Statistical Analysis of Electrophoretic Karyotype Variation Among Vegetative Compatibility Groups of *Fusarium oxysporum* f. sp. *cubense*

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This study tests whether variation in electrophoretic karyotype (EK) is correlated with presumptive clonal lineages as defined by vegetative compatibility groups (VCGs) in an asexual fungal plant pathogen. Highly reproducible EKs were generated for 118 isolates of the banana wilt pathogen, *Fusarium oxysporum* f. sp. *cubense*, representing 15 VCGs in a worldwide collection. Extensive EK differences were observed among the isolates: the chromosome number (CN) ranged from nine to 14 (median = 11; mode = 12), and the genome size (GS) ranged from 32.1 to 58.9 Mbp (mean ± standard deviation = 43.3 ± 5.8 Mbp). EK mean variation among 11 analyzed VCGs, however, was highly associated with VCGs, as determined by analysis of variance ($P < 0.0001$). Comparison of means for both CN and transformed GS data sets identified two groupings of EK types containing identical VCG constituencies: Type I is characterized by high CN and GS values and includes VCGs 0124, 0124-5, 0125, 1210, and 1214, members of which generally had been isolated from banana cultivars with ABB and AAB genotypes. Type II is characterized by low CN and GS values and includes VCGs 0120, 0121, 0122, 0123, 0129, and 1213, which usually had been isolated from cultivars with AAA genotypes. With the exception of VCG 0123, these two EK types correspond to preexisting groupings based on physiology, host specificity, and other molecular markers.

Additional keywords: chromosome polymorphisms; filamentous fungus; pulsed field gel electrophoresis; telomeres.

Determinations of fungal karyotypes with light microscopy have often been hindered by the presence of numerous small chromosomes and further confounded by peculiarities inherent to nuclear division among the higher fungi. These peculiarities include the retention of the nuclear envelope during karyokinesis, the absence of a typical metaphase plate, and asynchronous chromosome movement during anaphase. Nevertheless, definitive cytological karyotypes have been obtained among plant-pathogenic fungi from three-dimensional reconstructions of serially sectioned pachytene nuclei analyzed at the ultrastructural level (Boehm and McLaughlin 1990; Boehm *et al.* 1992; Boehm and Bushnell 1992). Although such studies have corrected earlier light-microscopic estimates of chromosome number and have provided comparable genome size estimates, they are labor-intensive and thus limited in scope. These methods have been largely superseded by pulsed field gel electrophoresis, a technique that allows the simultaneous comparison of numerous isolates to generate electrophoretic karyotypes (EKs), from which rapid determination of chromosome number and genome size is possible (Skinner *et al.* 1991).

Highly variable intraspecific EKs have been reported among isolates of plant-pathogenic fungi, altering our view of fungal genomes as typological entities (for review see Kistler and Miao [1992]). Usually, isolates chosen for analysis have belonged to distinct subspecific groupings based on, for example, single- and multilocus haplotypes (McDonald and Martinez 1991; Talbot *et al.* 1993), host specificities (Masel *et al.* 1990; Morales *et al.* 1993), and geographic origins (Kinscherf and Leong 1988). However, since such studies included only a small representational number of isolates, they may not have quantitatively measured the limits of intraspecific fungal EK variation.

The present study was undertaken as a first step toward measuring the relative level of EK variation within a *forma specialis* of a fungal plant pathogen. We were interested in whether pulsed field gel electrophoresis can be used to differentiate between inter- and intrapopulational differences in karyotype and whether these differences are correlated with presumptive clonal lineages as defined by vegetative compatibility groups (VCGs). Chosen for study was the asexual fungus *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cubense* (E. F. Sm.) W. C. Snyder & H. N. Hans., the pathogen causing Fusarium wilt of banana, or Panama disease. To generate a sufficiently large sample, 118 isolates of *F. o.* f. sp. *cubense* collected from sources worldwide, representing 15 VCGs, were analyzed to determine their chromosome number (CN) and genome size (GS). Isolates within a VCG are considered to be genetically distinct from those in other VCGs, since they can form heterokaryons in culture (Ploetz and Correll 1988; Puhalla 1985) and may share common physiological attributes (Moore *et al.* 1991, 1993; Pegg *et al.* 1993; Ploetz 1990a), cultivar specificities (Ploetz 1990a,b), and molecular markers (Kistler *et al.* 1991; H. C. Kistler, unpublished; Ploetz 1990a; Sorensen *et al.* 1993).

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RESULTS

Highly reproducible EKs were generated for each of the 118 *F. o.* f. sp. *cubense* isolates (Table 1 and Figs. 1 and 2). No changes in CN or significant differences in calculated GS values were observed for any isolate, despite repeated subculturing and sample preparation. Chromosome-sized DNAs were resolved under a single set of empirically derived running conditions (see Materials and Methods). To determine whether larger DNAs had not been resolved under these conditions, the run times were doubled, to 480 hr. No new DNA species were detected for a representative sampling of isolates (n = 13), which implies that the upper limits of the karyotype had been resolved (data not presented). Running 1% agarose gels at 4.5 V/cm with unramped 30-min switch intervals overnight, conditions which resolve the *Saccharomyces cerevisiae* karyotype, likewise did not resolve any new DNA species of lower molecular weight (n = 27), which implies that the full chromosome complement had been electrophoretically resolved (data not presented). Decreasing either switch intervals or total run times resulted in diminished resolution, especially for the higher molecular weight species of a given EK.

To confirm that chromosome-sized DNA molecules separated by pulsed field gel electrophoresis represent chromosomes, Southern blots were stringently hybridized with the *F. oxysporum* f. sp. *lycopersici* telomeric repeat $[TTAGGG]_{18}$ cloned in the plasmid pLD. Without exception, hybridization to pLD was detected for each chromosome-sized DNA from all isolates in VCGs 0120, 0121, and 0122 and one representative isolate from each of the remaining VCGs (Fig. 3 and additional data not presented). Thus, all separated chromosome-sized DNA molecules from *F. o.* f. sp. *cubense* will henceforth be referred to as chromosomes.

The CN values of the 118 *F. o.* f. sp. *cubense* isolates ranged from nine to 14, and the GS values from 32.1 to 58.9 Mbp (Table 1 and Fig. 1). The median CN was 11, with a modal value of 12, and the mean GS was 43.3 Mbp (standard deviation = 5.8; standard error = 0.54). Differences in both chromosome length and CN contributed to the variation in calculated genome size. With the sole exception of VCG 0121, every VCG had isolates with variable lower molecular weight chromosomes of less than 2 Mbp (Figs. 1–3). Interestingly, CN and GS were positively correlated when plotted (data not presented), and as GS increased, so did CN (R^2 = 0.995; coefficient of variance = 7.2; $P < 0.0001$).

Despite the differences observed in chromosome length and number, little variation was detected in hybridization to a plasmid containing the major ribosomal RNA genes from *Nectria haematococca* (Figs. 1 and 2). In most cases, hybridization was to a single chromosome of high molecular weight. However, among isolates in VCG 0124, hybridization was frequently to the two largest chromosomes and, in isolate 92 (VCG 0128), hybridization was to a single low molecular weight chromosome of 2.2 Mbp (Figs. 1 and 2).

CN variation within 14 of the 15 VCGs never exceeded plus or minus one chromosome, the exception being VCG 0120, members of which had four CN values (Fig. 4). Isolates in VCGs 0123, 0129, 1210, and 1213 had only two CN values, while isolates in VCGs 0121, 0126, 0128, 1212, and 1214 had only one CN value. This illustrates the limited distribution of CN values in each VCG, compared to the totals (Fig. 4). Multiple range tests of CN means for the 11 analyzed VCGs are presented in Figure 5. Four groups can be resolved, comprising the following VCGs: 1) VCG 0125; 2) VCGs 0124, 1210, 1214, and 0124-5; 3) VCGs 0120, 1213, and 0123; and 4) VCGs 0122, 0129, and 0121. The division between groups 2 and 3 is substantially larger than that separating the others (Fig. 5). We have subjectively used this division to form the basis of two greater EK groups: group I (VCGs 0125, 0124, 1210, 1214, and 0124-5) and group II (VCGs 0120, 1213, 0123, 0122, 0129, and 0121).

The extensive intraspecific EK variation in GS values was not due to chance distribution patterns. For the 11 VCGs tested, GS variation within a VCG was always less than that of the population as a whole, as determined by analysis of variance (ANOVA) on both transformed (see Materials and Methods) and untransformed GS data sets (Table 2). Approximately 72% of the variation in GS is accounted for by preexisting groupings based on VCG ($P < 0.0001$). Likewise, the transformed GS values gave equally high R^2 values with correspondingly lower coefficients of variance (Table 2). These findings support the conclusion that the GS values of the 11 analyzed VCGs deviate strongly from the null hypothesis and that differences do exist among VCGs with regard to GS value distributions. ANOVA was also used to compare the GS values of isolates from nine different geographic locations (see Materials and Methods). The highly significant effect of geographic location (F = 14.5, $P <$ 0.0001) indicates that the isolates were not randomly distributed geographically with respect to GS.

Multiple range tests for transformed and untransformed GS means are presented in Figure 6. The linear relationship between GS and CN explains the similarity in the ordering of the 11 VCGs in Figures 5 and 6. In the untransformed GS means, no groups are discernible, whereas in the GS means subjected to square root transformation (SQGS), distinct groups are evident (Fig. 6). In SQGS, two groups are clearly evident: group I (VCGs 0124, 0124-5, 1210, 0125, and 1214) and group II (VCGs 0120, 0123, 1213, 0121, 0122, and 0129). The groups identified by multiple comparison of the SQGS data produce group constituencies identical to those we have defined by comparison of the CN means, although the exact ordering of VCGs within the groups may differ slightly (compare Figs. 5 and 6). The same VCGs belong to both group I based on CN and group I based on SQGS (namely, VCGs 0124, 0124-5, 1210, 0125, and 1214), while the remaining six VCGs belong to group II in both cases (Figs. 5 and 6).

Assuming that our sampling of 118 *F. o.* f. sp. *cubense* isolates is representative of the population as a whole, we suggest two broad groupings or types for this fungal plant pathogen, based on EK variation: type I includes VCGs 0124, 0124-5, 0125, 1210, and 1214 and is characterized by high CN (11–14) and GS (39.9–58.9 Mbp) values, whereas type II includes VCGs 0120, 0121, 0122, 0123, 0129, and 1213 and is characterized by low CN (nine to 12) and GS (32.1–44.9 Mbp) values. Other features found in *F. o.* f. sp. *cubense* further support this proposed dichotomy (see Discussion).

The two EK types are not as readily resolved in a plot of GS means and associated standard deviations (Fig. 7) or 99% confidence interval spreads (data not presented) as they are in

Table 1. Chromosome number (CN) and genome size (GS) of *Fusarium oxysporum* f. sp. *cubense* isolates used in this study

VCG[a]	Isolate[b]	CN	GS	Cultivar[c]	Race[d]	Origin and collector[e]
0120	1. A2.132*	11	42.22	Mons mari	4	Australia, f
	2. 1220.117	11	41.87	Mons	?	Queensland, Australia, c
	3. 1222.130*	10	38.60	Mons	4?	Queensland, Australia, c
	4. 22424.128	12	44.37	Lady finger	?	Moorina, Queensland, Australia, g
	5. 22425.113	11	42.30	Cavendish	4?	Wamuran, Queensland, Australia, g
	6. 22411.129	11	42.25	Cavendish	4?	Wamuran, Queensland, Australia, g
	7. Pacovan.147	10	39.80	Pacovan	?	Bahia, Brazil, n
	8. Prata.126	10	40.35	Prata	?	Bahia, Brazil, n
	9. C1.127	10	39.55	Cavendish	4?	Canary Islands, f
	10. ADJ1.133	11	40.75	Dwarf Cavendish	4	Adeje, Canary Islands, d
	11. ADJ2.124	11	40.55	Dwarf Cavendish	4	Adeje, Canary Islands, d
	12. BUE1.140	11	40.85	Dwarf Cavendish	4	Buenavista, Canary Islands, d
	13. GAL1.139	9	33.50	Dwarf Cavendish	4	Las Galletas, Canary Islands, d
	14. GAL2.120	11	41.25	Dwarf Cavendish	4	Las Galletas, Canary Islands, d
	15. IC1.128	11	41.80	Dwarf Cavendish	4	Icod de los Vinos, Canary Islands, d
	16. ORT1.137	10	40.95	Dwarf Cavendish	4	La Orotava, Canary Islands, d
	17. ORT2.115	10	42.00	Dwarf Cavendish	4	La Orotava, Canary Islands, d
	18. PAJ1.136	11	44.90	Dwarf Cavendish	4	Pajalillos, Canary Islands, d
	19. FCJ7.146	11	43.00	Lacatan	?	Jamaica, q
	20. STGM1.135*	9	34.90	Gros Michel	1	Costa Rica, i
	21. 3S1.114*	11	42.80	Highgate	1	Honduras, i
	22. STH1.148	11	42.55	Highgate	1	Honduras, i
	23. 15638.145	11	41.60	?	?	Malaysia, a
	24. F9127.144*	11	42.65	Grande naine	4	South Africa, g
	25. NH.142	11	43.40	Williams	4?	South Africa, f
0121	26. F9130.150	9	37.08	Cavendish	4	Taiwan, g
	27. GM.149	9	37.28	Gros Michel	4?	Taiwan, h
	28. ML.164*	9	37.26	Cavendish	4	Taiwan, h
	29. H1.163	9	37.85	Cavendish	4	Taiwan, e
0122	30. Ph2.01	10	36.26	Cavendish	4?	Philippines, l
	31. P18.04	10	38.31	Cavendish	4	Philippines, h
	32. P79.05	9	33.00	Cavendish	4	Philippines, h
	33. SABA.07	9	32.21	Saba	2	Philippines, h
	34. PW3.08	9	33.20	Cavendish	4?	Philippines, l
	35. PW4.09	9	32.13	Cavendish	4?	Philippines, m
	36. PW6.11	9	36.06	Cavendish	4?	Philippines, m
	37. PW7.12	9	36.35	Cavendish	4?	Philippines, m
	38. PW5.10	11	43.40	Cavendish	4?	Philippines, m
0123	39. DAVAO.151	10	40.50	Silk	1?	Philippines, h
	40. Ph12.161	10	39.80	Latundan	?	Philippines, l
	41. PhL2.160	10	40.70	Latundan	?	Philippines, i
	42. T1.157	11	42.75	Gros Michel	1	Taiwan, f
0124	43. GMB.16	12	44.50	Gros Michel	?	Bahia, Brazil, n
	44. MACA.17	12	47.45	Maça (Silk)	?	Bahia, Brazil, n
	45. STD2.20	14	54.90	Highgate	1	Honduras, i
	46. BLUG.21	13	52.50	Bluggoe	2	Honduras, h
	47. STJ1.27	13	57.80	Grande naine	?	Jamaica, i
	48. MW43.30	13	53.85	Harare	?	Chitipa, Karonga, Malawi, b
	49. MW45.31	14	52.87	Harare	?	Chitipa, Karonga, Malawi, b
	50. MW47.32	13	48.07	Harare	?	Chesenga, Malawi, b
	51. MW50.34	13	48.65	Harare	?	Chitipa, Karonga, Malawi, b
	52. MW52.35	12	49.82	Sukali (Silk)	?	Karonga South, Malawi, b
	53. W54.36	12	45.35	Harare	?	Karonga South, Malawi, b
	54. MW55.37	12	43.32	Harare	?	Karonga, Malawi, b
	55. MW58.38	12	50.00	Harare	?	Karonga, Malawi, b
	56. MW64.39	13	52.20	Harare	?	Kaporo North, Malawi, b
	57. MW65.40	12	50.92	Harare	?	Hara Plain, Karonga, Malawi, b
	58. MW69.42	13	46.15	Kholobowa	?	Thyolo, Blantyre, Malawi, b
	59. MW71.43	12	50.75	Kholobowa	?	Mulanje, Blantyre, Malawi, b
	60. STN1.47	12	47.10	Bluggoe	?	Corinto, Nicaragua, i
	61. STPA2.55	12	48.10	Pisang awak	?	Tanzania, i
	62. B2-1.57	14	58.90	Burro (Bluggoe)	2	Florida, b
	63. JLTH3.199	13	48.85	Klue namwa	?	Smoeng Hwy. 1269, Thailand, v
	64. JLTH7.203	13	48.80	Klue namwa	?	Smoeng Hwy. 1269, Thailand, v
	65. JLTH4.200	12	45.75	Klue namwa	?	Smoeng Hwy. 1269, Thailand, v
	66. JLTH5.201	12	49.70	Klue namwa	?	Smoeng Hwy. 1269, Thailand, v

(continued on next page)

Table 1. (*continued from preceding page*)

VCG[a]	Isolate[b]	CN	GS	Cultivar[c]	Race[d]	Origin and collector[e]
0124-5	67. MW5.62	12	48.97	Zambia (Bluggoe)	?	Kaporo, Malawi, r
	68. MW11.64	12	48.97	Harare	?	Kaporo, Malawi, r
	69. MW39.66	13	54.75	Harare	?	Chitipa, Karonga, Malawi, b
	70. MW53.67	13	55.00	Sukali (Silk)	?	Karonga, Malawi, b
	71. MW56.68	12	55.30	Zambia	?	Karonga, Malawi, b
	72. MW61.71	11	48.70	Harare	?	Vinthukutu, Karonga, Malawi, b
	73. MW66.73	12	52.85	Kholobowa	?	Thyolo, Blantyre, Malawi, b
	74. MW70.75	12	52.75	Kholobowa	?	Thyolo, Blantyre, Malawi, b
	75. MW86.76	11	49.10	Mbufu (Bluggoe)	?	Chitipa, Karonga, Malawi, b
	76. JLTH1.197	12	39.95	Klue namwa	?	Smoeng Hwy. 1269, Thailand, v
	77. JLTH16.212	12	44.25	Klue namwa	?	Ban Nok, Thailand, v
	78. JLTH17.213	11	40.75	Klue namwa	?	Ban Nok, Thailand, v
	79. JLTH18.214	11	41.50	Klue namwa	?	Ban Nok, Thailand, v
0125	80. A1.78	13	48.20	Lady finger	1	Australia, f
	81. 8611.82	14	47.75	Lady finger	1	Currumbin, Queensland, Australia, g
	82. 22479.86	14	47.20	Ducasse	1	Bowen, Queensland, Australia, g
	83. 1S.92	13	43.50	Williams	?	Bodles, Jamaica, i
	84. STPA3.93	12	44.60	Pisang awak	?	Uganda, i
	85. STNP5.94	13	42.75	Ney poovan	?	Zaire, i
	86. JLTH20.216	14	44.40	Klue namwa	?	Ban Nok, Thailand, v
	87. JLTH21.217	14	44.70	Klue namwa	?	Ban Nok, Thailand, v
0126	88. STM3.165*	11	41.62	Maqueño	1	Honduras, i
	89. STA2.158	11	41.71	Highgate	1	Honduras, i
	90. STB2.172	11	40.85	Highgate	1	Honduras, i
0128	91. 22994.96	10	38.42	Bluggoe	2	Johnstone, Queensland, Australia, g
	92. A47.97	10	38.02	Bluggoe	?	Comoro Islands, j
0129	93. N5443.169	9	33.45	Cavendish	4?	Doonan, Queensland, Australia, g
	94. 8627.170	9	33.45	Cavendish	4?	N. Arm, Queensland, Australia, g
	95. 22401.171	9	33.47	Cavendish	4?	Wamuran, Queensland, Australia, g
	96. 1221.166	10	36.52	Mons	1	Queensland, Australia, c
	97. 22507.167	9	34.05	Lady finger	?	Queensland, Australia, g
1210	98. GG1.174	13	45.45	Apple (Silk)	1	Florida, b
	99. JC1.189	12	45.75	Apple	1	Florida, b
	100. JC4.186	12	45.75	Apple	1?	Florida, b
	101. JC7.181	12	45.75	Apple	1?	Florida, b
1211	102. SH3142.190	10	38.32	SH 3142	?	Queensland, Australia, g
1212	103. STNP1.98	10	36.40	Ney poovan	?	Pemba Island, Zanzibar, Tanzania, i
	104. STNP3.100	10	36.70	Ney poovan	?	Pemba Island, Zanzibar, Tanzania, i
1213	105. 1-2.119	11	41.65	Cavendish	4	Taiwan, u
	106. 2-2.195	11	42.30	Cavendish	4	Taiwan, u
	107. 4-1-1.194	10	39.15	Cavendish	4	Taiwan, u
	108. 4-2-1.193	10	35.75	Cavendish	4	Taiwan, u
	109. 5-1-1.192	11	41.20	Cavendish	4	Taiwan, u
	110. 6-2.191	11	41.00	Cavendish	4	Taiwan, u
1214	111. MW2.102	12	45.60	Harare	?	Misuku Hills, Karonga, Malawi, r
	112. MW7.103	12	45.60	Harare	?	Misuku Hills, Karonga, Malawi, r
	113. MW40.104	12	43.85	Harare	?	Misuku Hills, Karonga, Malawi, b
	114. MW41.105	12	43.85	Mbufu (Bluggoe)	?	Misuku Hills, Karonga, Malawi, b
	115. MW44.107	12	43.85	Harare	?	Misuku Hills, Karonga, Malawi, b
	116. MW46.108	12	43.85	Harare	?	Misuku Hills, Karonga, Malawi, b
	117. MW48.109	12	43.85	Harare	?	Misuku Hills, Karonga, Malawi, b
	118. MW51.110	12	43.85	Harare	?	Misuku Hills, Karonga, Malawi, b

[a] Vegetative compatibility groups (VCGs) were determined and numbered by R. C. Ploetz in Homestead, Florida, using nitrogen metabolism (nit) mutants according to the protocols of Cove (1976) as modified by Puhalla (1985).

[b] The accession numbers combine those of the collections at the Tropical Research and Education Center, Homestead, Florida, and at the University of Florida, Gainesville. Asterisks designate isolates for which partial electrophoretic karyotypes have been published by Miao (1990).

[c] Cultivars are inter- and intraspecific diploid or triploid hybrids of *Musa acuminata* (AA) and *M. balbisiana* (B). The ploidy levels and constitutions of the cultivars are as follows: AAA = Gros Michel, Highgate, Mons (Mons mari), Cavendish, Dwarf Cavendish, Grande naine, Williams, Lacatan; AA = SH 3142 (synthetic clone); AAB = Lady finger, Pacovan, Prata, Silk, Latundan, Maqueño; ABB = Saba, Bluggoe, Harare, Kholobowa, Pisang awak, Klue namwa, Ducasse; AB = Ney poovan.

[d] Race designations were provided by the donors of the isolates and are based on cultivar specificity. A question mark indicates that the race is unknown. A number followed by a question mark indicates the probable race.

[e] Collector or original source: a, American Type Culture Collection; b, R. C. Ploetz, Homestead, Florida; c, Paul E. Nelson, Fusarium Research Center, University Park, Pennsylvania; d, J. H. Hernandez, Tenerife, Canary Islands; e, S.-C. Hwang, Taiwan Banana Research Institute, Pingtung; f, B. Manicom, Nelspruit, South Africa; g, K. Pegg, Brisbane, Australia; h, S. Nash Smith, Alameda, California; i, R. H. Stover, La Lima, Honduras; j, IFRA, Montpellier, France (via R. C. Stover); l, A. M. Pedrosa, Phillippines; m, N. I. Roperos, Philippines; n, Z. J. M. Cordeiro, EMBRAPA, Cruz das Almas, Brazil (via E. D. Loudres); q, J. Ferguson-Conie, Banana Board, Kingston, Jamaica; r, B. Braunworth, Oregon State Unviersity, Corvallis; u, Tsai-young Chuang, National Taiwan University, Taipei; v, J. Leslie, Kansas State University, Manhattan.

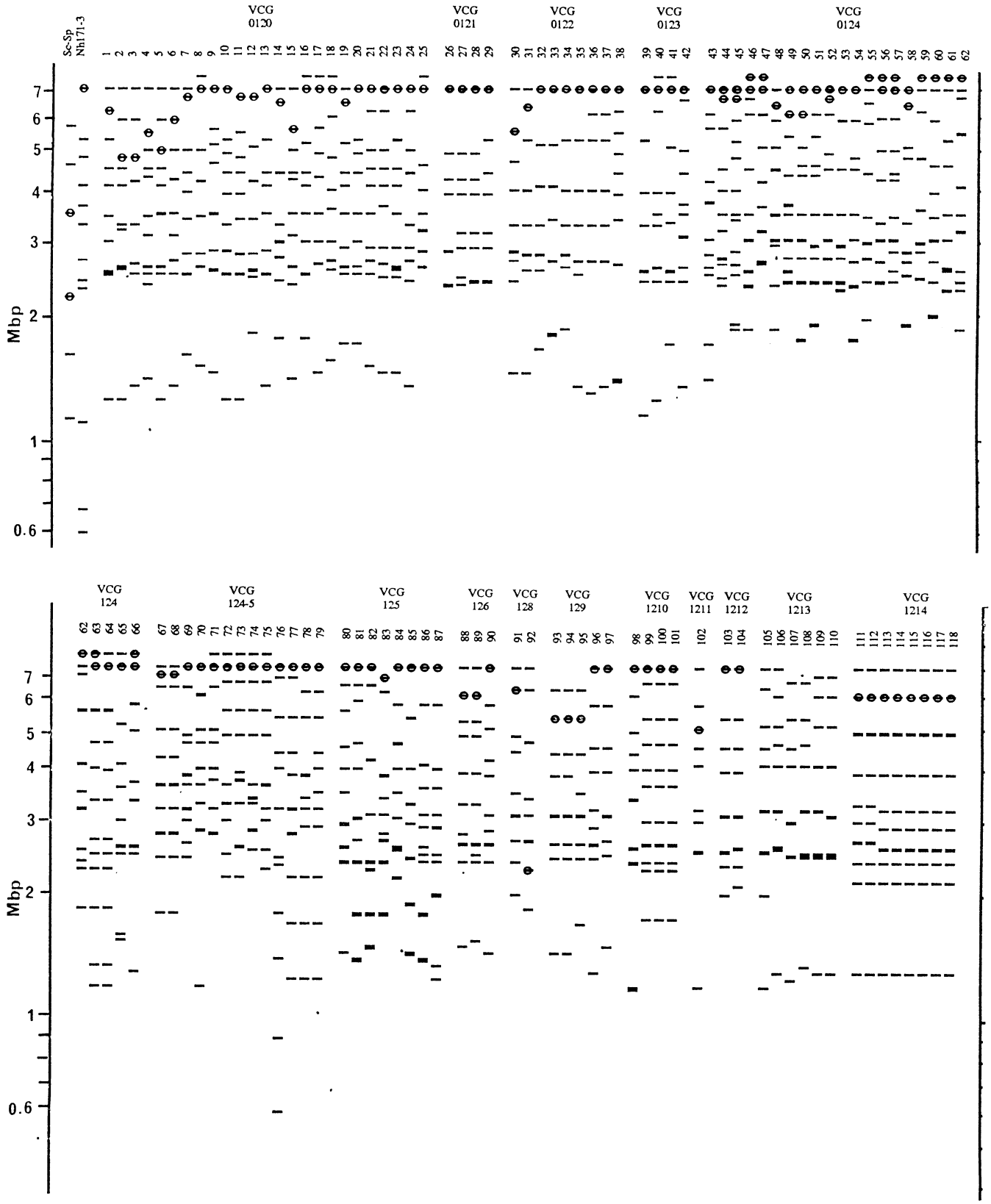

Fig. 1. Graphical representation of electrophoretic karyotypes generated for 118 isolates of *Fusarium oxysporum* f. sp. *cubense*, representing 15 vegetative compatibility groups (VCGs). The numbering of the isolates corresponds to that in Table 1. The three size standards used were *Schizosaccharomyces pombe* (Sp) and *Saccharomyces cerevisiae* (Sc), run in the same lane, and *Nectria haematococca* (Nh171-3). Hybridization to the *N. haematococca* ribosomal probe pUF8-3 is denoted by a circle over the chromosomal band. Isolates in VCG 1213 were not tested, and isolate 62 is represented twice.

the multiple comparison tests (Figs. 5 and 6). While Figure 7 indicates that most VCGs do not overlap, VCGs 0123 and 1213, in EK type II, have some degree of overlap with VCGs 1214 and 0125, in EK type I.

DISCUSSION

In this study we have been able to 1) measure the relative limits of EK variation within a *forma specialis* of an asexual fungal plant pathogen, 2) correlate this variation with genetically isolated lineages as defined by VCGs, and 3) discern two EK types, which correlate with VCG groupings previously established on the basis of host specificity, geographic origin, cultural physiology, and molecular markers.

The degree of EK variation within a VCG can be measured and compared to the absolute variance for the sample as a whole. CN values for the 118 isolates ranged from nine to 14. Therefore, the chromosome number of the smallest and largest differed by 56% of the smallest. Similarly, the smallest and largest GS values (32.1 and 58.9 Mbp) varied by 83% of the smallest, a span of nearly 27 Mbp. Within the most diverse VCG (0120), isolates varied by 33% in CN and 32% in GS. These values, as well as those for absolute variance of CN and GS, may be used to compare the level of EK diversity in other fungal collections when they become available.

The total genomic variation among the 118 *F. o.* f. sp. *cubense* isolates is large, but not so large as to suggest that isolates differ in ploidy. Indeed, the continuous nature of GS and CN variation suggests that isolates may differ because of aneuploidy. Preliminary evidence for aneuploidy in certain isolates is presented here. The ribosomal probe used in this study appeared to hybridize to two chromosomes in some strains of VCG 0124, the VCG with isolates having the largest mean genome size. Chromosomal length polymorphisms may also explain a degree of GS variation. For example, the genes for the major ribosomal RNAs are located on chromosomes of >5 Mbp in all isolates except isolate 92 (VCG 0128), in which they are found on a 2.2-Mbp chromosome. The NOR (nucleolus organizer region) chromosome, therefore, may vary more than twofold in size. Similar length polymorphisms for other chromosomes may be as yet undetected.

Additionally, a positive linear relationship exists between CN and GS in *F. o.* f. sp. *cubense*, meaning that increasing genome size is directly related to increasing number of chromosomes. Although this observation seems self-evident at first, such a relationship is by no means a general rule for genomic organization in fungi. For example, while the genome sizes of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are roughly equal (Fan *et al.* 1989), *S.*

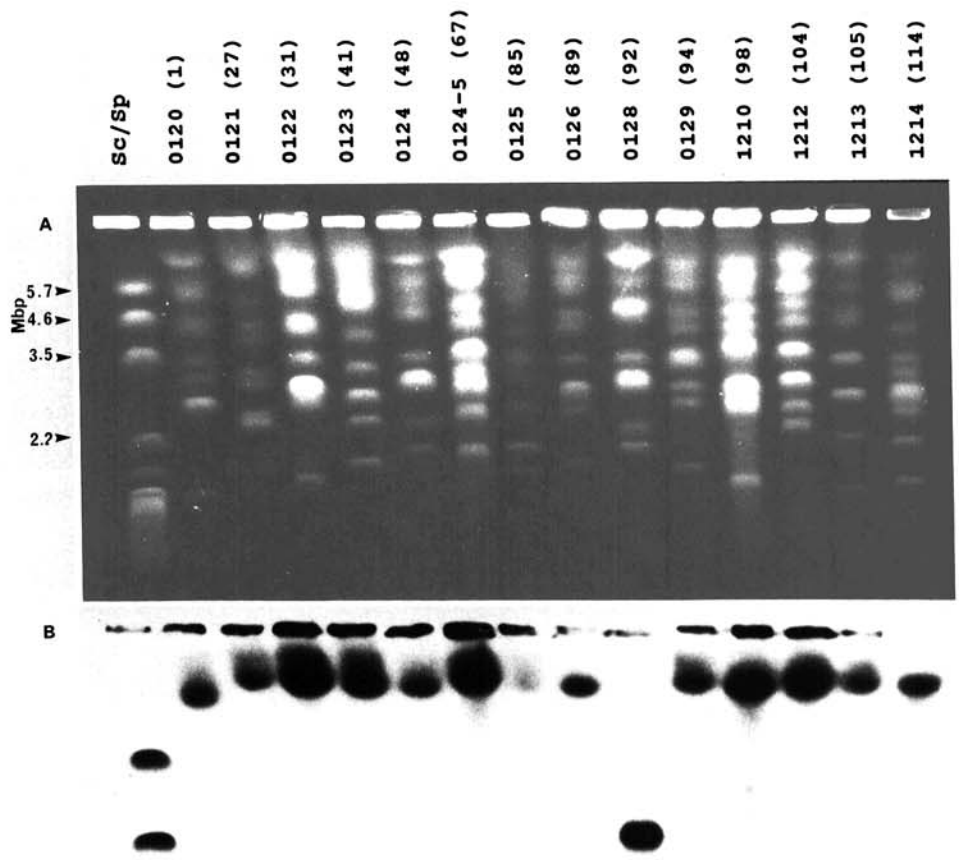

Fig. 2. Electrophoretic karyotypes of representative *Fusarium oxysporum* f. sp. *cubense* isolates from 14 of the 15 vegetative compatibility groups surveyed. The numbering of the isolates (in parentheses) corresponds to that in Table 1. The run parameters were as follows: 0.6% agarose gels were electrophoresed at 1.2 V/cm with a ramped switch time of $1.2–5.4 \times 10^3$ sec for 240 hr in 0.25× TBE (1× TBE is 89 mM Tris, 89 mM borate, and 2 mM EDTA, pH 8.0). **A,** Ethidium bromide–stained gel. **B,** Hybridization of the same gel to the *Nectria haematococca* ribosomal probe pUF8-3. The molecular weights indicated were obtained from *Schizosaccharomyces pombe* (Sp) and *Saccharomyces cerevisiae* (Sc) size standards run in the same lane.

cerevisiae has more than five times the number of chromosomes. We regard the positive relationship between CN and GS as further evidence of the aneuploid state of *F. o.* f. sp. *cubense*.

The extent of EK variation appears to differ among VCGs. For example, in VCG 0120 CN ranged from nine to 12 and GS from 33.5 to 44.9 Mbp, whereas in VCG 1214 CN was always 14 and GS ranged only from 43.9 to 45.6 Mbp. In general, diversity within a VCG seems to be related to the extent to which isolates of that group are distributed geographically. Large VCGs with cosmopolitan distributions (0120 and 0124) contain the greatest degree of polymorphism in karyotype, whereas VCGs with narrow geographic ranges (1210 and 1214) show little variation. The latter observation may be due to a founder effect. For example, although bananas have been commercially produced in Florida for about 100 years, Fusarium wilt has been observed on the cultivar Silk only within the last 20 years. The assumption that a single source or a small number of sources are responsible for this outbreak is corroborated by the minimal amount of variation noted in VCG 1210, a VCG which to date has been found only in Florida.

Some VCGs had remarkably uniform EKs despite different collection histories. For instance, the eight isolates in VCG 1214 from Misuku Hills, Malawi, collected by two different individuals, and the four isolates in VCG 0121 from Taiwan, collected by three different individuals, had single CN values of 14 and nine, respectively, and GS values ranging only from 43.8 to 45.6 Mbp and 37.0 to 37.8 Mbp, respectively (Table 1). To a lesser extent, characteristic EKs were observed among other isolates in VCGs 0122, 0123, 0126, 0129, 1210, and 1213 (Table 1 and Fig. 1). The EK diversity within a VCG may also reflect the amount of time during which the lineage has been genetically isolated. "Older" VCGs are more apt to have been spread by human activity to diverse geographic locations, whereas newly emerging VCGs may have had less time to spread and to accumulate divergent karyotypes. Alternatively, it is also possible that isolates of narrowly distributed VCGs represent genotypes uniquely suited for the environmental and host conditions found in that region.

There exist significant differences in GS values among the VCGs. Approximately 72% of this variation can be explained by *F. o.* f. sp. *cubense* groupings based on vegetative com-

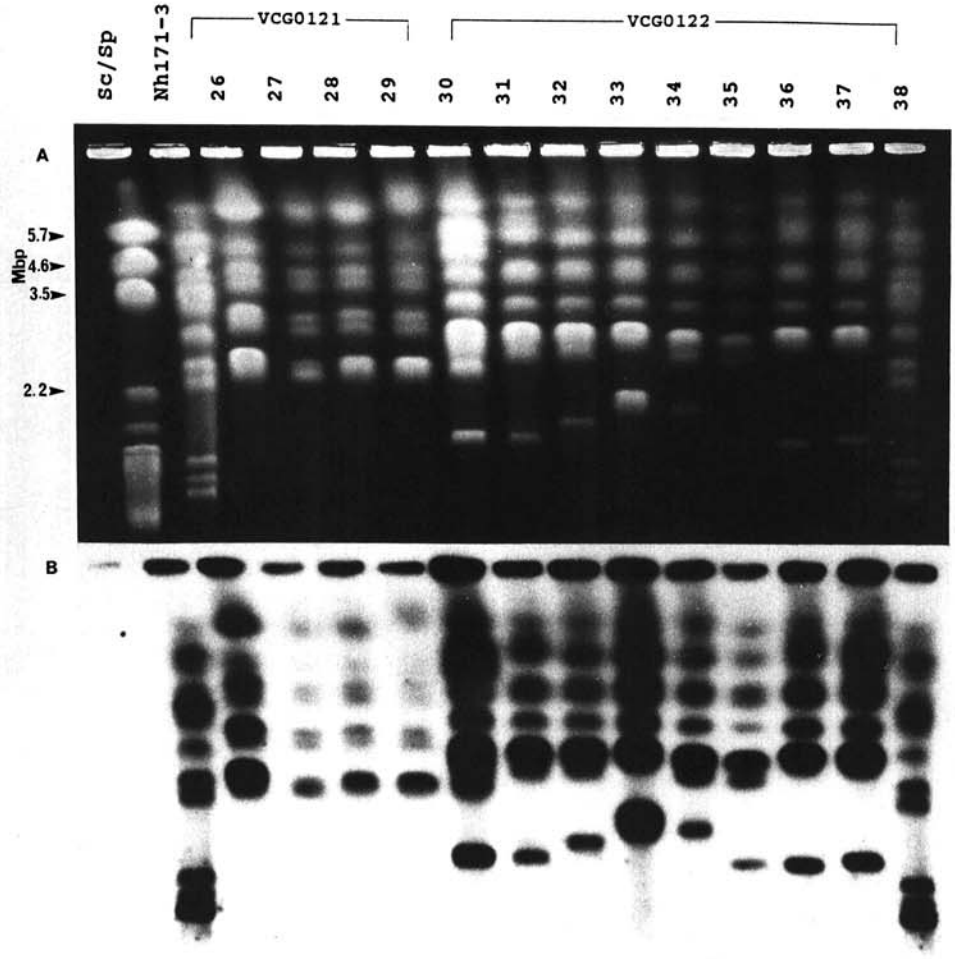

Fig. 3. Electrophoretic karyotypes for the *Fusarium oxysporum* f. sp. *cubense* isolates in vegetative compatibility groups (VCGs) 0121 and 0122. The numbering of the isolates corresponds to that in Table 1. **A,** Ethidium bromide gel. **B,** Hybridization of the same gel to the telomeric consensus sequence $[TTAGGG]_{18}$ isolated from *F. oxysporum* f. sp. *lycopersici* cloned in pLD (Kistler and Benny 1992). The same molecular weight standards and run parameters were used as in Figures 1 and 2. The probe did not hybridize to the two yeast standards.

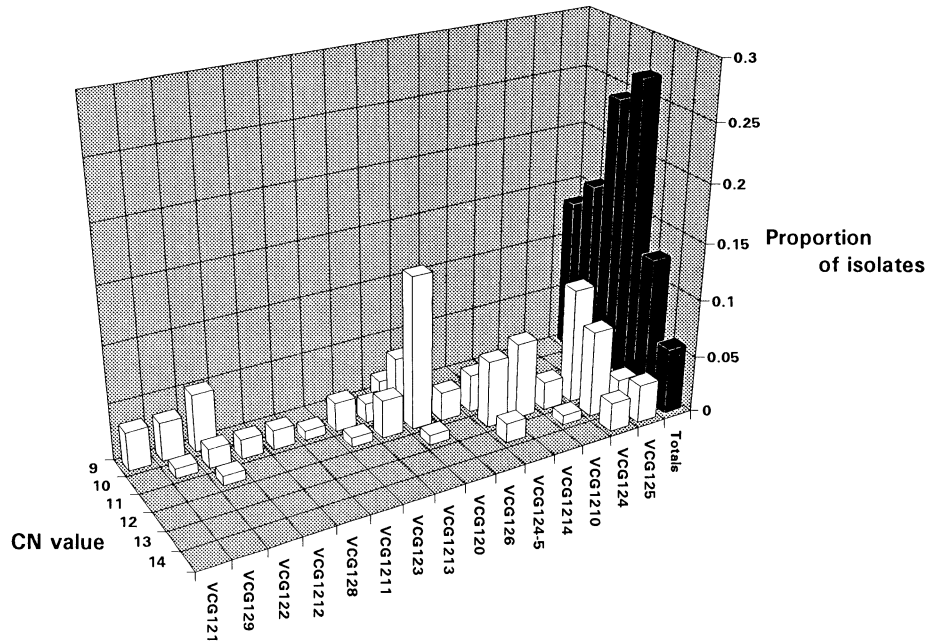

Fig. 4. Frequency distribution of chromosome number (CN) values for the 118 *Fusarium oxysporum* f. sp. *cubense* isolates surveyed. Vegetative compatibility groups (VCGs) are ordered from smallest to largest CN values.

Chromosome number (CN) means

		VCG	Mean	N
	(1)	125	98.56	8
I		124	85.62	24
		1210	78.37	4
	(2)	1214	72.50	8
		124-5	67.19	13
		120	37.06	25
	(3)	1213	36.83	6
		123	28.50	4
II		122	15.72	9
	(4)	129	11.50	5
		121	8.50	4

Fig. 5. Significance of chromosome number of the 11 vegetative compatibility groups (VCGs) of *Fusarium oxysporum* f. sp. *cubense* surveyed containing four or more isolates (a total of 110 isolates), as determined by the Waller-Duncan K ratio T test. Means connected by the same line are not significantly different at the 0.0001 probability level. K ratio = 100; df = 99; mean square error = 169.49; F = 52.54; critical T value = 1.76; minimum significant difference = 12.53.

patibility at highly significant probability levels. This suggests that VCGs may serve as effective barriers to genetic exchange in nature and may indeed be composed of clonal lineages. Additionally, since mutations that lead to chromosomal polymorphisms occur infrequently but persist abundantly in nature (Kinscherf and Leong 1988; Masel *et al.* 1990; McDonald and Martinez 1991; Morales *et al.* 1993; Talbot *et al.* 1993; Taylor *et al.* 1991), they may reflect selectively neutral genomic rearrangements (Kistler and Miao 1992). As such, they are as likely to correspond to clonal lineages as do other neutral mutations, such as those identified by anonymous restriction fragment length polymorphisms (Levy *et al.* 1991; Talbot *et al.* 1993).

Kistler and Miao (1992) presented the meiotic maintenance hypothesis, which predicts that EK variation should be more extensive in asexual fungal species than in species that have even a limited ability to undergo meiosis. In that regard, in the genus *Fusarium*, Yan and Dickman (1992) reported EK uniformity among geographically disparate isolates of *F. moniliforme* having different host ranges, but all having the ability to undergo meiosis in nature. Similarly, Xu and Leslie (1992), working with the same fungus, found identical EKs only among isolates within the same mating group. Whereas EK stability has been reported for the sexually reproducing *F. moniliforme*, the present report demonstrates a higher degree of EK variation among the asexually reproducing *F. oxysporum*. A more extensive comparison of quantitative levels of chromosomal variation in sexual and asexual species of *Fusarium* could determine the influence of meiosis in maintaining genomic organization.

We have proposed herein two broad groupings of *F. o.* f. sp. *cubense,* based on the extent of EK variation. Host specificity based on cultivar genotype lends further support to this proposed dichotomy. Edible bananas are inter- and intraspecific hybrids of two primitive diploid species, *Musa acuminata* (AA genome) and *M. balbisiana* (BB genome) (Kaemmer *et al.* 1992; Stover and Simmonds 1987). *F. o.* f. sp. *cubense* isolates in type I generally were isolated from cultivars with partial B genomes, such as Harare (ABB) and Silk (AAB), whereas type II isolates generally were recovered from dessert banana cultivars with pure A genomes, such as Cavendish and Gros Michel (both AAA) (see Table 1). Cultivar specificity among the two EK types broadly corresponds to race designations. For instance, all isolates from Taiwan (VCGs 0121, 0123, and 1213) belonged to type II and were mainly collected from AAA cultivars. Likewise, all isolates from Malawi (VCGs 0124, 0124-5, and 1214), Thailand (VCGs 0124, 0124-5, and 125), and Florida (VCGs 0124 and 1210) belonged to type I and were mainly from A × B hybrids. Approximately 89% of the isolates surveyed have

Table 2. Analysis of variance of the untransformed and transformed genome size data set for 110 isolates of *Fusarium oxysporum* f. sp. *cubense* in 11 vegetative compatibility groups (VCGs)

Genome size data set	Source	df	Sum of squares	Mean square	*F* value	*P* > *F*
Genome size	VCG	10	2,732.46	273.24	25.61	0.0001
	Error	99	1,056.45	10.67		
	Corrected total	109	3,788.91			
$R^2 = 0.721$	Coefficient of variance = 7.479					
Square root of genome size	VCG	10	15.870	1.587	26.94	0.0001
	Error	99	5.833	0.058		
	Corrected total	109	21.703			
$R^2 = 0.731$	Coefficient of variance = 3.681					

EK types which correspond with host specificity. However, cultivar support for EK types was not absolute. The Philippine isolates from VCG 0123, although having type II EKs, were all collected from type I hosts; other examples may also be found (Table 1). These exceptions, in which host genotype does not correlate with EK type, may reflect the evolutionary history of particular isolates or VCGs.

Our proposed grouping of *F. o.* f. sp. *cubense* based on EK types is also in agreement with those proposed by workers in Australia (Moore *et al.* 1991, 1993; Pegg *et al.* 1993; Sorensen *et al.* 1993). Sorensen *et al.* (1993) used random amplified polymorphic DNAs (RAPD) to analyze 10 *F. o.* f. sp. *cubense* isolates originating from Australia. When RAPD-generated DNA banding patterns were subjected to analysis by the unweighted pair group method with arithmetic mean, VCGs 0120, 0129, and 1211 clustered in one group, and VCGs 0124, 0124-5, 0125, and 0128 in another. These two groups broadly correspond to EK types II and I, respectively. Moore *et al.* (1991, 1993) studied volatiles produced by 245 *F. o.* f. sp. *cubense* isolates from Australia and two each from South Africa and Taiwan. When gas chromatograms of the headspace over steamed rice cultures were analyzed, all isolates in VCGs 0120, 0129, and 1211 produced characteristic volatile profiles; these were referred to as "odoratum" types. In contrast, isolates in VCGs 0123, 0124, 0124-5, 0125, and 0128 never produced volatile compounds and were referred to as "inodoratum" types. Pegg *et al.* (1993) also used gas chromatograms to investigate Asian isolates of *F. o.* f. sp. *cubense* and came to the same conclusions regarding VCG and volatile production as Moore *et al.* (1991, 1993); that is, the odoratum isolates were found consistently in the same VCGs. In general, their odoratum isolates were from the Philippines, Malaysia, and Indonesia, while the inodoratum isolates were from India, Thailand, and China. *M. acuminata* evolved in the rain forests of Southeast Asia, whereas *M. balbisiana* evolved in a monsoonal environment between the Indian subcontinent and Myanmar (Stover and Simmonds 1987). On the basis of their results and the origin and distribution of their Asian strains of *F. o.* f. sp. *cubense,* Pegg *et al.* (1993) suggested that the odoratum VCGs coevolved with *M. acuminata* in Southeast Asia and that the inodoratum VCGs coevolved with *M. balbisiana* in the region from India to Myanmar. Our proposal to divide *F. o.* f. sp. *cubense* populations into two EK types is in agreement with this view: Type II corresponds broadly to the odoratum group, in which VCG 0120 is central, while type I corresponds to the inodoratum group, in which VCGs 0124, 0124-5, and 0125 are central. Thus, two ancestral lines may exist in the pathogen causing Fusarium wilt of banana, each of which is differentiated by host specificity, cultural physiology, geographic origin, molecular markers, and variation in karyotype.

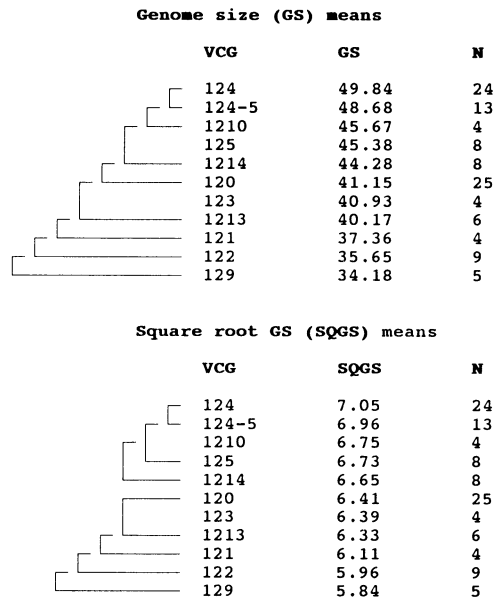

Fig. 6. Significance of untransformed genome size (GS) means and square root–transformed GS (SQGS) means of *Fusarium oxysporum* f. sp. *cubense*, as determined by the Waller-Duncan *K* ratio *T* test. Means connected by the same line are not significantly different at the 0.0001 probability level. *K* ratio = 100; df = 99; critical *T* value = 1.78. For GS, mean square error = 10.67; *F* = 25.60. For SQGS, mean square error = 0.05; *F* = 26.93.

MATERIALS AND METHODS

Isolates of *F. o.* f. sp. *cubense*.

Isolates of *F. o.* f. sp. *cubense* have been described previously (Ploetz 1990a,b; Ploetz and Correll 1988). The intraspecific sampling strategy used was to examine representative isolates of each of the 15 VCGs that have been identified in our worldwide collection of over 300 strains and also examine selected isolates within a VCG. VCGs with broad geographic ranges were sampled to include isolates from each country represented in the collection, housed at the Tropical Research and Education Center in Homestead, Florida. To

further investigate the degree of EK variation within particular geographic locales, efforts were made to include isolates from populations confined to single countries or banana-producing regions. For example, isolates from Australia, the Canary Islands, Taiwan, the Philippines, and, particularly, Malawi were extensively represented in the survey (Table 1).

Each culture was derived from a uninucleate microconidium from the original isolate, to rule out the possibility that polymorphisms in karyotype could be due to differences between nuclei of a heterokaryon. Conidia were obtained from cultures grown on potato-dextrose agar (Difco, Detroit, MI) slants, transferred to potato-dextrose broth (Difco) and grown for 5–8 days at 125 rpm to obtain high rates of conidial production. Conidia were harvested through four layers of cheesecloth, spun at 2,000 rpm (850 × *g*) in a Beckman TJ-6 clinical centrifuge (TH-4 rotor) for 10 min to pellet, and germinated in potato-dextrose broth overnight at 125 rpm. Germination rates were usually greater than 90% for most isolates, although some isolates had rates as low as 32%.

Protoplasting and pulsed field gel electrophoresis.

Germlings were spun as above to collect, washed twice in sterile water, and protoplasted by resuspending in a solution of 1.2 M $MgSO_4$ and 50 mM Na_3 citrate, pH 5.8, with Novozyme 234 (5 mg/ml) (BioSpecific Inc., Emeryville, CA). After 4–6 hr, usually more than 90% of germlings formed protoplasts. Protoplasts were spun as above, with the speed increasing to 3,000 rpm (1,500 × *g*). Microscopic examination of protoplasts which remained in solution after centrifugation indicated that they were much smaller than pelleted protoplasts, and they were considered to be anucleate. Pelleted protoplasts were washed in an osmoticum consisting of 1 M sorbitol, 50 mM $CaCl_2$, and 10 mM Tris, pH 7.4, and finally were resuspended in the same solution at concentrations ranging from 10^8 to 10^9 cells per milliliter. The preparation of agarose-embedded protoplasts for pulsed field gel electrophoresis followed preestablished protocols (Orbach *et al.* 1988). Protoplasts were briefly warmed to 42° C and mixed with 1.5 volumes of 1% low-melting-point agarose (IBI, New Haven, CT), 1 M sorbitol, 50 mM EDTA, and proteinase K (2.4 mg/ml) (GIBCO BRL Life Technologies Inc., Gaithersburg, MD) and immediately pipetted into prechilled sample molds. Solidified 0.6% agarose plugs were then incubated in a small volume of NDS buffer (0.5 M EDTA, 1% *N*-lauroylsarcosine, 10 mM Tris, pH 9.5) for 24–48 hr at 50° C and stored in 50 mM EDTA at 4° C.

Chromosome separations were performed by contour-clamped homogeneous electric field (CHEF) electrophoresis (Chu *et al.* 1986) with the CHEF-DRII apparatus (Bio-Rad Laboratories, Melville, NY). Typical runs were carried out on 15-welled, 0.6% FastLane agarose gels (FMC BioProducts, Rockland, ME), stacked two high, and electrophoresed at 4° C for 240 hr at 1.2 V/cm, with a ramped switch time of 1.2–5.4 × 10^3 sec. The running buffer was 0.25× TBE (1× TBE is 89 mM Tris, 89 mM borate, and 2 mM EDTA, pH 8.0) and was replaced daily. Other running conditions tested for representative isolates included 1) run times of 480 hr to resolve chromosomes of > 7.0 Mbp; 2) 1% agarose gels run for 18 hr at 4.5 V/cm with unramped 30-min switch times to resolve chromosomes of < 2.2 Mbp; and 3) 0.6% agarose gels run for 168 hr at 1.2 V/cm with ramped switch times of 1.2–3.6 × 10^3 sec. Molecular weight standards were run with each gel and included chromosomal preparations of *Schizosaccharomyces pombe* strain 972h- (Bio-Rad Laboratories) and *Saccharomyces cerevisiae* strain YNN295 (Bio-Rad Laboratories), run in the same lane, and *Nectria haematococca* strain 171-3, mating population VI (Kistler and Benny 1992). The gels were stained for 30 min in ethidium bromide (0.5 μg/ml), destained overnight in several changes of water, and photographed with Polaroid 55 film.

Southern hybridizations.

Fractionated chromosomal DNA was restained in ethidium bromide, nicked with 100 mJ of total energy (UV 254-nm

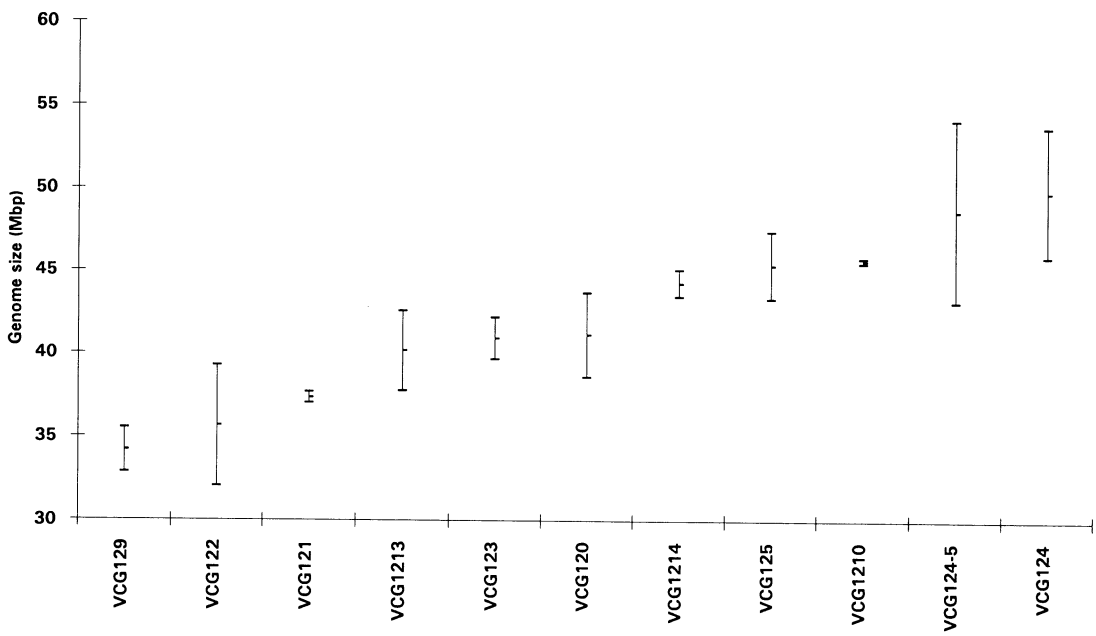

Fig. 7. *Fusarium oxysporum* f. sp. *cubense* genome size (GS) means and associated standard deviations for the same data set as in Figure 6.

crosslinker, model FB UVXL 1000, Fisher Scientific, Pittsburgh, PA), denatured for 30 min in a solution of 0.4 N NaOH and 1.5 M NaCl, and alkaline-transferred in the same solution to Nytran nylon membranes (Schleicher & Schuell, Keene, NH). Two probes were used in this study: pUF8-3 contains an 8-kb *Hin*dIII fragment from *N. haematococca* isolate T2, cloned in the *Hin*dIII site of pUC119, and hybridizes to 28S, 16S, and 5.8S ribosomal RNA (H. C. Kistler and U. Benny, unpublished data), and pLD contains the telomeric consensus sequence $[TTAGGG]_{18}$ isolated from *F. o.* f. sp. *lycopersici* strain 73 (Kistler and Benny 1992; Powell and Kistler 1990). All probes were labeled with α ^{32}P-dCTP by the random primer method (Sambrook *et al.* 1989). Hybridizations were carried out in a Hybaid hybridization oven (Dot Scientific Inc., Flint, MI), at 68° C in 6× SSC (20× SSC is 3 M NaCl and 0.3 M Na_3 citrate · $2H_2O$, pH 7.0), dried milk (2.5 mg/ml), 10% dextran sulfate, and denatured sheared herring sperm DNA (100 μg/ml) for pUF8-3, and at 65° C in 0.25 M Na_2HPO_4 and 7% SDS for pLD. High-stringency washes were done at the same temperatures as the hybridizations, in 2× SSC, 0.1% SDS, and dried milk (2.5 mg/ml) (two washes, 30 min each) and 0.1× SSC plus 0.1% SDS (two washes, 30 min each) for pUF8-3, and in 0.25 mM Na_2HPO_4 plus 5% SDS (2 washes, 30 min each) and 0.25 mM Na_2HPO_4 plus 1% SDS (2 washes, 30–60 min each) for pLD.

Data analysis.

CN and GS were derived directly from observation of photographic negatives. Initially, densitometry was used to confirm visual observations that some chromosome-sized bands migrated as doublets and triplets. Subsequent analysis of CN values, however, was based on visually determined band intensities. These methods were previously used to determine CN and GS for a variety of fungi (Kinscherf and Leong 1988; Masel *et al.* 1990; McDonald and Martinez 1991; Morales *et al.* 1993; Orbach *et al.* 1988; Talbot *et al.* 1993). As a test of the ability to arrive at reproducible counts, EKs were ascertained independently by two of the investigators, and identical CN values and negligible differences in calculated GS values were found. All isolates were run at least twice, and GS values were averaged. The calculated GS values may be subject to some experimental error, since from one to four chromosomes in all *F. o.* f. sp. *cubense* isolates exceeded our largest unambiguous *S. pombe* molecular weight marker of 5.7 Mbp. Likewise, the 7.0-Mbp chromosome of *N. haematococca*, our largest molecular weight marker, was extrapolated from *S. pombe* and may be subject to error. However, the majority of chromosome length polymorphisms and CN differences were associated with the lower molecular weight chromosomes of a given EK (Fig. 1).

Although both CN and GS values are presented for all 118 isolates (Table 1 and Fig. 1), statistical analysis was limited to VCGs in which four or more isolates were available. VCGs 0126, 0128, 01211, and 01212 were therefore excluded in this analysis. The low number of isolates available for these VCGs suggests that we might not have obtained adequate representation. The analysis, although not entirely inclusive, is nevertheless valid for the 110 isolates examined. CN is a meristic variable, and no assumptions were made concerning the nature of its distribution. CN means were computed for the 11 remaining VCGs and compared by means of the Waller-Duncan *K* ratio *T* test (a post hoc multiple comparison procedure).

To test the null hypothesis that the continuous variable GS was distributed by chance among the 110 isolates in the 11 selected VCGs, a number of ANOVA tests were performed. To determine whether GS values were randomly distributed for geographic origins, isolates from Jamaica, Malaysia, Nicaragua, South Africa, Tanzania, Uganda, and Zaire were excluded, and ANOVAs were performed for the isolates from the nine remaining countries for which there were four or more representatives.

A plot of residuals from the ANOVA of the untransformed GS data (n = 110) indicated a positive correlation of GS variance with mean that was satisfactorily corrected with either square root (SQGS) or natural logarithmic transformations. SQGS was considered the more appropriate transformation, since all values were within the same order of magnitude. Following a significant ANOVA, transformed and untransformed GS means were compared by means of the Waller-Duncan *K* ratio *T* test. The Statistical Analysis System package (SAS Institute 1982) on the mainframe at the Northeast Regional Data Center of the State University System of Florida in Gainesville was used for all statistical computations.

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