N_{e(m)}]$ and hermaphrodite frequency $[N_e]$ showed that sexual reproduction was more frequent among members of G. moniliformis population, following in order, G. $intermedia\ and\ G$. $subglutinans\ populations$, respectively. G. $moniliformis\ will\ be\ able\ to\ recombine\ by\ sexual\ reproduction\ so\ that\ it\ will\ have\ more\ adaptive\ advantages\ to\ the\ selection\ pressures.$ The present study contributes with valuable information on G. $fujikuroi\ species\ complex\ isolated\ from\ maize\ in\ Argentina\ from\ a\ population\ level\ point\ of\ view.$

Key words: Fusarium, Gibberella species complex, mating type, female fertility, effective population number, maize

INTRODUCTION

Maize (*Zea mays* L.) is an important crop in Argentina and its production, approximately 15 million tons is concentrated mainly in three provinces Buenos Aires, Santa Fe and Cordoba (SAGPyA, 2003).

Previous studies in Argentina have shown that Fusarium species were the predominant fungi isolated from maize ear rot and asymptomatic verticillioides (Sacc.) maize kernels. Fusarium Nirenberg (= F. moniliforme Sheldon =Gibberella fujikuroi mating population A; teleomorph, G. moniliformis Wineland), was the dominant species while the isolation frequency of F. proliferatum (Matsushima) Nirenberg (= G. fujikuroi mating population D; teleomorph, G. intermedia (Kuhlman) and F. subglutinans (Wollenweber and Nelson, Toussoun and Marasas (= G. fujikuroi mating E; teleomorph, subglutinans (Edwards) Nelson, Toussoun and Marasas) varied according to

the area under study (Chulze *et al.*, 1996; 2000; González *et al.*, 1995; Ramírez *et al.*, 1996; Sydenham *et al.*, 1993). Some isolates of these species can produce several mycotoxins including fumonisins, moniliformin, beauvericin and fusaproliferin (Bottalico, 1998; Reynoso *et al.*, 2004).

Effective population number (N_e) is a concept commonly used to compare different field populations with one another and with an idealized randomly mating population (Caballero, 1994; Wriht, 1931). N_e is also used to estimate the effects of drift and inbreeding. Leslie and Klein (1996) derived equations to extend this concept to fungi using mating type frequency and relative sexual fertility as the critical variables.

Species among the *G. fujikuroi* species complex vary so much under field conditions mainly in aspects such as secondary metabolites production, vegetative compatibility, female fertility and mating type ratio (Leslie, 1999).

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Previous studies on field populations of *G. fujikuroi* species complex have been done in different countries. Mansuetus *et al.* (1997) and Lim *et al.* (2001) have analyzed populations of *G. moniliformis*, *G. intermedia* and *G. thapsina* isolated from sorghum in Tanzania and Korea, respectively. Britz *et al.* (1998) also studied a population of *G. circinata* isolated from pine in South Africa.

Studies on population of *G. fujikuroi* species complex isolated from maize have been done in Mexico, USA and Korea (Leslie and Klein, 1996; Desjardins *et al.*, 1994; Park *et al.*, 1999). In Argentina a survey in a microgeographycal scale was done on strains that belong to *G. moniliformis* population isolated from maize cultivated under no-till conditions. This population showed a limited sexual reproduction as determined by the effective population number based on female fertility and mating type. Data obtained from the VCGs (vegetative compatibility groups) and AFLP analysis suggested that sexual reproduction was relatively common as evidenced by the high level of phenotypic diversity (Chulze *et al.*, 2000).

According to the above mentioned the aims of this study were to test (i) if mating type ratio and hermaphrodite frequency have an effect on effective population number; (ii) if sexual reproduction or vegetative propagation has an influence on the genetic structure of the populations.

Data will be useful to know more about the epidemiology of these pathogens and also to estimate the possibility of adaptation to the selection pressures. This knowledge will be relevant to improve strategies for controlling these pathogens, and for maize breeding programs.

MATERIALS AND METHODS

Sampling: Fusarium species were isolated from kernels from the same hybrid (PIONEER 32K61) planted in 11 commercial fields under conventional tillage in three main maize growing area from Argentina that included Santa Fe (SF), Cordoba (CO) and Buenos Aires (BA) Provinces, during the harvest season 1996/1997. The fields sampled were located within a 300-km-diameter region.

Sampling was done from each commercial field from 10 rows, 100 m length, and with distance between rows 70 cm, 5 plants by meter. Maize ears were taken with a sampling intensity 0.7%, 42 ears by sample. This sampling plan was chosen considering the high incidence of *Fusarium* species in maize in Argentina (Chulze et al., 1996).

Table 1: Distribution of *Gibberella fujikuroi* species complex recovered from maize at three locations within the main maize growing area in Argentina

	Main maize growing area®					
G. fujikuroi						
species complex	SF	CO	BA	Total		
G. moniliformis	57	80	66	203		
G. intermedia	24	10	44	78		
G. subglutinans	13	4	79	96		
Total	94	94	189	377		

^{*}Strain number isolated

Fusarium isolation and identification: From each sample, a sub-sample of 200 g of maize kernels was surface-disinfected with NaOCl (1%) for 1 min and then rinsed with distilled water (three times). One hundred kernels were plated (10 kernels per Petri dish) onto a modified pentachloronitrobenzene medium (PCNB). The PCNB plates were incubated at 24°C for 7 days with 12/12 h photoperiod under cold white and black fluorescent lamps (Nelson et al., 1983).

Identification of *Fusarium* species was done from single spore cultures on Carnation Leaf Agar (CLA) and Potato Dextrose Agar (PDA), and incubated under 12/12 h photoperiod under cold white and black fluorescent lamps according to Nelson *et al.*, 1983. From the total *Fusarium* species isolated (420 strains), 377 belonging to the *G. fujikuroi* species complex were chosen for the study (Table 1). The remain 43 strains belong to others *Fusarium* species that included *F. equiseti*, *F. oxysporum*, *F. semitectum* and *F. graminearum*.

The isolates are maintained as spore suspensions in 15% glycerol, frozen at -80°C. The strains are held in the culture collection at the Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto, Córdoba, Argentina

Mating type and female fertility: Sexual crosses to confirm mating population and to identify mating types were made in triplicate on carrot agar by using the protocol of Klittich and Leslie (1988) with standard tester strains A-00149 (MATA-1), A-00999 (MATA-2), D-04853 (MATD-2), D-04854 (MATD-1), E-03693 (MATE-2) and E-03696 (MATE-1) as female parents and the uncharacterised field isolates as male parents. A cross was scored positive only if we observed perithecia oozing a cirrhus of ascospores. Female fertility test of the field strains belonging to G. moniliformis, G. intermedia and G. subglutinans populations was done in crosses in which the field isolates were the female parents and the standard testers were the male parents.

Data analysis: Differences in frequencies were tested for significance using a paired t-test by Sigma Stat for Windows version 2.03 software (SPSS, Chicago). The

 N_e was calculated based on mating type ratios $[N_{e \text{ (ml)}}]$ and the proportion of female fertile strains $[N_{e \text{ (pl)}}]$ by using the equations of Leslie and Klein (1996) and also to determine the average number of asexual generations per sexual generation. The average number of female sterile mutations per strain was calculated using the frequency of the hermaphrodite strains as the zero term in the Poison distribution.

RESULTS

G. moniliformis was isolated in percentage ranging from 26 to 63%, G. intermedia from 4 to 14% and G. subglutinans from 4 to 39%. Maize samples from Buenos Aires showed higher levels of contamination with G. subglutinans.

Among the 203 strains belonging to *G. moniliformis*, the mating types *MATA-2/MATA-1* segregated 74:129, this ratio was significantly different from 1:1 (p<0.001) resulting in a decreases in the $N_{e\,(mt)}$ of 92.6% of the actual count (total population). Among the fertile isolates, 105 were hermaphrodites, giving a $N_{e\,(j)}$ of 89.8% of the actual count. The high proportion of female fertility among the strains of *G. moniliformis* observed result in less effect on $N_{e\,(j)}$ (Table 2).

If the population is at equilibrium, then these data can also be used to estimate the range in which the percentage of hermaphrodite strains can fluctuate and the relative number of asexual generations per sexual generation (Leslie and Klein, 1996). Considering that G. moniliformis population has completed a sexual cycle, then the number of hermaphrodite strains (N_h) ranged from 52 to 27% before the next round of sexual reproduction. Otherwise, if this population will go to the sexual cycle, then the N_h could increase from 52 to 72%. Based on the assumption that female fertility is lost due to selection and mutation rate during asexual reproduction, the average number of asexual generations per sexual generation ranges from 16 to 65 depending on the combined effects of mutation rate on female sterility and the selection against hermaphrodites during the asexual portion of the life cycle, i.e., $0.98 < \mu(1 - \theta) < 0.99$ (Table 3).

The populations of G. intermedia and G. subglutinans were analyzed following the same considerations as G. moniliformis. G. intermedia population showed a MATD-2/MATD-1 ratio 38:40, this ratio was near 1:1, therefore the $N_{e \, (ml)}$ was not reduced (99.9%). Among the 78 fertile isolates, 27 were hermaphrodites, giving a $N_{e \, (f)}$ of 76% of the actual count (total population) (Table 2).

Under the assumption that the G. intermedia population completed a sexual cycle the N_b ranged from

Table 2: Female fertility and inbreeding effective numbers for Gibberella moniliformis, Gibberella intermedia and Gibberella subglutinens populations recovered from maize in Argentina

			N_{ϵ}				
G. fujikuroi							
species complex	MT ^a	$N_{\mathrm{fs}}:N_{h}$	$N_{e(mt)}$	N_{e0}	$N_{e(\nu)}$	M	
G. moniliformis	74:129	98:105	92.6	89.8	65.9	0.6	
G. intermedia	38:40	51:27	99.9	76.4	74.2	1.1	
G. subglutinans	77:19	91:5	63.4	18.8	95.0	2.6	

 $^{\rm a}$ MT: MAT-2: MAT-1, $N_{\rm ps}$: Number of female-sterile male-fertile strains; $N_{\rm h}$, number of hermaphrodite strains, $N_{\rm e}$: Effective population number, $N_{\rm e(m)}$ Effective population number based on mating type and expressed as a percent of the actual count (equation derived by Leslie and Klein 1996), $N_{\rm e(p)}$: Inbreeding effective number based on numbers of males and hermaphrodites and expressed as a percentage of the actual count (equation derived by Leslie and Klein 1996), M: Mean number of female sterility mutations per strains

35 to 12% before the next round of sexual reproduction. On the other hand, if this population will go to the sexual cycle then the N_h ranged from 35 to 59%. Under equilibrium cycle, the average number of asexual generations per sexual generation ranged from 26 to 106, depending on the values for μ and θ (Table 3).

The mating types segregated 77:19 MATE-2/MATE-1 for isolates from the G. subglutinans population, this ratio was significantly different from 1:1 (p<0.001) resulting in a large decreases in the $N_{e\,(mt)}$ of 63% of the actual count (total population). This population showed a lower frequency of hermaphrodites strains in comparison with G. moniliformis and G. intermedia populations, giving a $N_{e\,(j)}$ of only 19% of the actual count (total population). Both the limited proportion (5%) of hermaphrodite strains in this population and the mating type ratio (77:19) had significant effect of reducing the N_e of the population (Table 2).

Considering that *G. subglutinans* population has completed a sexual cycle, then the N_h ranged from 5 to 22% before the next round of sexual reproduction. Otherwise if this population will go the sexual cycle, then the N_h could increase from 5 to 0.3%. Under equilibrium cycle the number of asexual generations per sexual generation ranged from 74 and 298, depending of the values for μ and θ (Table 3).

Differences were observed in the female sterile mutation (M) per genome among the mating populations, being these values 0.6, 1.1 and 2.6 for *G. moniliformis*, *G. intermedia* and *G. subglutinans* respectively (Table 2).

DISCUSSION

The isolation frequency of *G. fujikuroi* species complex isolated in the present study agrees with previous results on the recovery of *Fusarium* species from maize cultivated under no-till and conventional tillage conditions in Argentina (Chulze *et al.*, 1996, 2000; Ramírez *et al.*, 1996; Torres *et al.*, 2001). *G. moniliformis*

Table 3: Length and range in hermaphrodite for equilibrium cycles based on observed data from G. moniliformis, G. intermedia and G. subglutinans populations recovered from maize in Argentina

	Time *	Time *			Hermaphrodites			Time ^b		
G. fujikuroi										
species complex	0.98 °	0.99 °	0.999°	Maximum ^d	Observed	Minimum *	0.98 °	0.99°	0.999 °	
G. moniliformis	16	33	323	0.72	0.52	0.27	32	65	654	
G. intermedia	26	53	530	0.59	0.35	0.12	53	106	1.061	
G. subelutinans	74	149	1497	0.22	0.05	0.003	148	298	2994ª	

^a Time, in asexual generations, to cycle from h_a to the observed frequency given the value of $(1-\mu)\theta$, ^bTime, in asexual generations, to cycle from the observed frequency to h_b given the value of $(1-\mu)\theta$, ^c $(1-\mu)\theta$, ^d Value for h_a if the observed hermaphrodites frequency is used as fs_0 (Leslie and Klein, 1996), ^e Value for fs_0 if the observed hermaphrodites frequency is used as fs_0 (Leslie and Klein, 1996)

population was the predominant population isolated in the area evaluated, although *G. subglutinans* was the prevalent one (39%) isolated from Buenos Aires. This result could be explained by the climatic conditions of this region characterized by mid summer and moderate winter and the Atlantic winds that produce low temperature and humid conditions. The average annual rain fall, in this region, varied from 550 to 950 mm. The inverse ratio between *G. moniliformis* and *G. subglutinans* also has been observed in other regions from Germany, Austria, Poland and Argentina (Torres *et al.*, 2001; Lew *et al.*, 1990; Logrieco *et al.*, 1993).

In theory, mating type need to be at 1:1 ratio since the trait is known to be controlled by a single Mendelian locus in heterothallic Ascomycetes (Leslie *et al.*, 1996). As it was demonstrated by the effect of the $N_{e\ (mt)}$ a deviation from the ratio 1:1 could affect the sexual reproduction process.

Previous results on G. moniliformis population showed that the mating type ratio could be equally distributed like as in populations isolated from maize in USA (Leslie et al., 1992) and Italy (Moreti et al., 1995). In other populations the mating type ratio was significant different from 1:1, being the frequency of the MATA-1 higher than the MATA-2 (Leslie et al., 1996;1995). In the present study, the type MATA-1 from G. moniliformis mating population was more frequent that the MATA-2 (64 and 36%, respectively). This population showed an inverse behaviour that population from Tanzania, Korea and Argentina (Chulze 2000; Mansuetus et al., 1997; Park et al., 1999).

Leslie (1995) and Leslie and Klein (1996) provide experimental data that support the hypothesis that the proportion of female fertile strains in a population is an indicator of the frequency at which sexual reproduction occurs. They stated that the proportion of female fertile strains reaches a maximum following sexual reproduction, and that the number of female fertile strains decreases during vegetative propagation because of either mutations or selective advantage of the female sterile strains.

Data of $N_{e\,(ml)}$ and $N_{e\,(f)}$ of G. moniliformis population, with a high frequency of female fertile strains and a ratio MATA-2/MATA-1 (74:129) suggest that the sexual reproduction among members of this population will be favourable under field conditions. This will allow obtaining genetic variation by sexual recombination rather than of asexual recombination, selection and genetic drift. G. moniliformis population from Argentina showed similar behaviour in the female fertile strains frequency compared to populations from USA, Tanzania and Korea (Leslie and Klein 1996; Mansuetus $et\ al.$, 1997; Park $et\ al.$, 1999). Thus, even relatively discrepancies in mating type frequency do not reduce the effective population number to a degree such as drift can play a large role.

Results obtained from a population of G. moniliformis isolated from maize in a small region from Cordoba province, 50 km-diameter region, planted under no-till conditions, showed a $N_{e(m)}$ of 88% and $N_{e(f)}$ of 37% of the count (Chulze et al., 2000). Two factors could explain the differences in the values obtained in the present study, the origin of the strains, in this case from a wide region (300 km-diameter region) and the agricultural practices under which maize was planted (till and no-till conditions).

G. intermedia and G. subglutinans populations showed ratios MATD-2:MATD-1 and MATE-2:MATE-1 similar to those found in other populations from USA, Tanzania and Argentina (Leslie et al., 1996; Mansuetus et al., 1997; Torres et al. 2001; Leslie et al., 1992; Chulze et al., 1998).

G. intermedia population showed a lower hermaphrodite frequency than the G. moniliformis population, therefore the sexual reproduction could occur in this population, but at lower frequency than in the G. moniliformis population. The relative frequency of the different mating types does not appear to reduce the effective size of the population. The $N_{e(m)}$ and $N_{e(f)}$ values were similar to the values obtained from populations isolated from USA (Leslie et al., 1996).

 N_e values based on both mating type ratios and hermaphrodite frequencies are very low among members of G. subglutinans population. G. subglutinans population also has a lower percentage female fertile isolates than has been observed in most populations of

G. moniliformis or G. intermedia (Leslie and Klein, 1996), but it is comparable to that found in some populations of G. thapsinum or G. circinata (Leslie and Klein, 1996; Britz et al., 1998). The low estimate for N_e suggests that sexual reproduction is not common among members of this population. From these results we can assume that the vegetative propagation will be more important in this population than G. moniliformis and G. intermedia populations. Leslie and Klein (1996) explained the absence of sexual reproduction in local populations of the G. fujikuroi species complex by the presence of mutations that concomitantly resulted in female sterility with an increased vegetative propagation capability. Selection for an increased number of asexual propagules can result in a selective accumulation of female sterile strains, which could become prevalent even in large geographic areas. The relatively long cycle time (74 - 298) would provide sufficient time for the selection and dispersal of clones.

In conclusion, sexual reproduction in *G. moniliformis* population, with the lowest percentage of female sterile strains (48%), is nearly twice old frequent than in *G. intermedia* population and four fold than among members of *G. subglutinans* population. The mutation rate in the different mating populations analyzed (Table 3) suggests that members of *G. moniliformis* population will have reproduction advantages due to the less mutation probability from female fertile to female sterile strains.

The results obtained from *G. moniliformis* and *G. intermedia* suggest a high probability of sexual reproduction under field conditions, this could render the perithecia production in the field. At present we have not evaluated the presence of perithecia under field conditions.

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