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FUNGAL DISEASES

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Three evolutionary lineages of tomato wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, based on sequences of IGS, *MAT1*, and *pg1*, are each composed of isolates of a single mating type and a single or closely related vegetative compatibility group

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Abstract Three evolutionary lineages of the tomato wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* were found among a worldwide sample of isolates based on phylogenetic analysis of the ribosomal DNA intergenic spacer region. Each lineage consisted of isolates mainly belonging to a single or closely related vegetative compatibility group (VCG) and a single mating type (MAT). The first lineage (A1) was composed of isolates VCG 0031 and MAT1-1; the second (A2) included VCG 0030 and/or 0032 and MAT1-1; and the third (A3) included VCG 0033 and MAT1-2. Race 1 and race 2 isolates belonged to the A1 or A2 lineages, and race 3 belonged to A2 or A3 lineages, suggesting that there is no correlation between race and lineage. However, for the isolates from Japan, race 1 (with one exception), race 2, and race 3 isolates belonged to A2, A1, and A3 lineages, respectively. These results suggest that the races could have evolved independently in each lineage; and in Japan the present races were likely to have been introduced independently after they had evolved in other locations.

Key words *Fusarium oxysporum* · Phylogeny · rDNA IGS · MAT · *pg1* · VCG

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Introduction

Fusarium oxysporum Schlechtend.:Fr. is an asexual ascomycete fungus that causes severe soilborne vascular wilt diseases on many crops (Booth 1971). *F. oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & Hans. is a pathogenic form of the species that infects tomato (*Lycopersicon esculentum* Mill.) (Booth 1971). Three physiological races (1, 2, 3), named in order of discovery (Alexander and Tucker 1945; Booth 1971; Grattidge and O'Brien 1982), are distinguished by their specific pathogenicity to tomato cultivars. Monogenic race-specific genes conferring resistance to *F. oxysporum* f. sp. *lycopersici* have been identified in wild tomato and introgressed into commercial tomato cultivars (Sela-Buurlage et al. 2001). The *I*, *I2*, and *I3* loci confer resistance to race 1, race 2, and race 3, respectively. The appearance of new pathogenic races has been hypothesized to be the result of mutation and selection from preexisting races or nonpathogenic strains (Gordon and Martyn 1997).

Somatic fusion and heterokaryon formation can occur independently of sexual reproduction in fungi (Leslie 1993) but usually occurs only among isolates with similar genotypes (Kistler 1997). These exclusive networks of isolates capable of heterokaryosis have been called vegetative compatibility groups (VCGs) (Leslie 1993; Takehara 1992). Puhalla (1985) proposed the classification and identification of *F. oxysporum* on the basis of VCGs. Since that time, many researchers have classified isolates into formae speciales of *F. oxysporum* and have adopted Puhalla's numerical system for classification in the species. In *F. oxysporum* f. sp. *lycopersici*, four VCGs (0030—0033) have been reported in many countries to date (Elias and

Schneider 1991; Katan 1999; Katan and Di Primo 1999; Kuninaga and Yokosawa 1992; Marlatt et al. 1996).

An understanding of the evolutionary history of pathogenic forms in *F. oxysporum* requires knowledge of the phylogenetic relationships among the isolates (Appel and Gordon 1996). Because morphological apomorphies are not available for most fungi, comparisons of conserved DNA sequences is the standard approach to phylogenetic analyses at or above the species level. Variation in the intergenic spacer (IGS) of the ribosomal DNA has proven useful for resolving intraspecific relationships within *F. oxysporum* (Appel and Gordon 1996).

Recently, the mating type locus (*MAT1*), which regulates sexual reproduction in ascomycete fungi, was cloned from *F. oxysporum* (Arie et al. 2000). Each isolate had either one or the other of the two idiomorphs (*MAT1-1* or *MAT1-2*) at this locus. Turgeon (1998), Pöggeler (1999), and Barve et al. (2003) have demonstrated that *MAT*-based phylogenetic analyses can be useful for studying the evolution of closely related fungi.

Polygalacturonases (PGs) secreted by plant pathogenic fungi have received much attention owing to their potential role in various aspects of fungal pathogenicity (Arie et al. 1998; Di Pietro and Roncero 1996, 1998; García-Maceira et al. 2000; Gómez-Gómez et al. 2002; Huertas-González et al. 1999; Ruiz-Roldán et al. 1999). Peever et al. (2002) used *endo*polygalacturonase (*endo*PG) gene sequences to infer a phylogeny among closely related *Alternaria* spp. associated with citrus. Similarly, a sequence variation in *pg1*, which encodes the major extracellular *endo*PG (PG1) of *F. oxysporum* f. sp. *lycopersici* (Arie et al. 1998; Di Pietro and Roncero 1998), could provide insight into the phylogenetic relationships among *Fusarium* spp.

Phylogenetic analyses based on DNA sequences of the mitochondrial small subunit ribosomal RNA gene (mtSSU), rDNA, and translation elongation factor 1α (EF1α) have helped to elucidate the evolutionary relationships within several formae speciales of *F. oxysporum* (O'Donnell et al. 1998). They showed that the *F. oxysporum* complex is strongly supported as monophyletic, but that many formae speciales in the species were found to be polyphyletic, and suggested that host pathogenicity has evolved convergently. Various genetic markers including VCGs, isozymes, restriction fragment length polymorphisms (RFLP) of total genomic DNA and mitochondrial DNA, and random amplification polymorphic DNA (RAPD) have been used to study the relationships among race 1, race 2, and race 3 strains of *F. oxysporum* f. sp. *lycopersici* (Cai et al. 2003; Elias and Schneider 1992; Elias et al. 1993; Gale et al. 2003; Kuninaga and Yokosawa 1992; Marlatt et al. 1996; Mes et al. 1999). Results from these studies all demonstrated that VCG correlated with genetic similarity and that race did not correlate with VCG or genetic similarity.

The objective of this study was to determine the evolutionary and geographical relationships among isolates of *F. oxysporum* f. sp. *lycopersici* using phylogenetic analyses of partial sequences of IGS, *MAT1*, and *pg1* in combination with VCG and mating type (MAT). A secondary objective was to use these data to discuss how new races have occur in *F. oxysporum* f. sp. *lycopersici*.

Materials and methods

Fungal isolates

A total of 34 isolates of *F. oxysporum* f. sp. *lycopersici* were studied (Table 1). Thirteen isolates of *F. oxysporum* f. sp. *lycopersici* were collected in Japan, and 21 isolates were obtained from other countries (Table 1). Thirteen isolates of 10 other formae speciales of *F. oxysporum* were used for phylogenetic analysis. Additionally, two isolates of *Gibberella fujikuroi* (Sawada) Ito mating population B [= *Fusarium subglutinans* var. *subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas = *Fusarium sacchari* (Butler) W. Gams] were also used as outgroups for phylogenetic analyses in this study (Table 1). All fungal isolates were preserved at –80°C before use and cultured on potato-dextrose agar (PDA).

Race determination

Races were determined in planta using the tomato cvs. Ponderosa (*i i2 i3*, susceptible; Takayama Seed, Kyoto, Japan), Momotaro (*I i2 i3*, resistance to race 1; Takii Seed, Kyoto, Japan), Walter (*I I2 i3*, resistance to race 1 and race 2; gift from National Institute of Vegetable and Tea Science, Mie, Japan), and C1-303 (*I I2 I3*, resistance to races 1, 2, and 3; Sakata Seed, Kanagawa, Japan). Plants were inoculated with a spore suspension of each isolate as described previously by Kawabe et al. (2004). Briefly, roots of 3-week-old tomato seedlings were injured by peg insertion, and then 5 ml of spore suspension ($1–2 \times 10^7$ spores/ml) was poured into the soil. Inoculated plants were maintained in a growth chamber at 28°C under a 16-h photoperiod (16 h light, 8 h dark). Disease severity was assessed as yellowing and wilting as follows: 0, no symptoms; 1, yellowing of the second and third leaf; 2, yellowing to the fourth or fifth leaf; 3, wilting; 4, dead. Resistant cultivars generally did not exhibit any symptoms with avirulent races. Isolates causing wilt only on cv. Ponderosa were determined to be race 1, isolates causing wilting on cvs. Ponderosa and Momotaro were race 2, and isolates causing wilting on cvs. Ponderosa, Momotaro, and Walter were race 3. The experiment was repeated at least three times with four replicate seedlings per cultivar/isolate combination.

Vegetative compatibility testing

The VCG of each isolate was determined using nitrate nonutilizing (*nit*) mutants as previously described (Correll et al. 1987; Katan et al. 1991). Mutants were generated from each isolate, and their phenotypes (*nit1*, *nit2*, or NitM) were determined using the method described by Correll et al. (1987). The *nit1* and NitM mutants from each isolate were

Table 1. Fungal isolates used in this study

Forma specialis/race and isolate no.	Source[a]	Origin	VCG[b]	Mating type[b]	GenBank accession no.[e]		
					IGS	*MAT*	*pg1*
Fusarium oxysporum f. sp. *lycopersici* race 1							
6531	IFO	Kyushu, Japan	Incompatible[c]	MAT1-1	AB106018	AB105973	AB106063
16417	ATCC	USA	Incompatible	MAT1-1	AB106017	AB105972	AB106062
26034	NRRL	Italy	Incompatible	MAT1-1	AB106025	AB105980	AB106070
103036	MAFF	Mie, Japan	0032	MAT1-1	AB106020	AB105975	AB106065
305121	MAFF	Japan	Incompatible	MAT1-1	AB106021	AB105976	AB106066
727501	MAFF	Japan	0030	MAT1-1	AB106022	AB105977	AB106067
744006	MAFF	Fukuoka, Japan	Incompatible	MAT1-1	AB106023	AB105978	AB106068
CT-1	M. Bon	France	0030	MAT1-1	AB120970	AB120977	NA
F239	E. Vivoda	USA	Incompatible	MAT1-1	AB120971	AB120978	AB120985
MN-59	H. C. Kistler	Florida, USA	0030+0032[d]	MAT1-1	AB106024	AB105979	AB106069
OSU-451B[f]	H. C. Kistler	Ohio, USA	0031	MAT1-1	AB106026	AB105981	AB106071
F.o. lycopersici race 2							
281	A. Di Pietro	Spain	Incompatible	MAT1-1	AB109237	AB120979	U96456
1330	SUF	Fukuoka, Japan	Incompatible	MAT1-1	AB106035	AB105990	AB106080
2715	A. Di Pietro	Spain	Incompatible	MAT1-1	AB120972	AB120980	AB120986
4287	A. Di Pietro	Spain	0030	MAT1-1	AB120973	AB120981	AB120987
16605	ATCC	USA	Incompatible	MAT1-1	AB106028	AB105983	AB106073
26200	NRRL	Ohio, USA	0030+0032	MAT1-1	AB106033	AB105988	AB106078
26202	NRRL	Florida, USA	0030+0032	MAT1-1	AB106034	AB105989	AB106079
46933	ATCC	South Africa	0030+0032	MAT1-1	AB106029	AB105984	AB106074
103038	MAFF	Ibaraki, Japan	0031	MAT1-1	AB106031	AB105986	AB106076
103043	MAFF	Ichikai, Japan	0031	MAT1-1	AB106032	AB105987	AB106077
880621a-1	T. Arie	Imaich, Japan	0031	MAT1-1	AB106027	AB105982	AB106072
CT-2	M. Bon	France	Incompatible	MAT1-1	AB120974	AB120982	NA
MN-66[f]	H. C. Kistler	Arkansas, USA	0030+0032	MAT1-1	AB106036	AB105991	AB106081
F.o. lycopersici race 3							
26037[f]	NRRL	Florida, USA	0030+0032	MAT1-1	AB106041	AB105993	AB106085
26380	NRRL	Florida, USA	0033	MAT1-2	AB106042	AB106004	NA
26383	NRRL	Florida, USA	0033	MAT1-2	AB106043	AB106005	AB106086
F-1-1	Y. Hosobuchi	Fukuoka, Japan	0033	MAT1-2	AB106037	AB106001	AB106082
F167	E. Vivoda	USA	0030	MAT1-1	AB120975	AB120983	AB120988
F240	E. Vivoda	USA	0030+0032	MAT1-1	AB120976	AB120984	AB120989
H-1-4	Y. Hosobuchi	Hokkaido, Japan	0033	MAT1-2	AB106038	AB106002	AB106083
tomino1-c	This laboratory	Kumamoto, Japan	0033	MAT1-2	AB106044	AB106006	AB106087
tomino2-c	This laboratory	Kumamoto, Japan	0033	MAT1-2	AB106045	AB106007	AB106088
DA-1/7[f]	H. C. Kistler	Florida, USA	0033	MAT1-2	AB106047	AB106008	AB106090
F.o. apii							
1017	SUF	Chiba, Japan	NT	MAT1-2	AB106048	AB106009	AB106091
F.o. batatas							
103070	MAFF	Chiba, Japan	NT	MAT1-2	AB106049	AB106010	AB106092
O-17	K. Watanabe	Ibaraki, Japan	NT	MAT1-2	AB106050	AB106011	AB106093
F.o. conglutinans							
Cong:1-1	T. Yoshida	Tokyo, Japan	NT	MAT1-1	AB106051	AB105995	AB106094
F.o. cucumerinum							
Rif-1	T. Arie	Saitama, Japan	NT	MAT1-1	AB106052	AB105996	AB106095
F.o. fragariae							
851209e	T. Arie	Tochigi, Japan	NT	MAT1-1	AB106053	AB105997	AB106096
F.o. matthioli							
880116a	T. Arie	Chiba, Japan	NT	MAT1-1	AB106054	AB105998	AB106097
F.o. melongenae							
103051	MAFF	Ibaraki, Japan	NT	MAT1-1	AB106055	AB105999	AB106098
F.o. melonis							
26406	NRRL	California, USA	NT	MAT1-2	AB106056	AB106012	AB106099
F.o. niveum							
305608	MAFF	Japan	NT	MAT1-2	AB106057	AB106013	AB106100
F.o. radicis-lycopersici							
959	SUF	Japan	NT	MAT1-2	AB106060	AB106015	AB106103
103044	MAFF	Gifu, Japan	NT	MAT1-1	AB106058	AB106000	AB106101
103047	MAFF	Kochi, Japan	NT	MAT1-2	AB106059	AB106014	AB106102
Gibberella fujikuroi							
7610	FGSC	USA	NT	MAT1-2	AB106061	AF100296	AB106104
7611	FGSC	USA	NT	MAT1-1	NA	AB106016	AB106105

NT, not tested; NA, DNA fragment was not amplified by polymerase chain reaction (PCR); VCG, vegetative compatibility group; IGS, rDNA intergenic spacer; *MAT*, mating type gene; *pg1*, *endo*polygalacturonase gene

[a] IFO, Institute for Fermentation, Osaka (Osaka, Japan; now operated by National Institute of Technology and Evaluation Biological Resource Center); ATCC, American Type Culture Collection (Manassas, VA); NRRL, Agriculture Research Service Cultrue Collection of United State Department of Agriculture (Peolia, IL); MAFF, Microorganisms Section of the Gene Bank in the Ministry of Agriculture, Forestry and Fisheries of Japanese Government (Tsukuba, Ibaraki, Japan); SUF, Culture Collection of *Fusarium* in Shinshu University (Ueda, Nagano, Japan); FGSC, Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas city, KS)

[b] Determined in this study

[c] Incompatible with all testers and self

[d] Vegetatively compatible both with VCG 0030 and VCG 0032 testers

[e] Sequenced and deposited in this study

[f] Tester strain for determination of VCG

paired with the NitM and *nit1* mutants from tester isolates, respectively, on MM medium to evaluate heterokaryon formation (Katan et al. 1991). VCG-tester isolates, NRRL 26037 (VCG 0030), OSU-451B (VCG 0031), MN-66 (VCG 0032), and DA-1/7 (VCG 0033), were provided by H.C. Kistler (USDA, St. Paul, MN, USA). Heterokaryon formation was assessed after a 2-week incubation. The appearance of dense aerial mycelium at the line of contact of the two mutants was considered indicative of vegetative compatibility. Isolates that were vegetatively compatible with both VCG 0030 and 0032 testers were designated VCG 0030+0032 according to Mes et al. (1999). VCG assignment was based on at least three independent pairings.

Mating type determination

The mating type of each isolate was determined using a MAT-specific polymerase chain reaction (PCR) assay following the protocol of Arie et al. (2000). Isolates from which an approximately 370-bp fragment was amplified with primers Falpha1 + Falpha2 (Table 2) were determined to be MAT1-1, and isolates from which an approximately 190-bp fragment was amplified with primers FHMG11 + FHMG12 (Table 2) were determined to be MAT1-2.

DNA amplification and sequencing

Genomic DNA was extracted from mycelium as described previously (Arie et al. 1997) and used as a PCR template. PCR amplifications of the partial IGS region (ca. 600 bp), *pg1* (ca. 970 bp), *MAT1-1-1* α-box (ca. 370 bp), and *MAT1-2-1* HMG-box (ca. 190 bp) were conducted with primers FIGS11 + FIGS12 (Appel and Gordon 1996), PG1 + PG2 (Arie et al. 1998), Falpha1 + Falpha2 (Arie et al. 2000), and FHMG11 + FHMG12 (Arie et al. 1999), respectively (Table 2). Each PCR reaction mixture (50 µl) contained 20 ng genomic DNA, 1 × PCR buffer with 75 nmol $MgCl_2$ (Applied Biosystems, Foster City, CA, USA), 10 nmol (each) dNTPs, 100 pmol of each primer, and 1 U High fidelity PCR enzyme mixture (Applied Biosystems). Thermal conditions consisted of denaturation at 94°C for 2 min; 30 cycles at 94°C for 20 s, 58°C for 30 s, and 72°C for 1 min; final extension was at 72°C for 7 min. Amplified DNA fragments from 49 isolates were sequenced directly after treatment with EXOSAP-IT (USB, Cleveland, OH, USA); or when they could not been sequenced directly, amplified DNA fragments were sequenced after cloning into pCR2.1 (Invitrogen, Carlsbad, CA, USA) or pGEM-T easy (Promega, Madison, WI, USA) vector. DNAs were labeled using the dye terminator cycle sequencing kit (Amersham Bioscience, Uppsala, Sweden) and sequenced by the ABI 377 Auto sequencer (Applied Biosystems).

Phylogenetic analyses

DNA sequences were aligned with Clustal X 1.81 (Thompson et al. 1997). Indels were coded as single events. Three types of phylogenetic inference were implemented including neighbor-joining (NJ) (Saiou and Nei 1987) using Clustal X 1.81; maximum-parsimony (MP) (Fitch 1977) using MEGA 2.1 (Kumar et al. 2001) following the close-neighbor-interchange (CNI) algorithm (a branch swapping method); and maximum-likelihood (ML) (Felsenstein 1981) using DNAML, which is heuristic analysis in the Phylip 3.57c software package (Felsenstein 1993). The distance was computed with the two-parameter distance method (Kimura 1980), and was then used to construct a phylogenetic tree with the NJ program. The stability of groups analyzed by the NJ method was assessed using 1000 bootstrap replications of the data set. *G. fujikuroi*, a closely related species of *F. oxysporum* in the *Gibberella*/*Fusarium* species complex, FGSC 7611 (*MAT1-1*), and/or FGSC 7610 (*MAT1-2*), were used as outgroups in all phylogenetic analyses.

Table 2. Primers used for polymerase chain reaction

Name	Nucleotide sequence (5′ → 3′)[a]	Comment	Reference
FIGS11	GTAAGCCGTCCTTCGCCTCG	245 to 264 bp at *Fusarium oxysporum* intergenic spacer region, refered to sequences in Appel and Gordon (1996)	Appel and Gordon (1996)
FIGS12	GCAAAATTCAATAGTATGGC	1056 to 1037 bp at *F. oxysporum* intergenic spacer region, refered to sequences in Appel and Gordon (1996)	Appel and Gordon (1996)
PG1	GAYAAYGAYTTYRAYCCBATYG	336 to 357 bp at *F. oxysporum pg1* sequence (accession no. AB000124)	Arie et al. (1998)
PG2	CANGTRTTNGTVGGRTARTTRC	1308 to 1287 bp at *F. oxysporum pg1* sequence (accession no. AB000124)	Arie et al. (1998)
Falpha1	CGGTCAYGAGTATCTTCCTG	1575 to 1556 bp at *F. oxysporum MAT1-1* idiomorph (accession no. AB0011379)	Arie et al. (2000)
Falpha2	GATGTAGATGGAGGGTTCAA	1200 to 1219 bp at *F. oxysporum MAT1-1* idiomorph (accession no. AB0011379)	Arie et al. (2000)
FHMG11	TACCGYAAGGAGCGTCACC	30 to 48 bp at *Gibberella fujikuroi MAT1-2* idiomorph (accession no. AB00541)	Arie et al. (1999)
FHMG12	TTYWYCTSATCSGCSMKHWSCTTG	218 to 196 bp at *G. fujikuroi MAT1-2* idiomorph (accession no. AB00541)	Arie et al. (1999)

[a] B = C, G, and T; H = A, C, and T; K = G and T; M = A and C; N = A, C, G, and T; R = A and G: S = G and C; W = A and T; Y = C and T

Results

Race determination

Race designations for the isolates of *F. oxysporum* f. sp. *lycopersici* are shown in Table 1 and Figs. 2–4 (see below). Eleven isolates were determined to be race 1, thirteen were race 2, and ten were race 3. Among the 13 isolates from Japan, five were of race 1, four of race 2, and four of race 3. No isolates were able to wilt cv. C1-303, indicating that no additional races other than races 1, 2, and 3 were detected.

Vegetative compatibility group

The VCG could be determined only for 23 of 34 *F. oxysporum* f. sp. *lycopersici* isolates (Table 1). Race 1 isolates were assigned to VCG 0030 (two isolates), 0031 (one), 0032 (one), or 0030 + 0032 (one); race 2 isolates were assigned to VCG 0030 (one), 0031 (three), or 0030 + 0032 (four); and race 3 isolates were assigned to VCG 0030 (one), 0032 + 0032 (two), or VCG 0033 (seven). VCG 0033 consisted of only race 3 isolates (Table 1). Six race 1 isolates and five race 2 isolates were incompatible with all tester strains and with themselves (self-incompatible) (Kuninaga and Yokosawa 1992; Mes et al. 1999), even after prolonged incubation.

Mating type determination

The mating type designation for each isolate is shown in Table 1. All *F. oxysporum* f. sp. *lycopersici* race 1 and race 2 isolates and three (F167, F240, NRRL 26037) race 3 isolates were determined to be MAT1-1 (Table 1). Seven race 3 isolates were determined to be MAT1-2. No race 1 or race 2 isolates of MAT1-2 were found. No isolates showed amplification with both primer sets, and no isolates failed to show a band with both sets of primers (Table 1, Fig. 1).

Phylogenetic analyses

Phylogenetic analysis of rDNA IGS sequences by NJ methods revealed a large group composed of isolates of *F. oxysporum* f. sp. *lycopersici* and some formae speciales (indicated by asterisk in Fig. 2), and three well-supported groups (Fig. 2: A1, A2, A3) among the sample isolates in the large group (Fig. 2). All *F. oxysporum* f. sp. *lycopersici* isolates were in either of the three groups.

Group A1 was composed of VCG 0031 and several self-incompatible isolates. It contained race 1 and race 2 isolates obtained from Japan and a race 1 isolate from the United States.

Group A2 consisted of VCG 0030, 0032, and 0030 + 0032 isolates together with some self-incompatible ones. Race 1 isolates from Japan, race 1 and race 2 isolates from other countries, and race 3 isolates from the United States were found in this group.

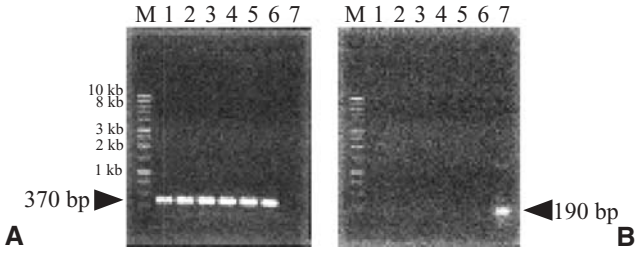

Fig. 1. Mating type determination of *Fusarium oxysporum* f. sp. *lycopersici* isolates by polymerase chain reaction (PCR) with MAT1-1 and MAT1-2 specific primers. **A** Products generated with primers Falpha1 + Falpha2 (Table 2). **B** Products generated with primers FHMG11 + FHMG12 (Table 2). All products were separated in a 0.8% gel. Lanes: *M*, 1-kb markers (Promega, Madison, WI); *1*, SUF 1330 (determined to be MAT1-1); *2*, ATCC 46933 (MAT1-1); *3*, IFO 6531 (MAT1-1); *4*, ATCC 16417 (MAT1-1); *5*, 880621a-1 (MAT1-1); *6*, F167 (MAT1-1); *7*, F-1-1 (MAT1-2)

Group A3 uniquely consisted of VCG 0033 isolates. It is the only group that included race 3 isolates obtained from Japan (Table 1, Fig. 2).

Groups A1 and A2 consisted of MAT1-1 isolates, and group A3 consisted of MAT1-2 isolates without exception (Table 1, Fig. 2). Two groups (B1, B2) were defined in MAT1-1 isolates by the phylogenetic analysis based on the *MAT1-1-1* α-box by NJ (Fig. 3A), which essentially did not contradict groups A1 and A2 in the IGS phylogeny (Fig. 2). An exception was found at MAFF 103036 (VCG 0032, race 1) which was a member of group A2 in the IGS tree but was classified in group B1 in the *MAT1-1-1* α-box tree. A separate group (C1) composed of seven isolates of *F. oxysporum* f. sp. *lycopersici* was defined in the phylogeny based on the *MAT1-2-1* HMG box (Fig. 3B), which corresponded to group A3 in the IGS tree (Fig. 2).

Phylogenetic analysis of *pg1* sequences by NJ resulted in a tree with two groups (D1, D2) (Fig. 4). Group D1 was composed of the groups A1 and A3 members in the IGS phylogenetic tree. Group D2 corresponded to group A2 in the IGS tree. MAFF 103036 (VCG 0032, race 1) and MAFF 305121 (self-incompatible, race 1) were independent from any groups in the *pg1* phylogeny. Combined sequences of IGS and *pg1* revealed a phylogeny that did not contradict the IGS phylogeny (data not shown).

Phylogenetic analyses of sequences of rDNA IGS (tree length 156, CI 0.897, RI 0.944), *MAT1-1-1* α-box (tree length 42, CI 0.976, RI 0.973), *MAT1-2-1* HMG box (tree length 15, CI 1.000, RI 1.000), and *pg1* (tree length 251, CI 0.904, RI 0.914) by MP, and phylogenetic analyses of sequences of rDNA IGS (Ln –1986.592), *MAT1-1* (Ln –706.087), *MAT1-2* (Ln –277.205), and *pg1* (Ln –2762.006) by ML methods were equivalent to the results by the NJ methods (data not shown). MP and ML searches yielded a single tree for each gene (IGS, *MAT1*, *pg1*). Each group (A1, A2, A3, B1, B2, C1, D1, D2) by the NJ method was supported by the MP and ML methods.

The results of this study are summarized in Table 3.

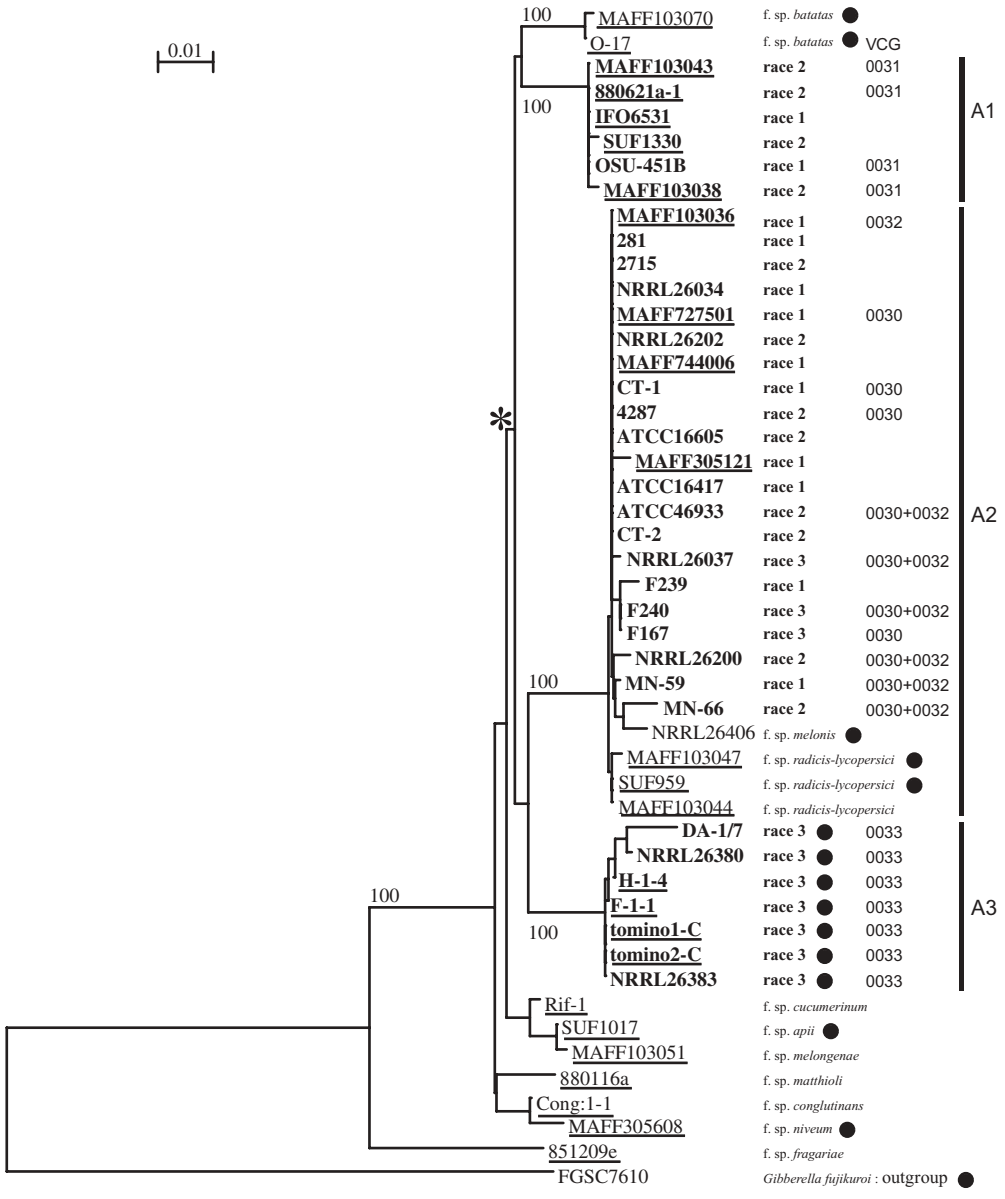

Fig. 2. Phylogenetic analysis of intergenic spacer (IGS) sequences of *Fusarium oxysporum* by neighbor-joining (Clustal X). Numbers on nodes represent bootstrap values estimated from 1000 replications of the data set when bootstrap values are higher than 50%. The three groups are labeled *A*1, *A*2, and *A*3, respectively. *Filled circles* represent MAT1-2 isolates; MAT1-1 isolates have no symbol. Isolates in *boldface* represent *F.o.* f. sp. *lycopersici*, others are isolates; other than *F.o.* f. sp. *lycopersici*. *Underlines* represent isolates from Japan. *Gibberella fujikuroi* FGSC 7610 was used as an outgroup. *Asterisk* shows the large cluster of *F. oxysporum* f. sp. *lycopersici*

Discussion

The phylogenetic analyses based on rDNA IGS, *MAT1*, and *pg1* revealed two or three groups in *F. oxysporum* f. sp. *lycopersici* (Figs. 2–4, Table 3). Mes et al. (1999) reported that isolates of VCG 0030 and of VCG 0032 were often compatible and suggested a close phylogenetic relationship between them. Similarly, our results demonstrated that tester isolate NRRL 26037 (VCG 0030) and some isolates of VCG 0030 were weakly compatible with tester isolate MN-66 (VCG 0032); and isolates of VCG 0030, 0032, and 0030 + 0032 were classified into group A2 in the IGS tree. These results suggested a close genetic relationship between VCGs 0030 and 0032, as did the results of Mes et al. (1999).

The results summarized in Table 3 supported the hypotheses that isolates within a VCG of *F. oxysporum* are genetically more similar than those in different VCGs regardless of race (Elias and Schneider 1991; Mes et al. 1999) and that isolates in each VCG may be clonally derived from a common ancestor (Kistler 1997). The *MAT1* phylogenies were congruent with the IGS phylogeny. Groups B1, B2, and C1 in *MAT1* phylogenies corresponded to groups A1, A2, and A3 in the IGS phylogeny, respectively. Even the two phylogenies based on *MAT1-1-1* and *MAT1-2-1* could not be combined. Only one group was defined in the phylogenetic tree based on *MAT1-2-1* because the *MAT1-2-1* sequences did not contain much phylogenetic signal.

Three groups were defined in the IGS and *MAT1* phylogenetic analyses, but two were defined in the *pg1* phylogenetic analysis. One (D1) of the groups in the *pg1* tree was

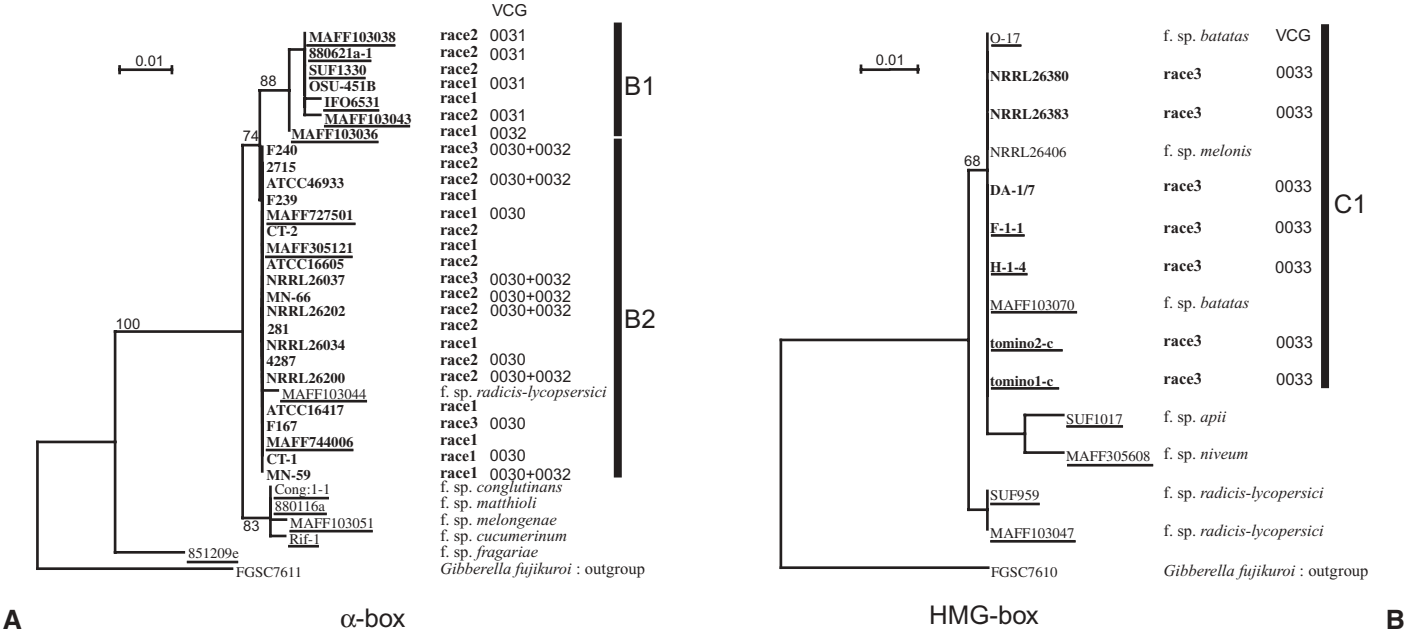

Fig. 3. Phylogenetic analysis of *MAT1-1-1* (**A**) and *MAT-1-2-1* (**B**) sequences of *Fusarium oxysporum* by neighbor-joining (Clustal X). *Numbers on nodes* represent bootstrap values estimated from 1000 replications of the data set when bootstrap values are higher than 50%. Isolates with *boldface* represent *F.o.* f. sp. *lycopersici*; others are isolates other than *F.o.* f. sp. *lycopersici*. *Underlines* represent isolates from Japan. *Gibberella fujikuroi* isolates FGSC 7611 or FGSC 7610 were used as outgroups

the combination of two groups (A1 + A3 and B1 + C3) in the IGS and *MAT1* trees, respectively. Therefore, IGS and *MAT1* sequences could be well-differentiated evolutionary units in preparing phylogenies.

Although *F. oxysporum* is asexual, Arie et al. (2000) suggested that the fungus carries functional mating type genes. Mating type determination of *F. oxysporum* f. sp. *lycopersici* isolates showed that groups A1 and A2 contained only MAT1-1 isolates and group A3 contained only MAT1-2 isolates (Table 3, Fig. 2). Isolates in each group would then be prevented from crossing within each group. Isolates in groups A1 and A2 (MAT1-1) were expected to cross with the isolates in group A3 (MAT1-2); however, successful crosses were not observed in pairings of any A1 or A2 isolates with any A3 isolates (data not shown). Based on these results, we infer that asexual reproduction has been a major driving force behind diversification of each of the three phylogenetic lineages of *F. oxysporum* f. sp. *lycopersici*. We postulated that *F. oxysporum* f. sp. *lycopersici* has evolved as three evolutionary lineages and appears to be reproducing asexually. Consequently, each lineage has been composed of one VCG and one mating type.

It is commonly assumed that in *F. oxysporum* f. sp. *lycopersici* race 2 derived from race 1, and race 3 derived from race 2, respectively. The relationships between races and any phylogenetically defined groups reported so far were not found in worldwide collections of *F. oxysporum* f. sp. *lycopersici* (Mes et al. 1999). This might be because the new race was derived from a preexisting race in each lineage. However, we found that race 1 isolates collected in Japan were members of group A2 with one exception (IFO 6531), race 2 isolates in Japan were members of group A1, and race 3 isolates in Japan were members of group A3 (Table 3). Based on these data, we concluded that race 2 and race 3 did not evolve from the preexisting races (race 1 and race 2, respectively) in Japan. In other words, the three races have phylogenetically distinct backgrounds and have likely been introduced independently into Japan. The only exception, IFO 6531 (race 1 but was classified into A1), was collected in Japan in 1959. All other A1 (race 2) isolates were collected from Japan during the late 1980s. Further studies with more isolates of race 1 and race 2 are needed to confirm the aforementioned assumption. However, no tomato cultivars that lack *I* are currently grown commercially in Japan; therefore, race 1 isolates of *F. oxysporum* f. sp. *lycopersici* may no longer be found on tomato in Japan.

Gale et al. (2003) reported that the current race 3 isolates in Florida and other states in the southern United States are in VCG 0033, which replaced the previous race 3 isolates in VCG 0030/0032 in Florida. The first report of race 3 in Japan was in 1998 (Hosobuchi 1998; Masunaga et al. 1998) and all the Japanese race 3 isolates in our collection are in VCG 0033, suggesting that race 3 in Japan has the same origin as the present race 3, VCG 0033 isolates in the United States.

Our tree suggested some relationships between isolates of *F. oxsporum* f. sp. *lycopersici* and isolates of some other formae speciales. Isolates of *F. oxysporum* ff. sp. *melonis* (NRRL 26046) and *radicis-lycopersici* (MAFF 103044,

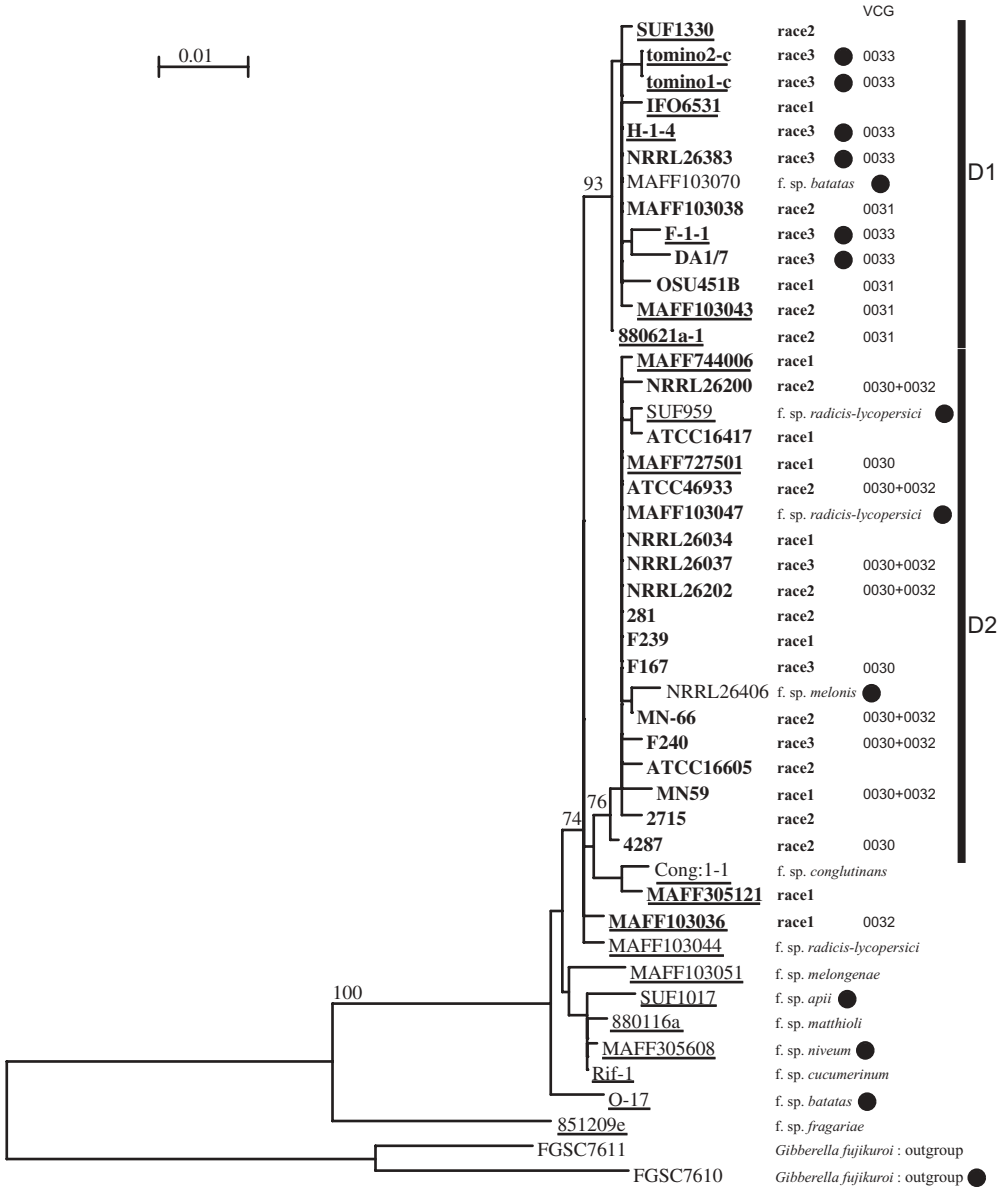

Fig. 4. Phylogenetic analysis of *pg1* sequences of *Fusarium oxysporum* by neighbor-joining (Clustal X). *Numbers* on nodes represent bootstrap values estimated from 1000 replications of the data set when bootstrap values are higher than 50%. *Filled circles* represent MAT1-2 isolates; others show MAT1-1 isolates. Isolates in *boldface* represent *F.o.* f. sp. *lycopersici*; others are isolates other than *F.o.* f. sp. *lycopersici*. *Underlines* represent isolates from Japan. *Gibberella fujikuroi* FGSC 7610 and FGSC 7611 were used as outgroups

Table 3. Summary of characters of *Fusarium oxysporum* f. sp. *lycopersici* isolates in phylogenetic groups determined in this study

Group			VCG	Mating type	Race and origin	
IGS group	*MAT1* group	*pg1* group			Isolates from Japan	Isolates from other Countries
A1	B1	D1	0031	MAT1-1	Race 1 (1) Race 2 (4)	Race 1 (1)
A2	B2[a]	D2[b]	0030 0032 0030+0032	MAT1-1	Race 1 (4)	Race 1 (5) Race 2 (9) Race 3 (2)
A3	C1	D1	0033	MAT1-2	Race 3 (4)	Race 3 (4)

Numbers in parentheses are the number of isolates

[a] MAFF 103036 is classified in *MAT1*-B1 group

[b] MAFF 103036 and 305121 are not included in *pg1*-D1 and D2 groups

MAFF 103047, and SUF 959) were classified within group A2. Other formae speciales were phylogenetically distinct from groups A1, A2, and A3 (Fig. 2). The limited isolates we examined in this study (NRRL 26406, MAFF 103047, and SUF 959, which were placed in group A2) were MAT1-2, whereas all isolates of *F. oxysporum* f. sp. *lycopersici* in the group were MAT1-1 (Table 1, Fig. 2). Furthermore, *F. oxysporun* ff. sp. *radicis-lycopersici* (SUF

959, MAFF 103044, MAFF 103047), *melonis* (NRRL 26406), and *batatas* (MAFF 103070, O-17) were classified within the *F. oxsporum* f. sp. *lycopersici* large cluster (indicated with asterisk in Fig. 2) in the IGS phylogeny. O'Donnell et al. (1998) revealed that *F. oxysporum* ff. sp. *lycopersici*, *batatas*, *melonis*, and *radicis-lycopersici* belonged to the same phylogenetic lineage generated by DNA sequences of translation elongation factor 1α and the mitochondrial small subunit ribosomal RNA gene. Our results coincide with their findings. Because the A2 group involved both MAT1-1 and MAT1-2 isolates, we thought the above relatedness may reflect the possibility that the ancestors in group A2 had reproduced sexually. Further analysis of the large cluster would help us to understand the diversity of formae speciales in *F. oxysporum*.

In this study, we were able to recognize three phylogenetic groups (= lineages) in *F. oxysporum* f. sp. *lycopersici* (Table 3). Among the worldwide samples, there were close correlations between mating type, VCG, and lineage but not between race and lineage. However, among isolates from Japan, there was a good correlation between race and lineage. This suggests the possibility that constant migration of *F. oxysporum* f. sp. *lycopersici* may occur on a worldwide scale and that each race was introduced independently to Japan relatively recently on seeds or seedlings.

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References

Alexander LJ, Tucker CM (1945) Physiologic specialization in the tomato wilt fungus *Fusarium oxysporum* f. *lycopersici*. J Agric Res 70:303–313

Appel DJ, Gordon TR (1996) Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. Mol Plant Microbe Interact 9:125–138

Arie T, Christiansen SK, Yoder OC, Turgeon BG (1997) Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved MAT HMG box. Fungal Genet Biol 21:118–130

Arie T, Gouthu S, Shimazaki S, Kamakura T, Kimura M, Inoue M, Takio K, Ozaki A, Yoneyama Y, Yamaguchi I (1998) Immunological detection of *endo*polygalacturonase secretion by *Fusarium oxysporum* in plant tissue and sequencing of its encoding gene. Ann Phytopathol Soc Jpn 64:7–15

Arie T, Yoshida T, Shimizu T, Kawabe M, Yoneyama K, Yamaguchi I (1999) Assessment of *Gibberella fujikuroi* mating type by PCR. Mycoscience 40:311–314

Arie T, Kaneko I, Yoshida T, Noguchi M, Nomura Y, Yamaguchi I (2000) Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. Mol Plant Microbe Interact 13:1330–1339

Barve MP, Arie T, Salimath S, Muehlbauer FJ, Peever TL (2003) Cloning and characterization of mating type (*MAT*) locus from *Ascochyta rabiei* (telemorph: *Didymella rabiei*) and a MAT phylogeny of legunum-associated *Ascochyta* spp. Fungal Genet Biol 39:151–167

Booth C (1971) The genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, UK

Cai G, Gale LR, Schneider RW, Kistler HC, Davis RM, Elias KS, Miyao EM (2003) Origin of race 3 of *Fusarium oxysporum* f. sp. *lycopersici* at a single site in California. Phytopathology 93:1014–1022

Correll JC, Klittich CJR, Leslie JF (1987) Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77:1640–1646

Di Pietro A, Roncero MIG (1996) Endopolygalacturonase from *Fusarium oxysporum* f. sp. *lycopersici*: purification, characterization, and production during infection of tomato plants. Phytopathology 86:1324–1330

Di Pietro A, Roncero MIG (1998) Cloning, expression, and role in pathogenicity of *pg1* encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. Mol Plant Microbe Interact 11:91–98

Elias KS, Schneider RW (1991) Vegetative compatibility groups in *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology 81:159–162

Elias KS, Schneider RW (1992) Genetic diversity within and among races and vegetative compatibility groups of *Fusarium oxysporum* f. sp. *lycopersici* as determined by isozyme analysis. Phytopathology 82:1421–1427

Elias KS, Zamir D, Lichtman-Pleban T, Katan T (1993) Population structure of *Fusarium oxysporum* f. sp. *lycopersici*: restriction fragment length polymorphisms provide genetic evidence that vegetative compatibility group is an indicator of evolutionary origin. Mol Plant Microbe Interact 6:565–572

Felsenstein J (1981) Evolutionary tree from DNA sequences: a maximum likelihood approach. J Mol Evol 39:783–791

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle

Fitch WM (1977) On the problem of discovering the most parsimonious tree. Am Nat 111:223–257

Gale LR, Katan T, Kistler HC (2003) The probable center of origin of *Fusarium oxysporum* f. sp. *lycopersici* VCG 0033. Plant Dis 87:1433–1438

García-Maceira FI, Di Pietro A, Roncero MIG (2000) Cloning and distribution of *pgx4* encoding an in planta expressed exopolygalacturonase from *Fusarium oxysporum*. Mol Plant Microbe Interact 13:359–365

Gómez-Gómez E, Ruíz-Roldán MC, Di Pietro A, Roncero MI, Hera C (2002) Role in pathogenesis of two endo-β-1,4-xylanase genes from the vascular wilt fungus *Fusarium oxysporum*. Fungal Genet Biol 35:213–333

Gordon TR, Martyn RD (1997) The evolutionary biology of *Fusarium oxysporum*. Annu Rev Phytopathol 35:111–128

Grattidge R, O'Brien RG (1982) The occurrence of a third race of Fusarium wilt of tomato in Queensland. Plant Dis 66:165–166

Hosobuchi Y (1998) *Fusarium oxysporum* infecting fusarium wilt resistant tomato cultivars. Ann Phytopathol Soc Jpn 64:434 (abstract, in Japanese)

Huertas-González MD, Ruiz-Roldán MC, Maceria FIG, Roncero MIG, Di Pietro A (1999) Cloning and characterization of *pl1* encoding an in planta-secreted pectate lyase of *Fusarium oxysporum*. Curr Genet 35:36–40

Katan T (1999) Current status of vegetative compatibility groups in *Fusarium oxysporum.* Phytoparasitica 27:51–64

Katan T, Di Primo P (1999) Current status of vegetative compatibility groups in *Fusarium oxysporum*: supplement. Phytoparasitica 27:273–277

Katan T, Zamir D, Sarfatti M, Katan J (1991) Vegetative compatibility groups and subgroups in *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology 81:255–262

Kawabe M, Mizutani K, Yoshida T, Teraoka T, Yoneyama K, Yamaguchi I, Arie T (2004) Cloning a pathogenicity-related gene, *FPD1*, in *Fusarium oxysporum* f. sp. *lycopersici*. J Gen Plant Pathol 70:16–20

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kistler HC (1997) Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. Phytopathology 87:474–479

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software, Bioinformatics 17:1244–1245

Kuninaga S, Yokosawa R (1992) Genetic diversity of *Fusarium oxysporum* f. sp. *lycopersici* in restriction fragment length polymorphisms of mitochondrial DNA. Trans Mycol Soc Jpn 33:449–459

Leslie JF (1993) Fungal vegetative compatibility. Annu Rev Phytopathol 31:127–150

Marlatt ML, Correll JC, Kaufmann P (1996) Two genetically distinct populations of *Fusarium oxysporum* f. sp. *lycopersici* race 3 in the United States. Plant Dis 80:1336–1342

Masunaga T, Shiomi H, Komada H (1998) Identification of race 3 of *Fusarium oxysporum* f. sp. *lycopersici* isolated from tomato in Fukuoka Prefecture. Ann Phytopathol Soc Jpn 64:435 (abstract, in Japanese)

Mes JJ, Weststeijn EA, Herlaar F, Lambalk JJM, Wijbrandi J, Haring MA, Cornelissen BJC (1999) Biological and molecular characterization of *Fusarium oxysporum* f. sp. *lycopersici* divides race 1 isolates into separate virulence groups. Phytopathology 89:156–160

O'Donnell K, Kistler HC, Cigelnik E, Ploetz R (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondria gene genealogies. Proc Natl Acad Sci USA 95:2044–2049

Peever TL, Ibañez A, Akimitsu K, Timmer LW (2002) Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. Phytopathology 92:794–802

Pöggeler S (1999) Phylogenetic relationships between mating-type sequences from homothallic and heterothallic ascomycetes. Curr Genet 36:222–231

Puhalla JE (1985) Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can J Bot 63:179–183

Ruiz-Roldán MC, Di Pietro A, Huertas-González MD, Roncero MIG (1999) Two xylanase genes of the vascular wilt pathogen *Fusarium oxysporum* are differentially expressed during infection of tomato plants. Mol Gen Genet 261:530–536

Saiou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic tree. Mol Biol Evol 4:406–425

Sela-Buurlage MB, Budai-Hadrian O, Pan Q, Carmel-Gore L, Vunsch R, Zamir D, Fluhr R (2001) Genome-wide dissection of *Fusarium* resistance in tomato reveals multiple complex loci. Mol Genet Genomics 265:1104–1111

Takehara T (1992) Nitrate-nonutilizing mutants of fungi and their use. Plant Protection 46:395–399 (in Japanese)

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Turgeon BG (1998) Application of mating type gene technology to problems in fungal biology. Annu Rev Phytopathol 36:115–137