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RESEARCH ARTICLE

Molecular phylogeny and diversity of *Fusarium* endophytes isolated from tomato stems

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One sentence summary: Tomato endophytic *Fusarium* obtained in this study were classified into the dominant soil fusaria, *Fusarium oxysporum* species complex, *F. fujikuroi* species complex and *F. solani* species complex.

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ABSTRACT

Plant tissues are a known habitat for two types of *Fusarium* species: plant pathogens and endophytes. Here, we investigated the molecular phylogeny and diversity of endophytic fusaria, because endophytes are not as well studied as pathogens. A total of 543 *Fusarium* isolates were obtained from the inside of tomato stems cultivated in soils mainly obtained from agricultural fields. We then determined partial nucleotide sequences of the translation elongation factor-1 alpha (EF-1α) genes of the isolates. Among the isolates from tomato, 24 EF-1α gene sequence types (EFST) were found: nine were classified as being from the *Fusarium oxysporum* species complex and its sister taxa (FOSC, 332 isolates), seven from the *F. fujikuroi* species complex (FFSC, 75 isolates) and eight from the *F. solani* species complex (FSSC, 136 isolates). To determine more characteristic details of the tomato isolates, we isolated 180 fusaria directly from soils and found 95% of them were nested within the FOSC (82 isolates; five EFSTs), FFSC (21 isolates; six FESTs) and FSSC (68 isolates; 11 EFSTs). These results suggested that the dominant *Fusarium* endophytes within tomato stems were members of the same three species complexes, which were also the dominant fusaria in the soils.

Keywords: endophyte; *Fusarium*; tomato; EF-1α gene; phylogeny; diversity

INTRODUCTION

Fusarium includes a large number of strains associated with agricultural productions, such as plant pathogens (Kistler 1997; Leslie and Summerell 2006), toxin producers on edible parts of plants (Desjardins 2006) and biological control agents for plant diseases (Alabouvette *et al.* 2001). In ecological perspective, *Fusarium* includes epiphytes (Inácio *et al.* 2002) and endophytes (Leslie *et al.* 1990; Kuldau and Yates 2000; Bacon and Yates 2006). In addition to these agriculturally and ecologically distinct strains, many are putative saprophytic. By virtue of their agricultural and ecological characteristics, *Fusarium* has become a model organism.

Fusaria have been classified historically on the basis of morphological characteristics. In recent decades, phylogenetic-based methods have moved taxonomy of *Fusarium* into a new phase based on molecular phylogenetics (Aoki 2009). Closely related phylogenetic species are grouped in species complexes. *Fusarium graminearum* species complex (FGSC) and *F. fujikuroi* species complex (FFSC) are examples: there are 16 species within the FGSC and over 50 species within the FFSC (Aoki, personal communication; O'Donnell, Cigelnik and Nirenberg 1998; O'Donnell *et al.* 2004, 2008b; Starkey *et al.* 2007; Aoki 2009). Although *F. oxysporum* and *F. solani* were described as single species, both comprise multiple species (Baayen *et al.* 2000; O'Donnell 2000; Enya *et al.* 2008). Based on these findings, *F. oxysporum* and *F. solani* are also now recognized as species complexes (FOSC and FFSC).

The plant pathogens in *Fusarium* cause root and stem rots, blights and wilts in a large number of cultivated plants. The

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FOSC includes more than 120 kinds of plant pathogens, which cause soil-borne diseases such as vascular wilt and root rot diseases. Each of pathogenic individual is highly host specific and their host range is limited to only one or a few plant species, called *forma specialis* (f. sp.) (Gullino, Katan and Garibaldi 2012). The FSSC includes pathogens which cause root rot diseases, and some of them are also classified as f. sp. (Aoki *et al.* 2003). The FFSC includes rice bakanae disease pathogen, which causes abnormal elongation of rice leaves by the production of gibberellin (Wulff *et al.* 2010).

Fusaria both positively and negatively affect crop cultivation: the harmful effects of pathogens and toxin producers and the beneficial effects of the biological control agents, which can be used as microbial pesticides, are easily understood. In contrast, the effects and potential of endophytes on crop cultivation are poorly understood. Furthermore, there is little information about the genetic and phylogenetic relationships between endophytes and plant pathogens, biological control agents or saprophytes. Although most endophytes are thought to be non-pathogenic (Kuldau and Yates 2000), further analyses of the ecological functions of *Fusarium* endophytes are needed to elucidate their roles in crop cultivation.

We hypothesized that *Fusarium* may be compatible with a broad range of plants, especially those in the FOSC although, in a few cases, slight disease symptoms such as discoloration and stunting were caused. This hypothesis is supported by evidence obtained in several previous studies. For example, Armstrong and Armstrong (1948) described the invasion of non-host plants by pathogenic strains of *F. oxysporum*; Banihashemi and deZeeuw (1975) reported that *F. oxysporum* f. sp. *melonis* can invade at least two non-host crops, corn and soybean; Gordon, Okamoto and Jacobson (1989) showed that the melon wilt pathogen can also invade five non-host crops (i.e. alfalfa, cotton, sugar beet, tomato and wheat); Katan (1971) found that *F. oxysporum* f. sp. *lycopercisi* could invade weeds that are non-hosts of this tomato pathogen; and Kuldau and Yates (2000) listed many plant species from which *Fusarium* endophytes were obtained. In the putative non-pathogenic members of the FOSC, the well-known biological control strain Fo47 could invade at least two crops, cucumber (Benhamou, Garand and Goulet 2002) and flax (Nagao, Couteaudie and Alabouvette 1990).

In this study, we aimed to characterize the phylogeny and diversity of *Fusarium* endophytes isolated from tomatoes (Table S1, Supporting Information). To improve the quality of the characterization, we also isolated fusaria directly from soils as a reference (Table S2, Supporting Information). Furthermore, we prepared morphologically and/or phytopathologically characterized reference strains. Some reference strains have been deposited in the MAFF gene bank system (Table S3, Supporting Information), National Institute for Agrobiological Sciences, Tsukuba, Japan. Nucleotide sequences of the translation elongation factor-1 alpha (EF-1α) gene were compared among the tomato isolates, soil isolates and reference strains. Furthermore, based our hypothesis, we confirmed the ability of some soil and tomato isolates to infect tomato and melon by means of inoculation and reisolation experiments.

MATERIALS AND METHODS

Location of soil sampling sites

Soils were obtained between March 2009 and June 2010 from six locations in Japan: a garden of the NARO Agricultural Research Center in Tsukuba, Ibaraki Prefecture, two commercial fields (fields A and B) in Ibaraki Prefecture, one commercial field (field C) in Chiba Prefecture and a field of a school in Ibaraki Prefecture (field D) and a field of Nagoya University in Togo, Aichi Prefecture.

Isolation of *Fusarium* endophytes from tomato stems

Soil-inhabiting *Fusarium* endophytes are thought to invade roots and then colonize stem vascular tissues. To obtain extensively colonizing endophytes, we isolated them from the inside of stems. Each of the soils was mixed with approximately the same weight of Kureha soil (Kureha, Tokyo, Japan), which is an artificial, aggregate-structured dry soil containing fertilizer that keeps field soils soft. The soil mixtures were dispensed into plastic baskets (33 $\times$ 25 $\times$ 10 cm deep) lined with two sheets of paper. Approximately 200 seeds of tomato cultivar Momotaro (Takii, Kyoto, Japan) were sown in the soils except for the soil sampled in Tsukuba where approximately 20 seeds were employed. After 3–6 weeks of cultivation in a greenhouse, a stem segment approximately 3.5 cm below the cotyledons was harvested from each plant and washed with tap water. Each piece was rinsed in 0.1% Tween 20 for a few seconds, then in sodium hypochlorite solution (2% effective chlorine) for 10 min and then washed four times in sterile distilled water. Each piece was then air-dried in a laminar flow chamber, and then placed on Fo-G1 agar medium (Nishimura 2007), followed by incubation at 26°C for 1–2 weeks. Fungal mycelia were transferred onto new Fo-G1 agar medium and incubated at 26°C for 2 weeks. Colonies were transferred onto synthetic low-nutrient agar media (Nirenberg and Aoki 1997). After 2 weeks incubation at 26°C, the cultures were stored at 8°C. Fungal isolates were named by a combination of two letters combined with four digits. 'SL' was the letter designation used to describe tomato isolates (Table S1, Supporting Information). Effectiveness of the surface sterilization was confirmed with the imprinting method (Shishido, Loeb and Chanway 1995): five randomly chosen pieces of surface-sterilized stems were imprinted onto fresh nutrient agar to confirm that no microbial growth was present after they had been incubated at 26°C for 2 weeks.

Isolation of *Fusarium* directly from soils

Soils used for isolating *Fusarium* were sampled from field A and Togo in September 2010 and June 2010. These soils were passed through a sieve with a 2-mm aperture, and a portion of each sample was used to determine moisture content by air-drying at 105°C for 24 h. Fungal isolates were named by a combination of two letters (TC) combined with four digits (Table S2, Supporting Information).

A total of 6 soil samples obtained on September 2010 from field A and 10 samples obtained on June 2010 from Togo were serially diluted 10-fold with sterile distilled water. Soil suspensions (100 μl) of each dilution were spread onto one plate of Fo-G1 agar medium. After 10 days incubation at 26°C, all fungal colonies that formed on each plate spread with a dilution equivalent of 1 mg soil (dry weight) per 100 μl aliquot were transferred onto fresh Fo-G1 agar medium and incubated for 2 weeks at 26°C. In addition, 17 fungal colonies (15 colonies from Togo and 2 colonies from Field A) were randomly chosen from the plates of the other soil suspension dilutions.

Partial EF-1α nucleotide sequences

Fungi were grown on potato dextrose agar medium at 26°C for 10 days. Each colony was transferred into 50 μl of TE buffer

(10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA) and then heated at 95°C for 10 min. The heated mycelial suspensions were used for templates in PCR for amplification of the EF-1α gene. PCR was performed with primers EF-1 and EF-2 (O'Donnell *et al.* 1998) in 50 μl containing 5 μl of the heated mycelium suspension, 0.3 μM each primer, 1.0 U KOD FX DNA polymerase (Toyobo, Osaka, Japan), 1× PCR buffer for KOD FX and 0.4 mM each dNTP. The PCR profile was as follows: an initial preheating at 94°C for 2 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s and extension at 68°C for 40 s, and a final extension at 68°C for 7 min. To confirm amplification, 5 μl of each sample was separated by electrophoresis using 1% agarose gels. PCR products were purified using a MinElute 96 UF PCR Purification Kit (Qiagen, Tokyo, Japan) following the manufacturer's directions. Nucleotide sequences of the purified fragments were determined directly using a BigDye Terminator Cycle Sequencing Kit version 3.1 (Life Technologies, Carlsbad, CA, USA) on a 3130 automated DNA sequencer (Life Technologies). Primer EF-22 (O'Donnell *et al.* 1998; Geiser *et al.* 2004) was used for cycle sequencing.

Unique partial sequences of the EF-1α gene were aligned using the Clustal X2 program (Jeanmougin *et al.* 1998; Larkin *et al.* 2007), and phylogenetic relationships were inferred based on the nucleotide sequence alignment of the gene among the *Fusarium* isolates using MEGA5 (Tamura *et al.* 2011). A neighbor-joining tree was constructed based on distances determined by the method of Jukes and Cantor (1969) using 1000 bootstrap replicates. Unique nucleotide sequences were compared with nucleotide sequences registered in FUSARIUM-ID (Geiser *et al.* 2004; Park *et al.* 2011) and the DDBJ/EMBL/GenBank databases using the BLAST program (Altschul *et al.* 1990), and the phylogenetic position within the genus was deduced based on sequences of their closest relatives.

Inoculation assays

Invasion of tomato plants (cultivar Momotaro) by the *Fusarium* isolates was confirmed by inoculation and reisolation experiments. Each cell of every white plastic tray (50 cells per tray ~75 ml each; Tokai Kasei, Mino, Japan) was filled with approximately 60 g of Kureha soil and sown with three tomato seeds. Each cell containing soil and tomato seeds received 20 ml of a conidial suspension (approximately 3E + 07 cells ml^{-1}) of an isolate, resulting in a density of approximately 5E + 06 cells g^{-1}. As a negative control, each cell received 20 ml of sterile distilled water. The tomato plants were cultivated in an air-conditioned greenhouse (28 ± 3°C) and thinned to two plants per cell. After 21–28 days of cultivation, a piece of the stem approximately 3.5 cm in length below the cotyledons of each plant was excised and then surface-sterilized as described above and incubated on Fo-G1 agar medium for 2 weeks at 28°C. To check for host specificity of the *Fusarium* isolates, their ability to colonize melon plants (cultivar Amus; Japan Horticultural Production and Research Institute, Matsudo, Japan) was tested. The method of inoculation and reisolation of the melon plants was the same as for tomato.

RESULTS

Isolation of *Fusarium* from tomato plants and soils and phylogenetic position of isolates by means of EF-1α sequence analysis

Soils used for tomato cultivation were sampled from six locations (Field A to D, Togo, and Tsukuba) between 9 March 2009 and 19 June 2010. A total of 17 615 tomato plants were used. A total of 543 fusaria were isolated from 542 of the 17 615 plants (Tables 1 and S1, Supporting Information); two isolates (SL0006 and SL0008) were isolated from the same plant. A total of 180 soil isolates were obtained from field A (64 isolates) and Togo (116 isolates) (Tables 2 and S2, Supporting Information).

Phylogenetic relationships among the tomato isolates, the soil isolates and the reference strains were investigated by constructing a phylogenetic tree (data not shown). The tomato isolates belonged to the following three species complexes: FOSC (332 isolates), FFSC (75 isolates) and FSSC (136 isolates) (Table 1). Most soil isolates belonged to the FOSC (82 isolates), the FFSC (21 isolates) and the FSSC (68 isolates), but 9 isolates belonged to other species/species complexes (Table 2).

Table 1. Isolation of *Fusarium* endophytes from stems of tomato plants.

Location of soil sampling[a]	No. of tomato plants cultivated	No. of isolates[b]		
		FOSC	FFSC	FSSC
Field A	8995	240	53	23
Field B	2657	12	0	72
Field C	2530	32	10	18
Field D	2785	45	11	14
Togo	631	2	1	6
Tsukuba	17	1	0	3
Total	17615	332	75	136

[a]Sampled soils were used for cultivating tomato plants.
[b]Species complex to which each isolate belongs was inferred based on partial nucleotide sequence of EF-1α gene.
FOSC, *F. oxysporum* species complex; FFSC, *F. fujikuroi* species complex; FSSC, *F. solani* species complex.

Table 2. Isolation of *Fusarium* directly from soils.

Location of soil sampling	No. of isolates			
	FOSC	FFSC	FSSC	Other *Fusarium*
Field A	40	1	22	1
Togo	42	20	46	8
Total	82	21	68	9

Comparison of EF-1α gene sequence types (EFSTs) among *Fusarium*

We divided *Fusarium* into EFSTs according to differences in nucleotide sequences of their EF-1α genes. Fungi that belonged to the FOSC accounted for 21 EFSTs: 332 tomato isolates were divided into nine EFSTs and 82 soil isolates comprised five EFSTs (Fig. 1, Table 3). Five EFSTs (FOSC-01, 03, 04, 05 and 09) were commonly detected in isolates from both tomato plants and soil, four EFSTs (FOSC-02, 06, 07 and 08) were detected only in tomato and no EFSTs were detected only in soil (Fig. 1, Table 3). Two tomato pathogens, *F. oxysporum* f. sp. *lycopersici* (15 strains) and *F. oxysporum* f. sp. *radicis-lycopersici* (four strains) (Table S3, Supporting Information), were represented in the reference strains. Strains pathogenic to tomato contained three EFSTs (FOSC-03, 10 and 11); FOSC-03 was also found in tomato (Fig. 1). In other reference strains belonging to the FOSC, there were 21 pathogens (formae

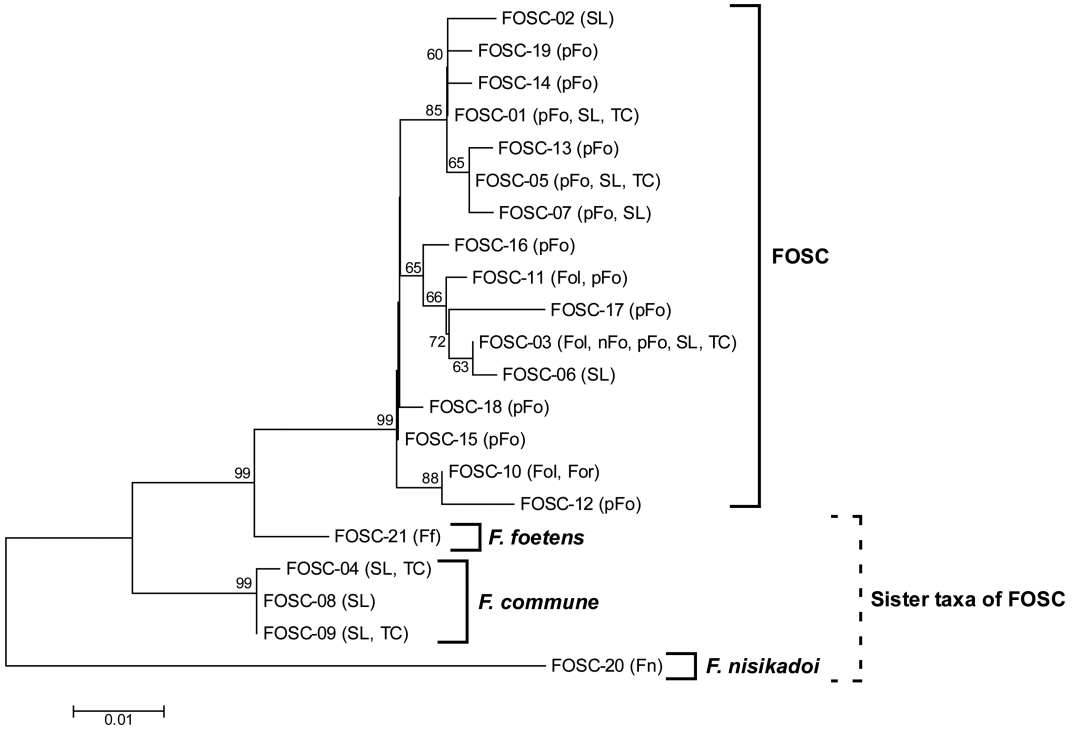

Figure 1. Neighbor-joining tree derived from 21 unique nucleotide sequences of the translation elongation factor 1α (EF-1α) gene found in 477 isolates and reference strains belonging to the FOSC and its sister taxa, *F. commune*, *F. foetens* and *F. nisikadoi*. Distances were determined by the maximum composite likelihood. Scale bar indicates a distance of 0.01 (one base changes per 100 nucleotide positions). Values on the branches represent bootstrap support of 60% or greater based on 1000 replicates. A total of 21 unique sequences (EFSTs) are indicated by FOSC-01 to 21. Information on the isolates and reference strains in each EFST is shown in parentheses: SL, tomato isolates; TC, soil isolates; *Ff*, *F. foetens*; *Fn*, *F. nisikadoi*; *Fol*, *F. oxysporum* f. sp. *lycopersici*; *For*, *F. oxysporum* f. sp. *radicis-lycopersici*; nFo, non-pathogenic *F. oxysporum*; and pFo, plant pathogenic *F. oxysporum* belonging to other formae speciales except for *lycopersici* and *radicis-lycopersici*.

speciales *asparagi*, *batatas*, *conglutinans*, *cubense*, *cucumerinum*, *dianthi*, *fragariae*, *gladioli*, *lactucae*, *lagenariae*, *matthiolae*, *melongenae*, *melonis*, *momordicae*, *niveum*, *phaseoli*, *raphani*, *spinaciae* and *tulipae*; a strain pathogenic to *Paulownia tomentosa*; and a strain pathogenic to *Alnus pendula*) (Table S3, Supporting Information). Of the 13 EFSTs found among these pathogens, 4 (EFST-01, 03, 05 and 07) were also found in tomato (Fig. 1). Of the 21 EFSTs, nucleotide sequences of the EF-1α gene of 5 EFSTs (FOSC-06, 08, 12, 20 and 21) represented novel EFSTs (O'Donnell *et al.* 2009; Table 3).

Fungi that belonged to the FFSC were divided into 26 EFSTs: 75 tomato isolates were divided into seven EFSCs; and 21 soil isolates were placed in six EFSTs (Fig. 2, Table 4). Two EFSTs (FFSC-03 and 07) were detected in both tomato and soil, five EFSTs (FFSC-01, 02, 04, 05 and 06) were detected only in tomato and four EFSTs (FFSC-08 to 11) were detected only in soil (Fig. 2, Table 4). Best matching isolates by homology searches in the FUSARIUM-ID sequence database were also shown in Table 4. All EFSTs found among tomato and soil, except for FFSC-07 and 11, suggested they were *F. fractiflexum*, *F. fujikuroi* or *F. proliferatum* (Fig. 2). FFSC-07 was associated with 13 isolates and was found among tomato and soil; FFSC-11 was associated with only one isolate from soil (Table 4). The phylogenetic identity of FFSC-07 and 11 could not be inferred from this analysis (Fig. 2). The present result suggests that it might represent a new species.

Members of the FSSC were divided into 16 EFSTs: 136 tomato isolates were divided into 8 EFSTs, and 68 soil isolates were divided into 11 EFSTs (Table 5). Six EFSTs (FSSC-01, 03–06 and 08) were detected in tomato and soil, two EFSTs (FSSC-02 and 07) were detected only in tomato and five EFSTs (FSSC-09 to 13) were detected only in soil. No reference strains belonging to the FSSC shared the same EFSTs detected in tomato and soil (Table 5). Of the 16 EFSTs, 9 (FSSC-01, 02, 07–09, 11 and 13–15) appeared to be new sequence types (O'Donnell *et al.* 2008a).

To add more FSSC reference strains, 27 EF sequences deposited in DDBJ/EMBL/GenBank were included in the phylogenetic analysis (Fig. 3, Table S4, Supporting Information). Of the thirteen EFSTs found in tomato and soil, seven were related to plant pathogens (Fig. 3): three EFSTs, FSSC-03, 08 and 10, related to pathogens of *Eustoma grandiflorum* (accession no. AB426618); three EFSTs, FSSC-01, 02 and 12, related to *F. solani* f. sp. *radicicola* (AB513841); and one EFSTs, FSSC-13, related to *F. solani* f. sp. *mori* (FSSC-16, the reference strain MAFF 840046) and *F. solani* f. sp. *pisi* (AF1788337 and AF178355). The other six EFSTs, 04–07, 09 and 11, were not closely related to pathogens used in the present study.

Nine soil isolates that formed a clade (*F. tricinctum* species complex) were resolved as four EFSTs (Other-01 to 04): tomato isolates were not detected in these EFSTs (Fig. 4, Table 6).

Inoculation and reisolation experiments using tomato and melon

To test for endophytic activity within tomato, and thus invasion ability, we performed inoculation and reisolation experiments using a total of 37 isolates and strains: 9 tomato isolates, 24 soil isolates, 1 tomato pathogen (*F. oxysporum* f. sp. *lycopersici* 880621a-1, a causal agent of vascular wilt disease of tomato), 1 melon pathogen (*F. oxysporum* f. sp. *melonis* Mel02010, a causal agent of vascular wilt disease of melon), 1 rice pathogen (*F. fujikuroi* AFM06–014A, a causal agent of bakanae disease) and 1 strain (*F. fujikuroi* MAFF 235151), of which pathogenicity was

Table 3. Identification of FOSC found in this study.

EFST[a]	Number of isolates/strains			Plant pathogens involved in the reference strains[b]	Representative			Identical EF sequence type (ST) of *F. oxysporum* species complex (O'Donnell *et al.* 2009)
	Tomato	Soil	Reference		Isolate/strain	Accession no.[c]	MAFF no.[d]	
FOSC-01	239	56	7	Cucumber pathogen (f. sp. *cucumerinum*), Melon pathogen (f. sp. *melonis*), Common bean pathogen (f. sp. *phaseoli*), *Alnus pendula* pathogen (f. sp. is not identified)	SL0001	AB916973	244588	ST11, ST20, ST47, ST68, ST73, ST81, ST171, ST204, ST247
FOSC-02	5	0	0	None	SL0014	AB916974	244591	ST224
FOSC-03	42	3	13	Sweet potato pathogen (f. sp. *batatas*), Tomato pathogen (f. sp. *lycopersici*)	SL0019	AB916975	244594	ST7, ST16, ST40, ST44, ST48, ST53, ST55, ST59, ST79, ST112, ST113, ST114, ST122, ST130, ST142, ST150, ST157, ST215, ST217, ST232, ST238, ST240, ST244, ST256
FOSC-04	2	4	0	None	SL0021	AB916976	244595	
FOSC-05	29	18	13	Cucumber pathogen (f. sp. *cucumerinum*), Strawberry pathogen (f. sp. *fragariae*), Lettuce pathogen (f. sp. *lactucae*), Bottle gourd pathogen (f. sp. *lagenariae*), Melon pathogen (f. sp. *melonis*), Bitter melon pathogen (f. sp. *momordicae*), Watermelon pathogen (f. sp. *niveum*), Spinach pathogen (f. sp. *spinaciae*)	SL0035	AB916977	244599	ST3, ST27, ST31, ST35, ST64, ST66, ST67, ST76, ST82, ST84, ST85, ST86, ST174, ST181, ST208, ST227, ST241, ST243
FOSC-06	1	0	0	None	SL0041	AB916978	244600	None
FOSC-07	12	0	1	An empress tree pathogen (f. sp. was not identified)	SL0054	AB916979	244601	ST187
FOSC-08	1	0	0	None	SL0350	AB916980	244605	None
FOSC-09	1	1	0	None	SL0580	AB916981	244610	ST164, ST180, ST182
FOSC-10	0	0	6	Tomato pathogens (ff. sp. *lycopersici* and *radicis-lycopersici*)	Reference strain CU1	AB916982	Not deposited	ST63, ST168, ST199, ST207
FOSC-11	0	0	4	Asparagus pathogen (f. sp. *asparagi*), Tomato pathogen (f. sp. *lycopersici*)	Reference strain GF1022	AB916983	Not deposited	ST21, ST51, ST56, ST75, ST83, ST161, ST216, ST245

Table 3. *continued*

EFST[a]	Number of isolates/strains			Plant pathogens involved in the reference strains[b]	Representative			Identical EF sequence type (ST) of *F. oxysporum* species complex (O'Donnell *et al.* 2009)
	Tomato	Soil	Reference		Isolate/strain	Accession no.[c]	MAFF no.[d]	
FOSC-12	0	0	7	Egg-plant pathogens (*melongenae*), Tomato pathogen (*radicis-lycopersici*)	Reference strain	AB916984	103047	None
FOSC-13	0	0	2	None	Reference strain	AB916985	103051	ST74
FOSC-14	0	0	1	*Brassica oleracea* pathogen (f. sp. *conglutinans*), Melon pathogen (f. sp. *melonis*), *Raphanus sativus* pathogen (f. sp. *raphani*)	Reference strain	AB916986	103057	ST2, ST4, ST19, ST28, ST29, ST32, ST37, ST42, ST50, ST65, ST71, ST87, ST88, ST89, ST90, ST101, ST110, ST117, ST133, ST151, ST159, ST169, ST170, ST176, ST188, ST189, ST190, ST191, ST194, ST196, ST210, ST254, ST255
FOSC-15	0	0	1	Carnation pathogen (f. sp. *dianthi*)	Reference strain	AB916987	103072	ST46
FOSC-16	0	0	2	*Matthiola* pathogen (f. sp. *matthiolae*), Melon pathogen (f. sp. *melonis*), Tulip pathogen (*tf. sp. ulipae*)	Reference strain	AB916988	235105	ST13, ST22, ST30, ST80, ST106, ST144, ST220, ST223
FOSC-17	0	0	1	Gladiolus pathogen (f. sp. *gladioli*)	Reference strain	AB916989	305610	ST12, ST92, ST93, ST104, ST146, ST148, ST155, ST246
FOSC-18	0	0	1	Banana pathogen (f. sp. *cubense*)	Reference strain	AB916990	306716	ST25
FOSC-19	0	0	1	Melon pathogen (f. sp. *melonis*)	Reference strain YU-1	AB916991	Not deposited	ST11, ST20, ST26, ST45, ST47, ST68, ST72, ST73, ST81, ST107, ST149, ST171, ST184, ST204, ST221, ST225, ST230, ST247, ST252
FOSC-20 (*F. nisikadoi*)	0	0	1	None	Reference strain	AB916992	237507	None
FOSC-21 (*F. foetens*)	0	0	2	*Begonia* × *hiemalis* pathogens (Leaf and stem rot disease; f. sp. is not identified)	Reference strain	AB916993	240179	None
Total	332	82	63					

[a]EF-1α gene sequence type.
[b]*Forma specialis* (f. sp.) was indicated in parentheses.
[c]DDBJ/EMBL/GenBank accession number.
[d]MAFF genebank system number, National Institute of Agrobiological Sciences, Tsukuba, Japan.

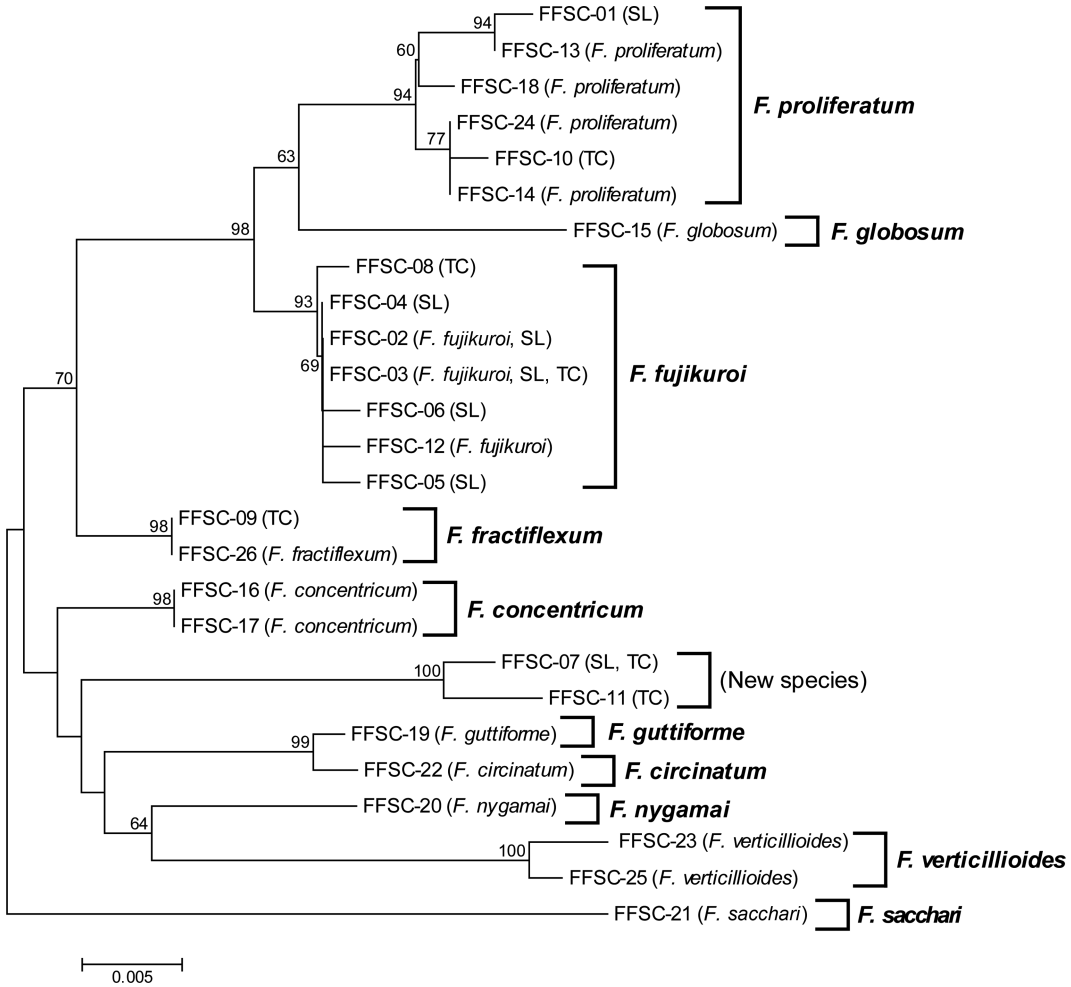

Figure 2. Neighbor-joining tree derived from 26 EFSTs found in 120 isolates and reference strains belonging to the FFSC. Distances were determined by the maximum composite likelihood. Values on the branches represent bootstrap support of 60% or greater based on 1000 replicates. The 26 unique sequences (EFTSs) are indicated by FFSC-01 to 26. Information on the isolates and reference strains in each EFST is shown in parentheses: SL, tomato isolates; TC, soil isolates.

unknown. These experiments were conducted seven different times: the first and second experiments used tomato isolates and plant pathogenic strains belonging to the FOSC; the third to fifth mainly used soil isolates; the sixth used soil isolates belonging to the FSSC; and the seventh used soil isolates belonging to the FFSC. All isolates and strains used were reisolated from surface-sterilized tomato stems except for soil isolate TC0058 (Table 7). Inoculation and reisolation experiments with melon were also performed using seven tomato isolates and two reference strains (one melon pathogen and one tomato pathogen). All isolates and strains used were reisolated from surface-sterilized melon stems (Table 8). In these experiments, wilt symptoms were not induced by any of the tomato and soil isolates tested. These experiments were performed several times in an air-conditioned greenhouse. However, the reisolation frequency appeared to be affected by factors other than temperature, such as day length or the strength of sunlight.

DISCUSSION

Fusaria have been targeted in a large number of research studies in areas such as disease control, ecology, methods and techniques, pathogenicity and taxonomy (Leslie and Summerell 2006). Development of media for specific isolation of Fusaria is an important achievement. Well-known selective media include Nash and Snyder medium and its derivatives such as Komada's medium (Nash and Snyder 1962; Komada 1975), malachite green agar (Castellá *et al.* 1997) and selective Fusarium agar (Burgess *et al.* 1988). Nash and Snyder medium and its derivatives contain pentachloronitrobenzene (PCNB). In 2007, two derivatives of Komada's medium were developed for isolating *F. oxysporum*. These media (Fo-G1 and Fo-G2) do not contain PCNB (Nishimura 2007). Our initial objective in the present study was to characterize FOSC endophytes within tomato stems phylogenetically. We used Fo-G1 medium and easily isolated fusaria belonging to the FFSC and FSSC in addition to FOSC. These results were consistent with those of Nishimura (2007), in which FFSC and FSSC strains could grow on Fo-G1 medium. We isolated FOSC more frequently from tomato than members of the other two species complexes. In other studies on *Fusarium* endophytes in roots and basal stems, FOSC was also reported as the dominant dweller in those habitats (Windels and Kommedahl 1974; Helbig and Carroll 1984; Gordon, Okamoto and Jacobson 1989). These results suggest that members of the FOSC were more compatible with plants tested or were more prevalent in the soils than those of the FFSC and FSSC.

Table 4. Identification of FFSC found in this study.

EFST	Number of isolates/strains			Plant pathogens involved in the reference strains	Representative			Best matching (FUSARIUM-ID)			Best matching (DDBJ/EMBL/GenBank)			Species inferred
	Tomato	Soil	Reference		Isolate strain	Accession no.	MAFF no.	Isolate ID	E-value	Identities (%)	Accession no.	E-value	Identities (%)	
FFSC-01	1	0	0		SL0018	AB916994	244593	FD_01379	0	99.25	KM873334	0	99.75	*F. proliferatum*
FFSC-02	3	0	1		SL0031	AB916995	244597	FD_01857	0	99.74	HQ622556	0	100	*F. fujikuroi*
FFSC-03	50	5	1		SL0033	AB916996	244598	FD_01857	0	100	LC009439	0	100	*F. fujikuroi*
FFSC-04	12	0	0		SL0089	AB916997	244602	FD_01857	0	99.74	JN695744	0	99.75	*F. fujikuroi*
FFSC-05	7	0	0		SL0273	AB916998	244603	FD_01857	0	99.74	LC009439	0	99.75	*F. fujikuroi*
FFSC-06	1	0	0		SL0293	AB916999	244604	FD_01857	0	99.74	LC009439	0	99.75	*F. fujikuroi*
FFSC-07	1	12[a]	0		SL0584	AB917000	244611	FD_01762	0	100	AF160306	0	100	n.i.[b]
FFSC-08	0	1	0		TC0066	AB917001	244617	FD_01857	0	99.74	LC009439	0	99.75	*F. fujikuroi*
FFSC-09	0	1	0		TC0083	AB917002	244619	FD_01162	0	100	AB917019	2E-165	94.26	*F. fractiflexum*
FFSC-10	0	1	0		TC0089	AB917003	244620	FD_01389	0	100	KC808223	0	100	*F. proliferatum*
FFSC-11	0	1	0		TC0108	AB917004	244625	FD_01151	0	99.74	AF160303	0	99.75	n.i.
FFSC-12	0	0	2	Rice bakanae disease pathogen	Reference strain AFM06–014A (*F. fujikuroi*)	AB917005	Not deposited	–	–	–	–	–	–	–
FFSC-13	0	0	2	*Cymbisium* leaf spot pathogen; Welsh onion seedling wilt pathogen (fumonisin B_1 production)	Reference strain (*F. proliferatum*)	AB917006	236459	–	–	–	–	–	–	–
FFSC-14	0	0	1		Reference strain (*F. proliferatum*)	AB917007	236871	–	–	–	–	–	–	–
FFSC-15	0	0	1		Reference strain (*F. globosum*)	AB917008	237511	–	–	–	–	–	–	–
FFSC-16	0	0	1		Reference strain (*F. concentricum*)	AB917009	237649	–	–	–	–	–	–	–
FFSC-17	0	0	1		Reference strain (*F. concentricum*)	AB917010	237650	–	–	–	–	–	–	–
FFSC-18	0	0	1		Reference strain (*F. proliferatum*)	AB917011	238030	–	–	–	–	–	–	–
FFSC-19	0	0	2		Reference strain (*F. guttiforme*)	AB917012	239055	–	–	–	–	–	–	–
FFSC-20	0	0	2		Reference strain (*F. nygamai*)	AB917013	239069	–	–	–	–	–	–	–
FFSC-21	0	0	1		Reference strain (*F. sacchari*)	AB917014	239074	–	–	–	–	–	–	–
FFSC-22	0	0	3		Reference strain (*F. circinatum*)	AB917015	239425	–	–	–	–	–	–	–
FFSC-23	0	0	1		Reference strain (*F. verticillioides*)	AB917016	240087	–	–	–	–	–	–	–
FFSC-24	0	0	2		Reference strain (*F. proliferatum*)	AB917017	410715	–	–	–	–	–	–	–
FFSC-25	0	0	1	Maize Fusarium ear or stalk rot pathogen	Reference strain (*F. verticillioides*)	AB917018	511481	–	–	–	–	–	–	–
FFSC-26	0	0	1	*Cymbidium* leaf spot pathogen	Reference strain (*F. fractiflexum*)	AB917019	237530	–	–	–	–	–	–	–
Total	75	21	24	–	–	–	–	–		–				

[a]Eleven of the 12 isolates were deposited in the NIAS Genebank (MAFF 244613 to 244616, 244623, 244624, 244626, 244627, 244629 to 244631).

[b]n.i., species could not be determined.

Table 5. Identification of FSSC found in this study.

EFST	Number of isolates/strains			Plant pathogens involved in the reference strain	Representative			Identical multilocus sequence type (MLST) of *F. solani* species complex (O'Donnell *et al.* 2008a)	Phylogenetically distinct species inferred[a]
	Tomato	Soil	Reference		Isolate/strain	Accession no.	MAFF no.		
FSSC-01	40	11	0		SL0002	AB917020	244589	None	5
FSSC-02	3	0	0		SL0009	AB917021	244590	None	5
FSSC-03	37	24	0		SL0016	AB917022	244592	5-c, 5-e, 5-f, 5-n, 5-d, 5-a	5
FSSC-04	33	19	0		SL0028	AB917023	244596	3+4-ddd, 3+4-ii, 3+4-nn, 3+4-rr	3+4
FSSC-05	11	3	0		SL0419	AB917024	244606	3+4-i, 3+4-z	3+4
FSSC-06	8	2	0		SL0460	AB917025	244607	3+4-k, 3+4-x, 3+4-y	3+4
FSSC-07	3	0	0		SL0465	AB917026	244608	None	3+4
FSSC-08	1	2	0		SL0573	AB917027	244609	None	5
FSSC-09	0	2	0		TC0068	AB917028	244618	None	3+4
FSSC-10	0	2	0		TC0095	AB917029	244622	5-h	5
FSSC-11	0	1	0		TC0157	AB917030	244632	None	11 or 17
FSSC-12	0	1	0		TC0159	AB917031	244633	5-g	5
FSSC-13	0	1	0		TC0163	AB917032	244634	None	11
FSSC-14	0	0	1	Lotus pathogen (f. sp. is not identified)	Reference strain	AB917033	240020	None	21
FSSC-15	0	0	1	Common bean pathogen (f. sp. *phaseoli*)	Reference strain	AB917034	305607	None	*F. cuneirostrum*
FSSC-16	0	0	1	White mulberry pathogen (f. sp. *mori*)	Reference strain	AB917035	840046	11-a	11
Total	136	68	4	–	–	–	–	–	–

[a]Phylogenetically distinct species shown in O'Donnell *et al.* (2008a) was inferred based on maximum parsimony and maximum likelihood tree analyses using MEGA5.

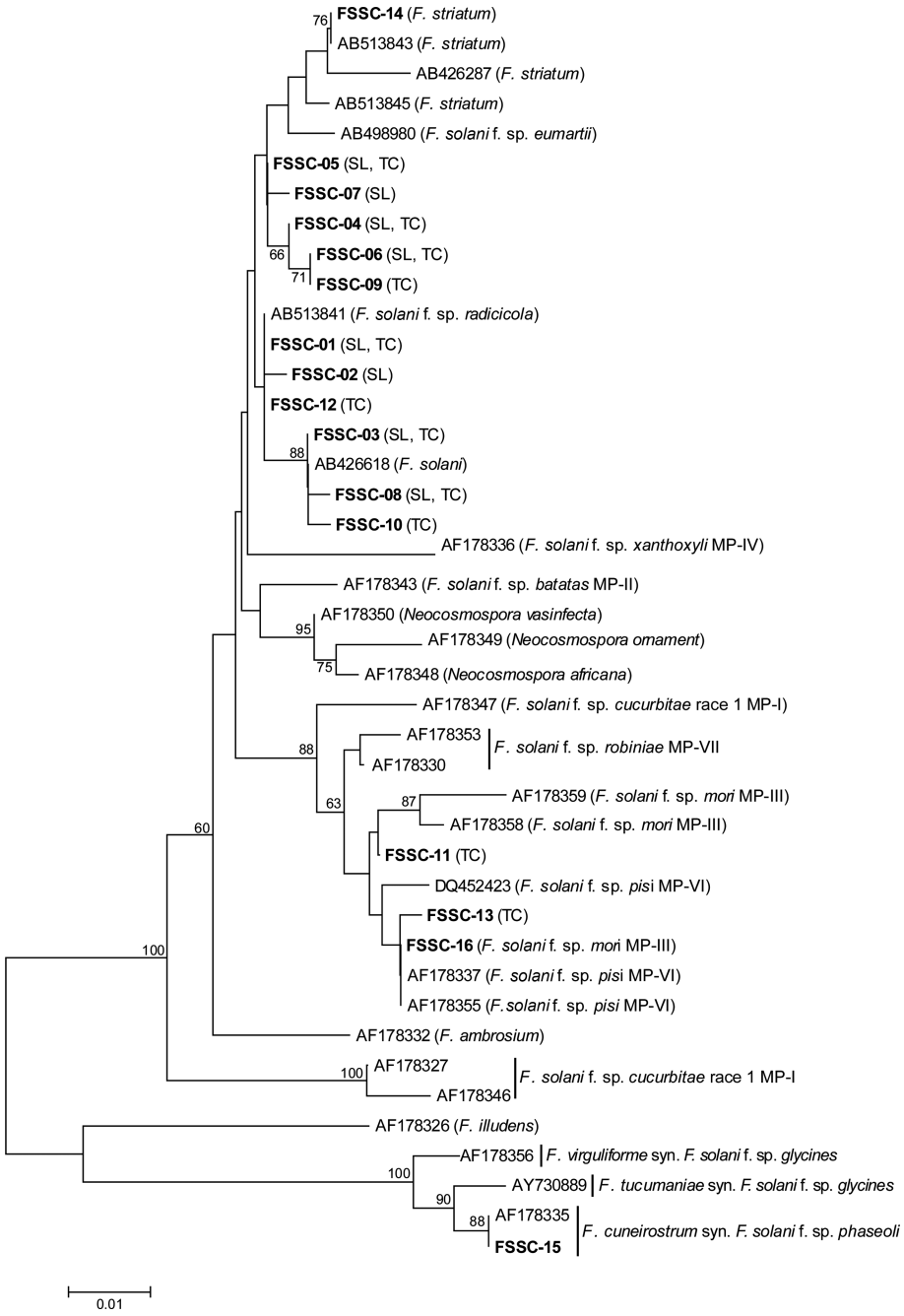

Figure 3. Neighbor-joining tree derived from 42 EFSTs found in 242 isolates, reference strains and database strains, of which EF sequences were deposited in DDBJ/EMBL/GenBank, belonging to the FSSC. Distances were determined by the maximum composite likelihood. Values on the branches represent bootstrap support of 60% or greater based on 1000 replications. A total of 42 unique sequences are indicated by FSSC-01 to 16 (original isolates in this study and reference strains) or DDBJ/EMBL/GenBank accession numbers (database strains). Information on the isolates, reference strains and database strains involved in each unique sequence is shown in parentheses: SL, tomato isolate(s); TC, soil isolate(s).

Tomato is a source of *Fusarium* endophytes. *Fusarium incarnatum/F. equiseti* species complex (Gordon, Okamoto and Jacobson 1989), FOSC (Gordon, Okamoto and Jacobson 1989; Hallman and Sikora 1994; Kim *et al.* 2007) and FSSC (Gordon, Okamoto and Jacobson 1989; Kavroulakis *et al.* 2007) endophytes have been obtained from tomato. The three *Fusarium* groups are widely distributed in the world (Backhouse, Burgess amd Summerell 2001). We obtained FFSC endophytes in addition to FOSC and FSSC endophytes from tomato but did not isolate *F. equiseti*. Fo-G1 medium might not be suitable for the growth of *F. equiseti*, or this fungus may be absent or present in too low number in the soils we used. To obtain *Fusarium* endophytes from tomato, we used a total of 17 615 tomato stems. From the 17 615 stems, 543 *Fusarium* endophytes were isolated (the proportion of stems from which fusaria were isolated was 0.031). Two isolates, SL0006 and SL0008, were obtained from the same stem and shared the same EFST. Thus, SL0006 and SL0008 might be clones. Kim *et al.* (2007) showed that the isolation frequency of

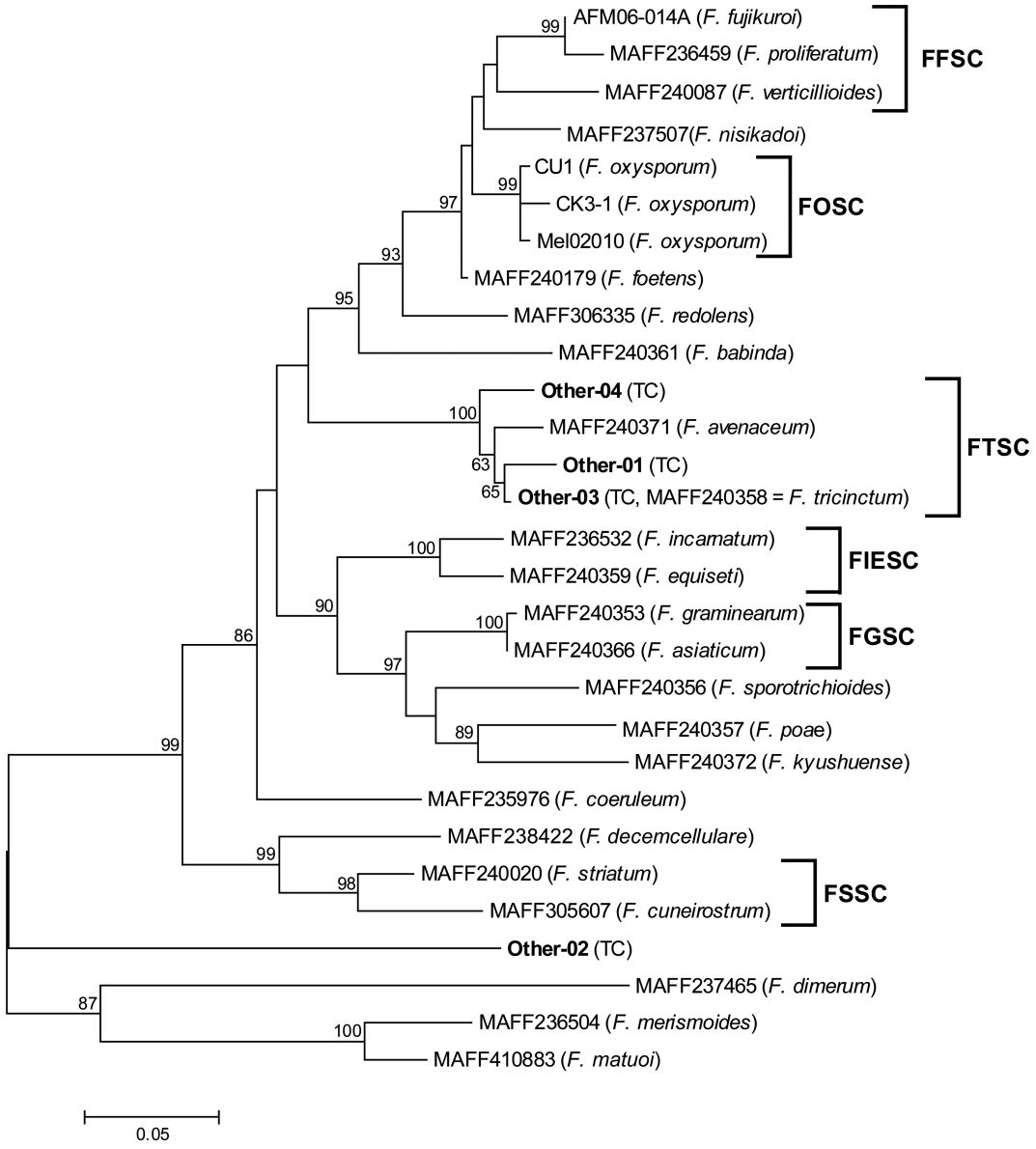

Figure 4. Neighbor-joining tree derived from 25 EFSTs found in reference strains and four EFSTs found in soil isolates belonging to species other than the FOSC, FFSC and FSSC. Distances were determined by the maximum composite likelihood. Values on the branches represent bootstrap support of 60% or greater based on 1000 replications. The 29 unique sequences are indicated by Other-01 to 04 and strain names. Information on the isolates and reference strains in each sequence is shown in parentheses: TC, soil isolates. FGSC, *F. graminearum* species complex; FIESC, *F. incarnatum*/*F. equiseti* species complex; FTSC, *F. tricinctum* species complex.

fungal endophytes from stems was lower than from roots. These results were inferred using five crops including tomato. Thus, if we had also isolated *Fusarium* endophytes from tomato roots, we might have obtained endophytes at a higher frequency than from stems.

We expected that the inoculation and reisolation experiments might reveal the degree of compatibility of fusaria with plants. In the experiments with tomato, tomato isolates SL0301, SL0303, SL0316 and SL0321 showed the high reisolation frequencies. These isolates were obtained from the same field and had the same EFST (FOSC-03). Thus, these isolates may be clones. Two significant results were apparent (Table 7). First, there were differences in the reisolation frequency among isolates. For example, the reisolation frequency of isolate SL0300 (0.267) was significantly higher than that of isolate TC0003 (0.008). Second, most soil isolates used in the experiments were also reisolated from tomato stems, with the exception of isolate TC0058. These two results suggested that the frequency of invasion of tomato stems differed among isolates and that most *Fusarium* isolates have the ability to invade tomato stems. Tomato isolates also showed the ability to invade melon (Table 8). Therefore, the results supported our hypothesis that *Fusarium* may be compatible with a broad range of plants, especially those especially in the FOSC.

As described in the section 'Results', a new species within the FFSC was suggested based on phylogenetic analysis of the EF-1α gene and searches of the FUSARIUM-ID database. The nucleotide sequences of the EF-1α genes of EFSTs FFSC-07 and 11 were almost identical to those of *Fusarium* sp. NRRL 26152 (isolate ID = FD_01762) and *Fusarium* sp. NRRL 26061 (FD_01151),

Table 6. Identification of other *Fusarium* species.

	Number of isolates/strains			Representative			Best matching (FUSARIUM-ID)			
EFST	Tomato	Soil	Reference	Isolate	Accession no.	MAFF no.	Isolate ID	E-value	Identities (%)	Species inferred
Other-01	0	6	0	TC0021	AB917036	244612	FD_01726	0	100	*F. acuminatum* (FTSC)
Other-02	0	1	0	TC0093	AB917037	244621	FD_00943	6e-34	97.43	Unknown
Other-03	0	1	1	TC0126	AB917038	244628	FD_01324	0	97.35	Unknown
Other-04	0	1	0	TC0265	AB917039	244635	FD_01846	0	100	*F. flocciferum* (FTSC)

FTSC, *F. tricinctum* species complex.

respectively. These strains were reported as a new, distinct species in an exhaustive phylogenetic analysis of the FFSC on the basis of nucleotide sequencing of six loci including the EF-1α gene (O'Donnell *et al.* 2000). NRRL 26152 and NRRL 26061 were initially reported in the year 2000. However, these strains have not been analyzed further phylogenetically or morphologically and have not been described as a new species because there are no strains closely related to them. However, our nucleotide sequence analysis of the EF-1α gene revealed that 14 isolates obtained from tomato and soil are closely related to NRRL 26152 and NRRL 26061. Thus, this set of isolates may be suitable for further characterizing this putatively novel *Fusarium*.

The FOSC has not been reclassified based on molecular phylogenetic and morphological traits. When analysis of these traits is finished, this complex very likely will be divided into more than one species. Two species, *F. foetens* (Schroers *et al.* 2004) and *F. nisikadoi* (Nirenberg and Aoki 1997; Aoki 2009), were identified based on comparison with *F. oxysporum*. Because the molecular phylogenetic traits of *F. foetens* and *F. nisikadoi* are closely related to *F. oxysporum*, they were reported as a member of or a sister taxon of the *F. oxysporum* species complex (Schroers *et al.* 2004; Aoki 2009). As a matter of practical convenience, we describe the two species in the FOSC. In Fig. 1, *F. oxysporum* pathogens, *F. oxysporum* biological control agents, *F. foetens* and *F. nisikadoi* were used as references. The phylogenetic position of FOSC-04 (six isolates), FOSC-08 (one isolate) and FOSC-09 (two isolates) was not closely related to *F. oxysporum* pathogens or biological control agents. Furthermore, these three EFSTs were obviously different from *F. foetens* and *F. nisikadoi*. BLAST searches of FUSARIUM-ID indicated that they are conspecific with *F. commune* NRRL 28058 (isolate ID = FD_01065; e-values = 0; and identities = 99.23 to 100%). This species is also relatively newly defined; it was first described in 2003 (Skovgaard *et al.* 2003).

We used 27 EF-1α gene sequences of the *F. solani* species complex from the DDBJ/EMBL/GenBank databases in addition to three reference strains within the FSSC in our molecular phylogenetic analysis. However, EFSTs FSSC-04 (52 isolates), FSSC-05 (14 isolates), FSSC-06 (10 isolates), FSSC-07 (3 isolates) and FSSC-09 (2 isolates) were not closely related to these reference strains, as shown in Fig. 3. We thus compared the nucleotide sequences of the five EFSTs with the sequences registered in the DDBJ/EMBL/GenBank databases. Sequences of five EFSTs respectively had high similarity with the nucleotide sequences of the EF-1α genes of reported NRRL strains 44906 (multilocus sequence type 3+4-lll), 52680 (3+4-mmm), 52832 (3+4-nnn), 53120 (3+4-ooo) and 53128 (3+4-ppp). These NRRL strains were presented in Migheli *et al.* (2010). The sequence identities were 99–100% between our isolates and the five NRRL strains. These NRRL strains were isolated from patients (toe, blood or cerebrospinal fluid). The five EFSTs found in our isolates also had a close relationship with four field isolates reported by Jiménez-Fernández *et al.* (2011) and two field isolates reported in Nitschke, Nihlgard and Varrelmann (2009). The four isolates of Jiménez-Fernández *et al.* (2011; cc20B, cc61C, cc41W and cc40A) were obtained from surface-sterilized stems of chickpea plants displaying Fusarium yellows (wilting syndrome); the two isolates of Nitschke, Nihlgard and Varrelmann (2009; sol-17 and sol-61) were obtained from surface-sterilized roots of sugar beets displaying root rot symptoms. Pathogenicity of the six isolates was not confirmed in the two reports, so we do not know whether plant pathogens were included in this phylogenetic group.

Molecular phylogenetic relationships between plant pathogenic and non-pathogenic *Fusarium* strains have been studied (Baayen *et al.* 2000; Bao *et al.* 2002; Fourie *et al.* 2009). However, pathogenic strains were not distinct from non-pathogenic strains based on molecular phylogenetic traits. These results were supported by studies on the molecular mechanisms of pathogenicity: accessory chromosomes that could have mobility to other strains and thus be able to confer pathogenicity to *F. oxysporum* (Ma *et al.* 2010). On the basis of molecular phylogenetic analyses, some tomato and soil isolates we obtained were closely related to plant pathogens. For example, the EFST FOSC-03 included tomato and sweet potato wilt pathogens (*F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *batatas*) in addition to tomato and soil isolates. As described above, this does not imply that these tomato and soil isolates were pathogenic. To be sure of their pathogenicity, it will be necessary to perform inoculation assays.

Biological control activity of non-pathogenic fusaria against Fusarium wilts has been reported since the 1980s (Ogawa and Komada 1984; Schneider 1984). Some *Fusarium* strains used for biological control could invade tomato stems (Amemiya, Koike and Hirano 1989; Hallman and Sikora 1994; Shishido *et al.* 2005). However, the relationship between their endophytic behavior and their biological control activity has not yet been revealed. Molecular characterization of the endophytic and biological control mechanisms of *Fusarium*, especially at the molecular level (Massart and Jijakli 2007), will be the next research target. If these mechanisms are identified, we may be able to develop new approaches and technologies for protecting plants from diseases. To accomplish our aim, we are now trying to screen isolates that can effectively control Fusarium wilt diseases and are also trying to develop a new disease control method using one of the isolates in the field. Our goal is to analyze biological control of *Fusarium* at the molecular level to improve this method of disease control.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

Table 7. Inoculation and reisolation of *Fusarium* strains from tomato plants[a].

Isolate strain	EFST	Species complex (species)	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Exp. 6		Exp. 7		Total		
			T	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	Proportion
SL0303	FOSC-03	*F. oxysporum*	19	19	–	–	–	–	–	–	–	–	–	–	–	–	19	19	1.000
SL0301	FOSC-03	*F. oxysporum*	20	18	–	–	–	–	–	–	–	–	–	–	–	–	20	18	0.900
SL0321	FOSC-03	*F. oxysporum*	20	16	–	–	–	–	–	–	–	–	–	–	–	–	20	16	0.800
SL0316	FOSC-03	*F. oxysporum*	19	13	–	–	–	–	–	–	–	–	–	–	–	–	19	13	0.684
880621a-1 (Tomato pathogen)	FOSC-03	*F. oxysporum* f. sp. *lycopersici*	–	–	20	11	–	–	–	–	–	–	–	–	–	–	20	11	0.550
SL0317	FOSC-03	*F. oxysporum*	19	7	–	–	–	–	–	–	–	–	–	–	–	–	19	7	0.368
TC0289	FFSC-03	*F. fujikuroi* (*F. fujikuroi*)	–	–			–	–	–	–	–	–	–	–	70	25	70	25	0.357
TC0100	FFSC-03	*F. fujikuroi* (*F. fujikuroi*)					–	–	–	–	–	–	–	–	72	25	72	25	0.347
SL0271	FFSC-03	*F. fujikuroi* (*F. fujikuroi*)					–	–	–	–	–	–	–	–	69	19	69	19	0.275
SL0300	FOSC-03	*F. oxysporum*	20	12	20	3	–	–	–	–	50	9	–	–	–	–	90	24	0.267
TC0091	FFSC-03	*F. fujikuroi* (*F. fujikuroi*)	–	–	–	–	–	–	–	–	–	–	–	–	70	17	70	17	0.243
Mel02010 (Melon pathogen)	FOSC-01	*F. oxysporum* f. sp. *melonis*	20	6	20	2	–	–	–	–	–	–	–	–	–	–	40	8	0.200
TC0089	FFSC-10	*F. fujikuroi* (*F. proliferatum*)	–	–	–	–	10	2	–	–	–	–	–	–	–	–	10	2	0.200
TC0095	FSSC-10	*F. solani*	–	–	–	–	10	2	–	–	–	–	–	–	–	–	10	2	0.200
TC0108	FFSC-11	*F. fujikuroi* (New species)	–	–	–	–	10	0	–	–	50	9	–	–	–	–	60	9	0.150
TC0083	FFSC-09	*F. fujikuroi* (*F. fractiflexum*)	–	–	–	–	7	1	–	–	–	–	–	–	–	–	7	1	0.143
TC0074	FSSC-08	*F. solani*	–	–	–	–	10	0	20	0	50	9	–	–	–	–	80	9	0.113
TC0073	FSSC-04	*F. solani*	–	–	–	–	–	–	–	–	–	–	49	5	–	–	49	5	0.102
SL0364	FOSC-01	*F. oxysporum*	19	5	20	1	–	–	20	0	–	–	–	–	–	–	59	6	0.102
TC0008	FOSC-04	*F. oxysporum*	–	–	–	–	10	1	–	–	–	–	–	–	–	–	10	1	0.100
TC0031	FFSC-07	*F. fujikuroi* (New species)	–	–	–	–	10	1	–	–	–	–	–	–	–	–	10	1	0.100
TC0068	FSSC-09	*F. solani*	–	–	–	–	10	1	–	–	–	–	–	–	–	–	10	1	0.100
TC0111	FOSC-03	*F. oxysporum*	–	–	–	–	10	1	–	–	–	–	–	–	–	–	10	1	0.100
TC0066	FFSC-08	*F. fujikuroi* (*F. fujikuroi*)	–	–	–	–	10	0	20	0	50	0	–	–	70	10	150	10	0.067
TC0078	FFSC-03	*F. fujikuroi* (*F. fujikuroi*)	–	–	–	–	10	0	–	–	50	3	–	–	–	–	60	3	0.050
SL0370	FSSC-04	*F. solani*	–	–	–	–	–	–	–	–	–	–	50	2	–	–	50	2	0.040
MAFF 235151	FFSC-02	*F. fujikuroi* (*F. fujikuroi*)	–	–	–	–	–	–	–	–	–	–	–	–	51	2	51	2	0.039
AFM06–014A (Rice pathogen)	FFSC-12	*F. fujikuroi* (*F. fujikuroi*)	–	–	–	–	10	0	20	1	–	–	–	–	–	–	30	1	0.033
TC0001	FOSC-01	*F. oxysporum*	–	–	–	–	10	0	20	1	–	–	–	–	–	–	30	1	0.033
TC0005	FOSC-05	*F. oxysporum*	–	–	–	–	10	0	20	1	–	–	–	–	–	–	30	1	0.033
TC0007	FSSC-03	*F. solani*	–	–	–	–	10	0	20	1	–	–	–	–	–	–	30	1	0.033
TC0070	FOSC-09	*F. oxysporum*	–	–	–	–	10	0	20	0	50	2	–	–	–	–	80	2	0.025
TC0179	FSSC-04	*F. solani*	–	–	–	–	–	–	–	–	–	–	50	1	–	–	50	1	0.020
TC0245	FSSC-04	*F. solani*	–	–	–	–	–	–	–	–	–	–	50	1	–	–	50	1	0.020
TC0010	FSSC-01	*F. solani*	–	–	–	–	10	0	20	0	50	1	–	–	–	–	80	1	0.013
TC0003	FSSC-04	*F. solani*	–	–	–	–	10	0	20	0	50	0	50	1	–	–	130	1	0.008
TC0058	FSSC-04	*F. solani*	–	–	–	–	–	–	–	–	–	–	50	0	–	–	50	0	0.000
Uninoculated	–	–	20	0	20	0	20	0	20	0	50	0	50	0	70	0	250	0	0.000

[a]Exp.1 was performed from 10 June 2010 to 1 July 2010; Exp. 2 was from 25 August 2010 to 15 September 2010; Exp. 3 was from 14 September 2010 to 6 October 2010; Exp. 4 was from 25 October 2010 to 22 November 2010; Exp. 5 was from 15 December 2010 to 9 January 2011; Exp. 6 was from 25 January 2011 to 18 February 2011; and Exp. 7 was from 4 February 2011 to 3 March 2011.

T, number of tested plants; *D*, number of plants from which *Fusarium* was recovered.

Table 8. Inoculation and reisolation of *Fusarium* strains from melon plants.[a]

			Exp. 1		Exp. 2		Exp. 3		Total		
Isolate/strain	EFST	Species complex	*T*	*D*	*T*	*D*	*T*	*D*	*T*	*D*	Proportion reisolated
SL0303	FOSC-03	*F. oxysporum*	–	–	20	18	–	–	20	18	0.900
Mel02010 (Melon pathogen)	FOSC-01	*F. oxysporum* f. sp. *melonis*	–	–	–	–	20	17	20	17	0.850
SL0301	FOSC-03	*F. oxysporum*	–	–	20	17	–	–	20	17	0.850
SL0317	FOSC-03	*F. oxysporum*	–	–	20	15	–	–	20	15	0.750
SL0321	FOSC-03	*F. oxysporum*	–	–	20	15	–	–	20	15	0.750
SL0316	FOSC-03	*F. oxysporum*	–	–	20	13	–	–	20	13	0.650
SL0300	FOSC-03	*F. oxysporum*	9	6	20	16	20	4	49	26	0.531
880621a-1 (Tomato pathogen)	FOSC-03	*F. oxysporum* f. sp. *lycopersici*	–	–	20	13	20	3	40	16	0.400
SL0364	FOSC-01	*F. oxysporum*	–	–	–	–	20	5	20	5	0.250
Uninoculated	–	–	9	0	20	0	20	0	49	0	0.000

[a]Exp.1 was performed from 28 April 2010 to 26 May 2010; Exp. 2 was from 14 June 2010 to 5 July 2010; and Exp. 3 was from 25 August 2010 to 15 September 2010.

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REFERENCES

Alabouvette C, Edel V, Lemanceau P, *et al.* Diversity and interactions among strains of *Fusarium oxysporum*: application to biological control. In: Jeger MJ, Spence NJ, *et al.* (eds), *Biotic Interactions in Plant-pathogen Associations*. Oxfordshire, UK: CABI Publishing, 2001, 131–58.

Altschul SF, Gish W, Miller W, *et al.* Basic local alignment search tool. *J Mol Biol* 1990;**215**:403–10.

Amemiya Y, Koike M, Hirano K. Suppression of *Verticillium* wilt in tomato by non-pathogenic isolates of *Fusairum oxysporum*. *Soil Microorg* 1989;**33**:27–34.

Aoki T. Taxonomic system of the genus *Fusarium*. *Microbiol Cult Coll* 2009;**25**:1–12 {Written in Japanese}.

Aoki T, O'Donnell K, Homma Y, *et al.* Sudden-death syndrome of soybean is caused by two morphologically and phylogenetically distinct species within the *Fusarium solani* species complex—*F. virguliforme* in North America and *F. tucumaniae* in South Americavol. *Mycologia* 2003;**95**:660–84.

Armstrong GM, Armstrong JK. Nonsusceptible hosts as carriers of wilt Fusaria. *Phytopathology* 1948;**38**:808–26.

Baayen RP, O'Donnell K, Bonants PJM, *et al.* Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. *Phytopathology* 2000;**90**:891–900.

Backhouse D, Burgess LW, Summerell BA. Biogeography of *Fusarium*. In: Summerell BA, Leslie JF, Backhouse D, *et al.* (eds) *Fusarium*. St. Paul, MN: APS Press, 2001, 122–37.

Bacon CW, Yates IE. Endophytic root colonization by *Fusarium* species: histology, plant interactions, and toxicity. In: Schulz BJE, Boyle CJC, Sieber TN (eds), *Microbial Root Endophytes*. Heidelberg, Germany: Springer, 2006, 133–52.

Banihashemi Z, deZeeuw DJ. The behavior of *Fusarium oxysporum* f. sp. *melonis* in the presence and absence of host plants. *Phytopathology* 1975;**65**:1212–7.

Bao JR, Fravel DR, O'Neill NR, *et al.* Genetic analysis of pathogenic and nonpathogenic *Fusarium oxysporum* from tomato plants. *Can J Bot* 2002;**80**:271–9.

Benhamou N, Garand C, Goulet A. Ability of nonpathogenic *Fusarium oxysporum* strain Fo47 to induce resistance against *Pythium ultimum* infection in cucumber. *Appl Environ Microb* 2002;**68**:4044–60.

Burgess LW, Nelson PE, Toussoun TA, *et al.* Distribution of *Fusarium* species in sections Roseum, Arthrosporiella, Gibbosum and Discolor recovered from grassland, pasture and pine nursery soils of eastern Australia. *Mycologia* 1988;**80**:815–24.

Castellá G, Bragulat MR, Rubiales MV, *et al.* Malachite green agar, a new selective medium for *Fusarium* spp. *Mycopathologia* 1997;**137**:173–8.

Desjardins AE. *Fusarium Mycotoxins – Chemistry, Genetics, and Biology*. St. Paul, MN, USA: APS Press, 2006.

Enya J, Togawa M, Takeuchi T, *et al.* Biological and phylogenetic characterization of *Fusarium oxysporum* complex, which causes yellows on *Brassica* spp., and proposal of *F. oxysporum* f. sp. *rapae*, a novel forma specialis pathogenic on *B. rapa* in Japan. *Phytopathology* 2008;**98**:475–83.

Fourie G, Steenkamp ET, Gordon TR, *et al.* Evolutionary relationships among the *Fusarium oxysporum* f. sp. *cubense* vegetative compatibility groups. *Appl Environ Microb* 2009;**75**:4770–81.

Geiser DM, Jiménez-Gasco MM, Kang S, *et al.* FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. *Eur J Plant Pathol* 2004;**110**:473–9.

Gullino ML, Katan J, Garibaldi A. The genus *Fusarium* and the species that affect greenhouse vegetables and ornamentals. In: Gullino ML, Katan J, Garbaldi A (eds). *Fusarium Wilts of Greenhouse Vegetable and Ornamental Crops*. St. Paul, MN: APS Press, 2012, 5–9.

Gordon TR, Okamoto D, Jacobson DJ. Colonization of muskmelon and nonsusceptible crops by *Fusarium oxysporum* f. sp. *melonis*

and other species of *Fusarium*. *Phytopathology* 1989;**79**:1095–100.

Hallman J, Sikora RA. Influence of *Fusarium oxysporum*, a mutualistic fungal endophyte, on *Meloidogyne incognita* infection of tomato. *J Plant Dis Protect* 1994;**101**:475–81.

Helbig JB, Carroll RB. Dicotyledonous weeds as a source of *Fusarium oxysporum* pathogenic on soybean. *Plant Dis* 1984;**68**:694–6.

Inácio J, Pereira P, de Carvalho M, *et al*. Estimation and diversity of phylloplane mycobiota on selected plants in a Mediterranean-type ecosystem in Portugal. *Microb Ecol* 2002;**44**:344–53.

Jeanmougin F, Thompson JD, Gouy M, *et al*. Multiple sequence alignment with Clustal X. *Trends Biochem Sci* 1998;**23**:403–5.

Jiménez-Fernández D, Navas-Cortés JA, Montes-Borrego M, *et al*. Molecular and pathogenic characterization of *Fusarium redolens*, a new causal agent of Fusarium yellows in chickpea. *Plant Dis* 2011;**95**:860–70.

Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN (ed). *Mammalian Protein Metabolism*. New York, NY: Academic Press, 1969, 21–132.

Katan J. Symptomless carriers of the tomato Fusarium wilt pathogen. *Phytopathology* 1971;**61**:1213–7.

Kavroulakis N, Ntougias S, Zervakis GI, *et al*. Role of ethylene in the protection of tomato plants against soil-borne fungal pathogens conferred by an endophytic *Fusarium solani* strain. *J Exp Bot* 2007;**58**:3853–64.

Kim H-Y, Choi GJ, Lee HB, *et al*. Some fungal endophytes from vegetable crops and their anti-oomycete activities against tomato late blight. *Lett Appl Microbiol* 2007;**44**:332–7.

Kistler HC. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. *Phytopathology* 1997;**87**:474–9.

Komada H. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. *Rev Plant Protec Res* 1975;**8**:114–25.

Kuldau GA, Yates IE. Evidence for *Fusarium endophytes* in cultivated and wild plants. In: Bacon CW, White JF (eds), *Microbial Endophytes*. New York, NY: Marcel Dekker, 2000, 85–117.

Larkin MA, Blackshields G, Brown NP, *et al*. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;**23**:2947–8.

Leslie JF, Pearson CAS, Nelson PE, *et al*. *Fusarium* spp. from corn, sorghum and soybean fields in the central and eastern United States. *Phytopathology* 1990;**80**:343–50.

Leslie JF, Summerell BA. *The Fusarium Laboratory Manual*. Ames, IA: Blackwell Publishing, 2006.

Ma LJ, van der Does HC, Borkovich KA, *et al*. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature* 2010;**464**:367–73.

Massart S, Jijakli HM. Use of molecular techniques to elucidate the mechanisms of action of fungal biocontrol agents: a review. *J Microbiol Meth* 2007;**69**:229–41.

Migheli Q, Balmas V, Harak H, *et al*. Molecular phylogenetic diversity of dermatologic and other human pathogenic fusarial isolates from hospitals in northern and central Italy. *J Clin Microbiol* 2010;**48**:1076–84.

Nagao H, Couteaudie Y, Alabouvette C. Colonization of sterilized soil and flax roots by strains of *Fusarium oxysporum* and *Fusarium solani*. *Symbiosis* 1990;**9**:343–54.

Nash SM, Snyder WC. Quantitative estimations by plate counts of propagules of the bean root rot Fusarium in field soils. *Phytopathology* 1962;**52**:567–72.

Nirenberg NI, Aoki T. *Fusarium nisikadoi*, a new species from Japan. *Mycoscience* 1997;**38**:329–33.

Nishimura N. Selective media for *Fusarium oxysporum*. *J Gen Plant Pathol* 2007;**73**:342–8.

Nitschke E, Nihlgard M, Varrelmann M. Differentiation of eleven *Fusarium* spp. isolated from sugar beet, applying restriction fragment analysis of polymerase chain reaction-amplified translation elongation factor 1α gene fragment. *Phytopathology* 2009;**99**:921–9.

O'Donnell K. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia* 2000;**92**:919–38.

O'Donnell K, Cigelnik E, Nirenberg HI. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* 1998;**90**:465–93.

O'Donnell K, Gueidan C, Sink S, *et al*. A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal Genet Biol* 2009;**46**:936–48.

O'Donnell K, Kistler HC, Cigelnik E, *et al*. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *P Natl Acad Sci USA* 1998;**95**:2044–9.

O'Donnell K, Nirenberg HI, Aoki T, *et al*. A Multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience* 2000;**41**:61–78.

O'Donnell K, Sutton DA, Fothergill A, *et al*. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. *J Clin Microbiol* 2008a;**46**:2477–90.

O'Donnell K, Ward TJ, Aberra D, *et al*. Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the *Fusarium graminearum* species complex from Ethiopia. *Fungal Genet Biol* 2008b;**45**:1514–22.

O'Donnell K, Ward TJ, Geiser DM, *et al*. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genet Biol* 2004;**41**:600–23.

Ogawa K, Komada H. Biological control of Fusarium wilt of sweet potato by non-pathogenic *Fusarium oxysporum*. *Ann Phytopathol Soc Jpn* 1984;**50**:1–9.

Park B, Park J, Cheong KC, *et al*. Cyber infrastructure for *Fusarium*: three integrated platforms supporting strain identification, phylogenetics, comparative genomics and knowledge sharing. *Nucleic Acids Res* 2011;**39**:D640–6 {Database issue}.

Schneider RW. Effects of nonpathogenic strains of *Fusarium oxysporum* on celery root infection by *F. oxysporum* f. sp. *apii* and a novel use of the Lineweaver-Burk double reciprocal plot technique. *Phytopathology* 1984;**74**:646–53.

Schroers H-J, Baayen RP, Meffert JP, *et al*. *Fusarium foetens*, a new species pathogenic to begonia elatior hybrids (*Begonia* × *hiemalis*) and the sister taxon of the *Fusarium oxysporum* species complex. *Mycologia* 2004;**96**:393–406.

Shishido M, Loeb BM, Chanway CP. External and internal root colonization of lodgepole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. *Can J Microbiol* 1995;**41**:707–13.

Shishido M, Miwa C, Usami T, *et al*. Biological control efficiency of Fusarium wilt of tomato by nonpathogenic *Fusarium oxysporum* Fo-B2 in different environments. *Phytopathology* 2005;**95**:1072–80.

Skovgaard K, Rosendahl S, O'Donnell K, *et al*. *Fusarium commune* is a new species identified by morphological and molecular phylogenetic data. *Mycologia* 2003;**95**:630–6.

Starkey DE, Ward TJ, Aoki T, *et al.* Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. *Fungal Genet Biol* 2007;**44**:1191–204.

Tamura K, Peterson D, Peterson N, *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;**28**:2731–9.

Windels CE, Kommedahl T. Population differences in indigenous *Fusarium* species by corn culture of prairie soil. *Am J Bot* 1974;**61**:141–5.

Wulff EG, Sørensen JL, Lübeck M, *et al.* *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. *Environ Microbiol* 2010;**12**:649–57.