Evolutionary Applications www.evolutionaryapplications.org

Evolutionary Applications ISSN 1752-4571

ORIGINAL ARTICLE

Local origin of two vegetative compatibility groups of *Fusarium oxysporum* f. sp. *vasinfectum* in Australia

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Keywords
agriculture, cotton, fungal, fusarium wilt, host shift, host–pathogen, pathogen emergence, plant disease.

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Received: 12 May 2010
Accepted: 18 May 2010
First published online: 7 July 2010

doi:10.1111/j.1752-4571.2010.00139.x

Abstract

Pathogenicity and genetic diversity of *Fusarium oxysporum* from geographically widespread native *Gossypium* populations, including a cotton growing area believed to be the center of origin of VCG 01111 and VCG 01112 of *F. oxysporum* f. sp. *vasinfectum* (*Fov*) in Australia, was determined using glasshouse bioassays and AFLPs. Five lineages (A–E) were identified among 856 isolates. Of these, 12% were strongly pathogenic on cotton, 10% were weakly pathogenic and designated wild *Fov*, while 78% were nonpathogenic. In contrast to the occurrence of pathogenic isolates in all five lineages in soils associated with wild *Gossypium*, in cotton growing areas only three lineages (A, B, E) occurred and all pathogenic isolates belonged to two subgroups in lineage A. One of these contained VCG 01111 isolates while the other contained VCG 01112 isolates. Sequence analyses of translation elongation factor-1α, mitochondrial small subunit rDNA, nitrate reductase and phosphate permease confirmed that Australian *Fov* isolates were more closely related to lineage A isolates of native *F. oxysporum* than to *Fov* races 1–8 found overseas. These results strongly support a local evolutionary origin for *Fov* in Australian cotton growing regions.

Introduction

Widespread and increasing human impacts on all levels of biological organization (e.g. changes in land-use patterns, fragmentation of natural ecosystems) suggests that there is value in the application of evolutionary principles to emerging issues relating to these processes (Thompson 2005). One clear example where this is of direct relevance is with regard to the incidence and prevalence of disease in agro-ecosystems, particularly in the context of the role that interactions between production and native components of these landscapes might play in the emergence and spread of new diseases (Anderson et al. 2004; Burdon and Thrall 2008). This is not only with respect to understanding the underlying epidemiological and evolutionary processes, but also with regard to identifying appropriate control strategies (e.g. Ewald 1994; Jeger et al. 2006; Gilligan 2008). Here, we present results from extensive studies of Fusarium wilt disease in Australian cotton growing regions, with the aim of evaluating likely evolutionary origins and agronomic management implications for the pathogen. Of particular note is the fact that there are a number of native *Gossypium* species in Australia, raising the possibility that these wild relatives have played a role in the evolution of the pathogen, as has been demonstrated in other systems (e.g. Burdon et al. 1983; Oates et al. 1983; Frenkel et al. 2007).

Fusarium oxysporum f. sp. *vasinfectum* (*Fov*) is a soilborne fungal pathogen of cotton (*Gossypium hirsutum* L.) characterized by a parasitic phase within the vascular tissue and a saprophytic phase in the soil or plant residue after host death. Worldwide, eight races have been characterized based on pathogenicity on differential host sets (Chen et al. 1985; Hillocks 1992), and 12 vegetative compatibility groups (VCGs) identified, each presumably representing a clonal lineage (Fernandez et al. 1994; Bentley et al. 2000). Genetic evidence has demonstrated that the eight pathogenic races of *F. oxysporum* f. sp. *vasinfectum* are polyphyletic with at least two independent evolutionary origins (Skovgaard et al. 2001).

In Australia, Fusarium wilt was first recorded in the Brookstead, Cecil Plains and Boggabilla regions of Queensland/New South Wales in 1993/1994 from which it subsequently spread to most major cotton growing regions. While Australian isolates of *Fov* are pathogenically similar to race 6 on the standard differential hosts, they belong to VC groups 01111 and 01112 which are vegetatively incompatible with all non-Australian isolates of the

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pathogen, including race 6 isolates (Davis et al. 1996; Bentley et al. 2000). Furthermore, phylogenetic analysis of multigene sequences and pathogenicity has shown that Australian *Fov* are distinct from all races and VCGs found in California and China (Kim et al. 2005).

In Australia, the two VCGs have different geographic distributions with VCG 01111 occurring in all infected cotton growing regions, while VCG 01112 is restricted to the Boggabilla region where it was first detected (Wang et al. 2006). Despite genetic variation among isolates, no clear spatial population structure has been found at the largest spatial scale. However, both the greatest genetic diversity and some indication of local population differentiation was observed in the Boggabilla region, which, when coupled with the first reports of Fusarium wilt of cotton originating from this area (Kochman 1995), suggests that this may be the centre of origin of the two VCGs of *Fov* in Australia (Wang et al. 2006).

Understanding the evolutionary origin of new pathogens is important for effective disease management as strategies to control introduced pathogens may differ from those for pathogens that arise locally. New occurrences of Fusarium wilt pathogens are frequently the result of recent introductions rather than independent local origins (Gordon and Martyn 1997), but the simultaneous appearance of two distinct VCGs of *F. oxysporum* f. sp. *vasinfectum* suggests that this may not be the case in Australia. Given the clonal nature of *Fov*, distinguishing between long-distance migration and local evolution as a source of origin should be relatively straightforward. An introduced pathogen is likely to be genetically distinct from the pre-existing pool of local *F. oxysporum* isolates, while a locally derived pathogen should be more closely related to sympatric nonpathogen types.

Cotton was introduced to Australia with European settlement in 1788, but not grown extensively until the early 1960s. However, 17 wild *Gossypium* species are indigenous to Australia, four of which (*G. australe*, *G. bickii*, *G. nelsonii*, *G. sturtianum*) have native ranges that overlap or abut areas where the majority of cultivated cotton is grown (Craven et al. 1994). Interestingly, a survey of *Fusarium* species associated with these wild cottons detected a number of *F. oxysporum* isolates that caused mild, but typical, foliar and vascular symptoms of Fusarium wilt on cultivated cotton (Wang et al. 2004), which suggests that *Fov* may have existed in Australia before cotton was introduced. This raises the possibility that the two *Fov* VCGs found in commercial cotton fields evolved locally. Such evolution of pathogenicity in *F. oxysporum* has previously been documented in other crops including melon and tomato (Katan et al. 1994; Rosewich et al. 1999; Cai et al. 2003).

Uncultivated areas within agricultural production systems may represent reservoirs of native microflora similar to those that would have been present in adjacent agricultural soils prior to cultivation (Gordon et al. 1992). This suggests that if a new crop pathogen arises *in situ*, it is likely to show close relatedness to nonpathogenic isolates occurring in such nearby uncultivated areas. For example, a local origin for Fusarium root rot of pea in Denmark was implicated by the close DNA sequence homology of pathogenic strains with nonpathogenic isolates collected from the same fields (Skovgaard et al. 2002).

The primary goal of this study was to assess the hypothesis that VCG 01111 and VCG 01112 of *F. oxysporum* f. sp. *vasinfectum* evolved from local *F. oxysporum* populations in Australia. To do this, we determined genetic relationships between *Fov* isolates found in cotton fields, in nearby uncultivated soils, and indigenous *F. oxysporum* isolates found in a range of soils associated with native *Gossypium* species.

Materials and methods

Reference isolates of Australian *F. oxysporum* f. sp. *vasinfectum*

Isolates 24500 and 24595 of VCG 01111 and isolates 24492 and B/96/02 of VCG 01112, provided by Natalie Moore and Wayne O'Neil (Queensland Department of Primary Industries, Indooroopilly, Australia), were used as references of pathogenic Australian *Fov* in this study.

Sample collection

Soil was collected from a total of 90 populations of four native *Gossypium* species (*G. australe*, *G. bickii*, *G. nelsonii*, *G. sturtianum*) in 2001–2002 in the eastern and central parts of Australia (Table 1). At each site *c.* 200 g of soil was taken from the rhizosphere of 3–10 plants after the surface 2 cm layer was removed.

Within the cotton growing region, 200 g of soil was collected from each of five randomly chosen points in an uncultivated plot of native vegetation in the Boggabilla region in 2002 (Fig. 1). This site comprised a fenced minimally disturbed grassy woodland of *c.* 1.5 km^2 that had never been cultivated.

Soil and plant samples were collected in 2002 and 2004 from three cultivated cotton crops in fields (7, 5, and E2; Fig. 1) in which cotton had been grown in wheat or fallow rotation since the 1980s. These fields were all within *c.* 1 km of the native vegetation site. In each field, 200 g of soil was collected from each of five positions that were >50 m from field margins and 10 rows apart. At the same time, 20–35 symptomatic plants were randomly sampled in the same fields by cutting a 10-cm stem section from the main shoot. Both soil and plant samples were air-dried at ambient temperature. Soils were ground,

Table 1. Number of *Gossypium* populations sampled in this study and incidence of *Fusarium oxysporum* and wild *F. oxysporum* f. sp. *vasinfectum* (*Fov*) in populations summarized by *Gossypium* species and geographic regions respectively.

Sources of populations	Number of populations sampled	Number (%)* of populations associated with *F. oxysporum*	Number (%) of populations associated with wild *Fov*
By *Gossypium* species			
G. australe	33	16 (48)	11 (33)
G. bickii	13	8 (62)	4 (31)
G. nelsonii	11	6 (55)	4 (36)
G. sturtianum	33	30 (91)	20 (61)
By geographic regions			
Mount Isa (QLD) 20°15′–32°05′S; 139°00′–150°59′E	14	8 (57)	4 (29)
Longreach-Theodore (QLD) 20°15′–32°05′S; 139°00′–150°59′E	12	11 (92)	8 (67)
Alice Springs-Tennant Creek (NT) 19°17′–23°49′S; 132°44′–138°00′E	51	30 (59)	17 (33)
Leigh Creek-Arkaroola (SA) 30°00′–31°02′S; 137°46′–139°26′E	13	11 (85)	10 (77)
Total	90	60 (67)	39 (43)

*Percentage of the populations in the total sampled.

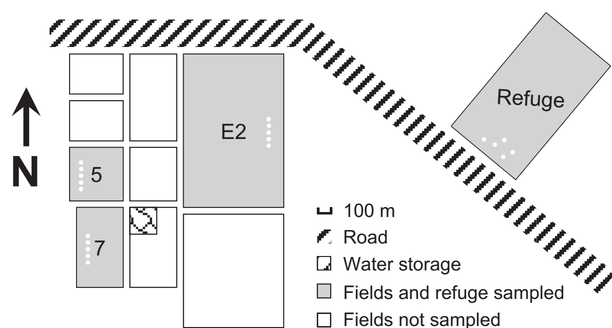

Figure 1 Diagram showing spatial relationships among the uncultivated land (refuge) and the three cultivated fields sampled in the Boggabilla region of New South Wales. White spots in the fields and refuge indicate the source of the plant and soil samples.

passed through a 710-μm sieve and then stored at 4°C until assayed.

Fungal isolation

Isolation was conducted on Peptone PCNB agar (Burgess et al. 1994). For isolation of *F. oxysporum* from soils, 0.5 g of soil was sprinkled onto five plates and incubated at 25°C for 1 week. All colonies morphologically resembling *F. oxysporum* were re-grown from single spores and subcultured. Finally, isolates were grown on carnation leaf agar plates at 25°C with a 12-h photoperiod, and *F. oxysporum* identified following Leslie and Summerell (2006). For isolation of *Fov* from plant samples, stem sections were surface sterilized in 0.5% sodium hypochlorite for 5 min and peeled under aseptic conditions. Small pieces of discoloured vascular tissue were placed on plates and incubated at 25°C for 1 week. Fungal hyphae growing out of tissue pieces were subcultured. The above procedure was repeated if *F. oxysporum* was not recovered in the initial attempt. Samples were considered free of *F. oxysporum* if both attempts were unsuccessful.

All isolates were grown on 10% potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) slants at 25°C for 1 week. Conidia were washed off by adding 1.5 mL of sterile 15% glycerol into each tube and pipetting the liquid several times. Conidial suspensions were stored in 2.0 mL cryogenic vials at −80°C.

Pathogenicity screening tests

Strains were tested for pathogenicity against a highly susceptible cotton cultivar, Siokra 1–4. Inoculum ranging in concentration from 2.5×10^5 to 8.5×10^7 spores/mL was prepared by growing strains on an orbital shaker in 75 mL of 25% potato dextrose broth (PDB; Difco) at 18–23°C for 1 week. Two-week-old seedlings were inoculated by dipping the roots in inocula for 5 min. Distilled water and a conidial suspension of *Fov* isolate 24500 (VCG 01111), were used as noninoculated and positive controls, respectively. Treated plants were transplanted into fresh potting mix (compost and perlite; 50/50, v/v) and grown at 18–23°C in a naturally lit glasshouse. A total of nine plants in three pots were challenged with each strain. Fusarium wilt was identified by the appearance of dark-brown discoloration in the vascular tissue and foliar necrosis 6 weeks after inoculation. Disease severity was assessed on a 0–4 scale (0 = asymptomatic; 1 = vascular discolouration only; 2 = necrosis on ≤50% of the foliage; 3 ≥ 50% but <100% foliar necrosis; 4 = 100% foliar necrosis).

The entire test was repeated and strains causing a mean disease severity of >1.5 were putatively identified as *F. oxysporum* f. sp. *vasinfectum* as suggested by Armstrong and Armstrong (1981). Isolates showing pathogenicity in both tests and producing a mean disease severity in the range of 0.1–1.5 were designated as wild *Fov*, i.e. weakly pathogenic on cotton.

Virulence comparison tests

The virulence (i.e. severity of disease symptoms) of wild *Fov* from soils associated with wild *Gossypium* populations and that found in cotton fields was compared on a moderately tolerant cotton cultivar Sicot 189 and a susceptible wild cotton (*G. sturtianum, Gos*-5250). Virulence comparison and pathogenicity screening tests used the same methodology except that in the former tests *G. sturtianum* seedlings were inoculated when 4 weeks old with a conidial suspension ($1.0 \pm 0.2 \times 10^6$ conidia/mL) from which hyphae had been removed by straining through tissue. All tests were conducted twice with three replicates for each strain. For each replicate, 30 plants were used in each trial involving cotton, but due to a lack of seeds only seven and nine plants, respectively, were used in the first and second trials involving *G. sturtianum*.

DNA extraction

Strains were grown for 3 days in 12 mL of 80% PDB in 15 mL sterile test tubes at 25°C after which mycelium was harvested by centrifuging cultures (2800 g for 15 min), decanting liquid, and transferring the pellet onto Whatman No.1 filter paper to remove excess water. Genomic DNA was extracted from lyophilized mycelia using DNeasy Plant kits (Qiagen Pty Ltd, Clifton Hill, Australia). DNA concentrations were determined using a GeneQuant II spectrophotometer (Pharmacia Biotech, Cambridge, England) and adjusted to 50 ng/μL.

AFLP analysis

AFLP fingerprints were generated using the protocol described by Vos et al. (1995). DNA (250 ng) was co-digested with *Mse*I and *Eco*RI at 37°C for 2 h and oligomer adapters ligated to DNA fragments at 37°C for 3 h in 40 μL of digestion-ligation buffer. Preselective amplification was performed with 5 μL of digestion-ligation reaction in 50 μL of polymerase chain reaction (PCR) buffer containing nonselective primers *Mse*I+0 and *Eco*RI+0 (20 cycles of 30 s at 94°C, 60 s at 56°C, and 60 s at 72°C). Selective amplification was performed with 5 μL of 1:30 diluted preselective amplification reaction in 20 μL of PCR buffer containing primers *Mse*I + A and ^{33}P-labelled *Eco*RI + AGG (one cycle of 30 s at 94°C, 30 s at 65°C, and 60 s at 72°C; 12 cycles of 65°C with annealing temperature lowered by 0.7°C during each cycle; and 23 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 72°C).

Amplified DNA fragments were separated on a 6% polyacrylamide gel electrophoresed at 50 W for 2.5 h on an AFLexpress automatic sequencer (Amersham Pharmacia Biotech, Roosendaal, the Netherlands) flanked by a 30–330 plus 1668 bp AFLP DNA ladder. Autoradiographs were obtained by exposing Kodak BioMax MR film (Eastman Kodak Co., Rochester, NY, USA) to dried gels. All AFLP bands of medium to dark intensity were scored manually from the autoradiographs. Fragment sizes were inferred using Gene Profiler Eval. 4.03 (Scanalytics, Rockville, MD, USA). A common set of four reference strains were included on each gel to maintain consistency of scoring across gels. Identical profiles were obtained from different DNA preparations of the same isolates, confirming the reproducibility of the AFLP fingerprints.

AFLP bands were scored as dominant markers (present/absent). The binary data matrix was analyzed using NTSYSpc 2.11X (Exeter Software, Setauket, NY, USA). Haplotypes were determined by calculating the Dice coefficient of genetic similarity in the SIMQUAL module and constructing an unweighted pair-group with arithmetic averages (UPGMA) dendrogram in the SAHN module. Bootstrap values (10 000 replicates) for each branch (%) of the dendrogram were calculated using Winboot (International Rice Research Institute, Manila, Philippines).

Sequence analysis

Amplification and sequencing primers are listed in Table 2. Portions of the translation elongation factor-1α (EF-1α) gene, the mitochondrial small subunit (mtSSU) rDNA, the nitrate reductase (NIR) gene, and the phosphate permease (PP) gene were amplified and sequenced from representative isolates (Table 3). The genes were amplified in 50 μL reaction mixtures containing 100 ng template DNA, 1.5 mM MgCl, 2 mM dNTPs, 10 pM primer, and 2 U *Amplitaq* DNA Polymerase (Applied Biosystems, Foster City, CA, USA) in 1× *GeneAmp* buffer (Applied Biosystems). PCR amplifications were performed in a Hybaid Express cycler (Thermo, San Diego, CA, USA) with the following program: initial denaturing (2 min at 95°C), 35 cycles of denaturing (30 s at 94°C), primer annealing, primer extension (45 s at 72°C), and final extension (5 min at 72°C). PCR products were purified using Amicon *Montage* PCR clean-up columns (Millipore, Bedford, MA, USA) and re-suspended in 100 μL of 10 mM TRIS.

Sequencing reactions were conducted on the purified PCR products with 3.2 pM of the forward or reverse

Table 2. Primers used in this study.

Locus	Primer sequence (5′ to 3′)	Length	T_m (°C)	References	Use
Translation elongation factor-1α (EF-1α)					
EF-1	ATG GGT AAG GAA GAC AAG AC	20	50	O'Donnell et al. 1998b	Amp.; Seq.
EF-2	GGA AGT ACC AGT GAT CAT GTT	21	50	O'Donnell et al. 1998b	Seq.
Mitochondrial small subunit (mtSSU)					
MS1	CAG CAG TCA AGA ATA TTA GTC AAT G	25	50	White et al. 1990	Amp.; Seq.
MS2	GCG GAT TAT CGA ATT AAA TAA C	22	55	White et al. 1990	Amp.; Seq.
Nitrate reductase (NIR)					
NIR 1F	CCG CGG GAT CAG ACC AGA GCC C	22	60	Skovgaard et al. 2001	Amp.; Seq.
NIR 2R	TTT GGA GGT AGA GGA TAA GGC	21	60	Skovgaard et al. 2001	Amp.; Seq.
Phosphate permease (PP)					
PHO1	ATC TTC TGG CGT GTT ATC ATG	21	50	O'Donnell et al. 2000	Amp.; Seq.
PHO3	TTC CAG CAC TAC AGC AAG TGG	21	65	This study	Seq.
PHO4	GTG CTG GAA GAA GTC TCT CC	20	55	O'Donnell et al. 2000	Seq.
PHO6	GAT GTG GTT GTA AGC AAA GCC C	22	50	O'Donnell et al. 2000	Amp.; Seq.

T_m, annealing temperature; Amp., amplification; Seq., sequencing.

primer using the fluorescent-labeled BigDye kits v3.1 (Perkin-Elmer, Boston, MA, USA) in a Hybaid Express cycler (Thermo) with the program recommended by the manufacturer. Products were cleaned up by isopropanol precipitation and run on an ABI PRISM Genetic Analyzer capillary sequencer (Applied Biosystems).

Forward and reverse sequences were assembled, edited using Sequencher 4.2 (Gene Codes, Ann Arbor, MI, USA), and deposited in GenBank (Table 3). Alignments were conducted using ClustalW as implemented in BioEdit 7.0.5.2 (Hall 1999). In addition to the sequences generated in this study, representatives of *F. oxysporum* f. sp. *vasinfectum* race 1–8 and representative taxa from the order *Hypocreales* were downloaded from GenBank to augment the alignments (Table 4). Three sequence alignments were constructed: (i) concatenated EF-1α and mtSSU sequences from representative strains of *Fov* from Boggabilla and native *F. oxysporum* from soils associated with wild *Gossypium* populations to explore the genetic relationships between pathogenic and nonpathogenic (against *G. hirsutum* cotton) Australian strains (deposited in TreeBASE under the accession numbers SN2747-10816); (ii) concatenated EF-1α, mtSSU, NIR, and PP sequences from pathogenic and nonpathogenic Australian isolates and representatives of *Fov* races 1–8 to determine the genetic relationships between Australian *F. oxysporum* strains and *Fov* occurring elsewhere in the world (deposited in TreeBASE under the accession numbers SN3665-16634); and (iii) EF-1α sequences from all lineages of Australian *F. oxysporum* identified by the AFLP analyses and representatives of other key *Fusarium* lineages to assess the phylogenetic relationships of the Australian *F. oxysporum* to other *Fusarium* species and taxa (deposited in TreeBASE under the accession numbers SN3665-16635).

Parsimony optimized topologies, partition homogeneity estimates, and bootstrap values were generated using PAUP 4.0 beta 10 (Sinauer Associates, Sunderland, MA, USA). Unweighted maximum parsimony was conducted using the heuristic search option and 100 random addition sequences with the tree-bisection-reconnection branch swapping and the MULTREES option on. Bayesian inference was used to estimate posterior probabilities for consensus nodes using MRBAYES 3.1 (Ronquist and Huelsenbeck 2003) and the most appropriate models of sequence evolution for the Bayesian analysis were identified using Modeltest 3.7 (Posada and Crandall 1998). Trees were visualized using TreeView 1.6.6 (Page 1996).

VCG tests

The vegetative compatibility of native *F. oxysporum* isolates derived from soils associated with wild *Gossypium* and reference *Fov* strains were tested using the method described by Puhalla (1985). For each strain, three nitrate nonutilizing mutants (*nit* 1, *nit* 3, and Nit M) were generated on a minimal medium amended with 1.5–4.0% (w/v) of potassium chlorate. Pairing tests were performed in 96 cell plates by growing different mutants of two isolates at 25°C for 2 weeks in a minimal medium containing sodium nitrate as the sole nitrogen source. Heterokaryon formation was identified by wild-type growth.

Results

Fungal isolation

A total of 856 *F. oxysporum* isolates were recovered, including 562 isolates from soils associated with wild *Gossypium*, 35 from uncultivated refuge soil, 178 from

Table 3. Representative isolates from which translation elongation factor (EF-1α), mitochondrial small subunit (mtSSU) rDNA, nitrate reductase (NIR), and phosphate permease (PP) sequences were generated in this study to assess the phylogenetic relationships between Australian *Fusarium oxysporum* and *F. oxysporum* f. sp *vasinfection* (*Fov*) race 1–8 and between Australian *F. oxysporum* and those found elsewhere in the world.

Accession identifier	Pathogenicity on *Gossypium hirsutum*	Lineage	Subgroup/ haplotype*	Origin, race or VCG group	EF-1α GenBank acc no.	mtSSU GenBank acc no.	NIR GenBank acc no.	PP GenBank acc no.
Accessions from Boggabilla site								
7080	Pathogenic	A	A-I/A02	Field soil	DQ435339	DQ435357	–†	–
251104	Pathogenic	A	A-I/A05	Diseased plant	DQ435340	DQ435358	–	–
241117	Pathogenic	A	A-II/A14	Diseased plant	DQ435341	DQ435359	–	–
7110	Pathogenic	A	A-II/A16	Field soil	DQ435342	DQ435360	–	–
7094	Nonpathogenic	A	A-I/A06	Field soil	DQ435343	DQ435361	–	–
7135	Nonpathogenic	A	A-I/A08	Field soil	DQ435344	DQ435362	–	–
6521	Nonpathogenic	A	A-I/A11	Refuge soil	DQ435345	DQ435363	–	–
7099	Nonpathogenic	A	A-I/A12	Field soil	DQ435436	DQ435364	–	–
6543	Nonpathogenic	A	A-I/A13	Refuge soil	DQ435347	DQ435365	–	–
7081	Nonpathogenic	A	A-II/A19	Field soil	DQ435348	DQ435366	–	–
7108	Nonpathogenic	B	/B26	Field soil	DQ435349	DQ435367	–	–
7070	Nonpathogenic	E	/E32	Field soil	DQ435350	DQ435368	–	–
Accessions from soil associated with wild Gossypium								
2613	Slightly pathogenic	A	A-I/A14	Wild *Gossypium* soil	DQ435351	DQ435369	EU246622	EU246656
3545	Nonpathogenic	A	–	Wild *Gossypium* soil	EU246540	EU246587	EU246623	EU246657
3546	Slightly pathogenic	A	–	Wild *Gossypium* soil	EU246541	EU246588	EU246624	EU246658
3547	Slightly pathogenic	A	–	Wild *Gossypium* soil	EU246542	EU246589	EU246625	EU246659
3549	Nonpathogenic	A	A-I/A15	Wild *Gossypium* soil	DQ435352	DQ435370	EU246626	EU246660
3556	Slightly pathogenic	A	–	Wild *Gossypium* soil	EU246543	EU246590	EU246627	EU246661
3608	Nonpathogenic	A	–	Wild *Gossypium* soil	EU246544	EU246591	EU246628	EU246662
6510	Nonpathogenic	A	–	Wild *Gossypium* soil	EU246545	EU246592	EU246629	EU246663
6632	Nonpathogenic	A	–	Wild *Gossypium* soil	EU246546	EU246593	EU246630	EU246664
1517	Slightly pathogenic	B	–	Wild *Gossypium* soil	EU246586	EU246619	–	–
1537	Nonpathogenic	B	–	Wild *Gossypium* soil	EU246585	EU246620	–	–
2631	Slightly pathogenic	E	–	Wild *Gossypium* soil	EU246562	EU246597	EU246634	EU246668
3506	Slightly pathogenic	E	–	Wild *Gossypium* soil	EU246563	EU246598	EU246635	EU246669
3522	Nonpathogenic	E	–	Wild *Gossypium* soil	EU246564	EU246599	EU246636	EU246670
3544	Nonpathogenic	E	–	Wild *Gossypium* soil	EU246565	EU246621	–	–
3552	Slightly pathogenic	E	–	Wild *Gossypium* soil	EU246566	EU246600	EU246637	EU246671
4511	Slightly pathogenic	E	–	Wild *Gossypium* soil	EU246567	EU246601	EU246638	EU246672
4590	Slightly pathogenic	E	–	Wild *Gossypium* soil	EU246568	EU246602	EU246639	EU246673
6519	Nonpathogenic	E	–	Wild *Gossypium* soil	EU246569	EU246603	EU246640	EU246674
Reference accessions obtained from public collections								
SC1‡	*Fov*	–	–	Race 1	EU246574	EU246608	EU246645	EU246679
IMI-141148‡	*Fov*	–	–	Race 2	EU246571	EU246605	EU246642	EU246676
IMI-338122‡	*Fov*	–	–	Race 3	EU246573	EU246607	EU246644	EU246678
IMI-141112‡	*Fov*	–	–	Race 4	EU246570	EU246604	EU246641	EU246675

Table 3. (*Continued*)

Accession identifier	Pathogenicity on *Gossypium hirsutum*	Lineage	Subgroup/ haplotype*	Origin, race or VCG group	EF-1α GenBank acc no.	mtSSU GenBank acc no.	NIR GenBank acc no.	PP GenBank acc no.
IMI-325576‡	*Fov*	–	–	Race 5	EU246572	EU246606	EU246643	EU246677
ATCC-16611‡	*Fov*	–	–	Race 6	EU246549	EU246596	EU246633	EU246667
Ag6‡	*Fov*	–	–	Race 7	EU246547	EU246594	EU246631	EU246665
Ag85‡	*Fov*	–	–	Race 8	EU246548	EU246595	EU246632	EU246666
24500§	*Fov*	–	–	VCG 01111	EU246575	EU246609	EU246646	EU246680
24595§	*Fov*	–	–	VCG 01111	EU246576	EU246610	EU246647	EU246681
041101	*Fov*	–	–	VCG 01111	EU246578	EU246611	EU246648	EU246682
051101	*Fov*	–	–	VCG 01111	EU246579	EU246612	EU246649	EU246683
X1§	*Fov*	–	–	VCG 01111	EU246577	EU246613	EU246650	EU246684
24492§	*Fov*	–	–	VCG 01112	EU246584	EU246618	EU246655	EU246689
24597§	*Fov*	–	–	VCG 01112	EU246580	EU246614	EU246651	EU246685
24598§	*Fov*	–	–	VCG 01112	EU246581	EU246615	EU246652	EU246686
24646§	*Fov*	–	–	VCG 01112	EU246582	EU246616	EU246653	EU246687
B/96/02§	*Fov*	–	–	VCG 01112	EU246583	EU246617	EU246654	EU246688

*Identified based on results illustrated in Fig. 6.

†Data not available.

‡Provided in the form of DNA by Linda Smith (Queensland Department of Primary Industries, Indooroopilly, Australia).

§Provided in culture by Natalie Moore, Linda Smith, and Wayne O'Neil (Queensland Department of Primary Industries, Indooroopilly, Australia).

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Table 4. Translation elongation factor (EF-1α), mitochondrial small subunit (mtSSU) rDNA, nitrate reductase (NIR), and phosphate permease (PP) sequences from representative *Fusarium*, *Gibberella*, *Nectria*, and *Neocosmospora* species that were used to assess the relationships of Australian *F. oxysporum* to other major phylogenetic groups within the order *Hypocreales*.

Accession identifier	Genus	Species	EF-1α GenBank acc no.	mtSSU GenBank acc no.	NIR GenBank acc no.	PP GenBank acc no.
NRRL25300	*Fusarium*	*begoniae*	AF160293	–*	–	–
NRRL31238	*Fusarium*	*brasilicum*	AY452963	–	–	–
NRRL31281	*Fusarium*	*brasilicum*	AY452964	–	–	–
NRRL22678	*Fusarium*	*brasiliense*	AY320144	–	–	–
NRRL22743	*Fusarium*	*brasiliense*	AY320145	–	–	–
NRRL13618	*Fusarium*	*bulbicola*	AF160294	–	–	–
NRRL13721	*Fusarium*	*cerealis*	AF212464	–	–	–
NRRL25491	*Fusarium*	*cerealis*	AF212465	–	–	–
NRRL28387	*Fusarium*	*commune*	AF246832	–	–	–
NRRL26434	*Fusarium*	*concentricum*	AF333933	–	–	–
NRRL31171	*Fusarium*	*cortaderiae*	AY452961	–	–	–
NRRL31205	*Fusarium*	*cortaderiae*	AY452960	–	–	–
NRRL25475	*Fusarium*	*culmorum*	AF212463	–	–	–
NRRL3288	*Fusarium*	*culmorum*	AF212462	–	–	–
NRRL22275	*Fusarium*	*cuneirostrum*	AY320158	–	–	–
NRRL31104	*Fusarium*	*cuneirostrum*	AY320159	–	–	–
NRRL31044	*Fusarium*	*foetens*	AY320072	–	–	–
NRRL31045	*Fusarium*	*foetens*	AY320073	–	–	–
NRRL28854	*Fusarium*	*fractiflexum*	AF333932	–	–	–
NRRL22945	*Fusarium*	*guttiforme*	AF160297	–	–	–
NRRL29642	*Fusarium*	*hostae*	AF324322	–	–	–
NRRL29643	*Fusarium*	*hostae*	AF324323	–	–	–
NRRL25200	*Fusarium*	*lactis*	AF160272	–	–	–
VI01268	*Fusarium*	*langsethiae*	AJ420822	–	–	–
VI01271	*Fusarium*	*langsethiae*	AJ420823	–	–	–
NRRL26231	*Fusarium*	*miscanthi*	AF324331	–	–	–
NRRL26239	*Fusarium*	*miscanthi*	AF324332	–	–	–
NRRL13604	*Fusarium*	*napiforme*	AF160266	–	–	–
NRRL25179	*Fusarium*	*nisikadoi*	AF324329	–	–	–
NRRL25183	*Fusarium*	*nisikadoi*	AF324330	–	–	–
BBA65634	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 1)	AF362145	AF362178	AF362145	AF362178
BBA64495	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362144	AF362177	AF362144	AF362177
BBA65633	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362146	AF362179	AF362146	AF362179
BBA65635	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362147	AF362180	AF362147	AF362180
BBA65636	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362148	AF362181	AF362148	AF362181
BBA65653	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362141	AF362174	AF362141	AF362174
BBA65655	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362149	AF362182	AF362149	AF362182

Table 4. (*Continued*)

Accession identifier	Genus	Species	EF-1α GenBank acc no.	mtSSU GenBank acc no.	NIR GenBank acc no.	PP GenBank acc no.
BBA66844	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362150	AF362183	AF362150	AF362183
BBA69405	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 2)	AF362151	AF362184	AF362151	AF362184
BBA62374	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 3)	AF362142	AF362175	AF362142	AF362175
BBA62375	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 3)	AF362143	AF362176	AF362143	AF362176
BBA64496	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 3)	AF362159	AF362192	AF362159	AF362192
BBA66845	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 3)	AF362153	AF362186	AF362153	AF362186
BBA67521	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 3)	AF362152	AF362185	AF362152	AF362185
BBA69712	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 3)	AF362162	AF362195	AF362162	AF362195
BBA66846	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 4)	AF362164	AF362197	AF362164	AF362197
BBA69518	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 4)	AF362160	AF362193	AF362160	AF362193
BBA69519	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 4)	AF362157	AF362190	AF362157	AF362190
BBA69520	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 4)	AF362140	AF362173	AF362140	AF362173
BBA69521	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 4)	AF362139	AF362172	AF362139	AF362172
BBA65650	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 5)	AF362154	AF362187	AF362154	AF362187
BBA65654	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 5)	AF362155	AF362188	AF362155	AF362188
BBA66847	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 6)	AF362158	AF362191	AF362158	AF362191
BBA69716	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 7)	AF362163	AF362196	AF362163	AF362196
BBA69050	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 7)	AF362156	AF362189	AF362156	AF362189
BBA69711	*Fusarium*	*oxysporum* f. sp. *vasinfectum* (race 8)	AF362161	AF362194	AF362161	AF362194
NRRL22276	*Fusarium*	*phaseoli*	AY220186	–	–	–
NRRL31156	*Fusarium*	*phaseoli*	AY220187	–	–	–
NRRL31071	*Fusarium*	*proliferatum*	AF291058	–	–	–
NRRL22946	*Fusarium*	*pseudocircinatum*	AF160271	–	–	–
NRRL25208	*Fusarium*	*ramigenum*	AF160267	–	–	–
NRRL25600	*Fusarium*	*redolens*	AF324294	–	–	–
NRRL28181	*Fusarium*	*redolens*	AF077391	–	–	–
NRRL22400	*Fusarium*	*solani* f.sp. *batas*	AF178343	–	–	–
NRRL22402	*Fusarium*	*solani* f.sp. *batas*	AF178344	–	–	–
VI01313	*Fusarium*	*sporotrichioides*	AJ420818	–	–	–
VI01319	*Fusarium*	*sporotrichioides*	AJ420819	–	–	–
NRRL13613	*Fusarium*	*succisae*	AF160291	–	–	–
NRRL31085	*Fusarium*	*tucumaniae*	AY220170	–	–	–
NRRL31086	*Fusarium*	*tucumaniae*	AY220171	–	–	–
NRRL22292	*Fusarium*	*virguliforme*	AY220188	–	–	–
NRRL22489	*Fusarium*	*virguliforme*	AY220189	–	–	–
NRRL26432	*Gibberella*	*circinata*	AF333929	–	–	–
NRRL28894	*Gibberella*	*moniliformis*	AF273313	–	–	–
NRRL29169	Gibberella	*zeae*	AF212461	–	–	–

Table 4. (*Continued*)

Accession identifier	Genus	Species	EF-1α GenBank acc no.	mtSSU GenBank acc no.	NIR GenBank acc no.	PP GenBank acc no.
NRRL34079	Gibberella	*zeae*	AY452958	–	–	–
NRRL22141	Nectria	*haematococca*	AF178329	–	–	–
NRRL22161	Nectria	*haematococca*	AF178330	–	–	–
NRRL22436	*Neocosmospora*	*africana*	AF178348	–	–	–
NRRL22468	*Neocosmospora*	*ornamentata*	AF178349	–	–	–
NRRL22166	*Neocosmospora*	*vasinfecta*	AF178350	–	–	–

*Data not available.

cultivated field soils, and 81 from diseased cotton plants (Table 5). The incidence of *F. oxysporum* varied among species and regions, occurring in 91% of soils associated with *G. sturtianum* populations, but in only 48–62% of populations of the other three species. Among the four regions, *F. oxysporum* occurred at a high frequency in the Longreach-Theodore (92%) and Leigh Creek-Arkaroola (85%) regions, but at a lower frequency in the Alice Spring-Tennant Creek (59%) and Mount Isa regions (57%) (Table 1). *Fusarium oxysporum* was isolated from all uncultivated and cultivated soils collected from the Boggabilla region, and *Fov* was isolated from all diseased *G. hirsutum* plants.

Pathogenicity screening tests

Fifteen percent of the 562 *F. oxysporum* isolates from soils associated with native *Gossypium*, were weakly pathogenic (*i.e.* causing mild stunting, foliar necrosis, and vascular discoloration) on Siokra 1–4, one of the most susceptible Australia cotton cultivars, with a mean disease severity of 0.3 (range: 0.1–0.6). This group was therefore putatively designated as wild *Fov* (Table 5). In contrast to the *Fov* found in cotton fields, no isolate of wild *Fov* associated with wild *Gossypium* soils was able to kill inoculated plants during the 6-week experimental period.

The incidence of wild *Fov* among *F. oxysporum* isolates varied by *Gossypium* species as well as geographic region. The greatest incidence occurred in isolates derived from soils associated with *G. sturtianum* (18%), with lower numbers among isolates from the other three species (Table 5). The incidence of wild *Fov* also appeared to vary geographic, ranging from 27% of the Leigh Creek-Arkaroola region isolates to only 5% of those from the Mount Isa region (Table 5).

Eighteen (10%) of the 178 *F. oxysporum* isolates recovered from cultivated field soils were *Fov* causing severe wilt symptoms in both trials (mean disease severity = 2.5; range = 1.8–3.1). However, none of the isolates from the uncultivated soil was pathogenic on cotton (Table 5). All isolates from diseased cotton plants were confirmed to be *Fov* as they consistently caused severe disease symptoms in both pathogenicity screening trials.

Virulence comparison tests

Wild *F. oxysporum* f. sp. *vasinfectum* (strains 2613 and 3556 from soils associated with *G. sturtianum*) was less aggressive on cotton but similar, or even more aggressive, on *G. sturtianum* relative to the performance of the reference *Fov* strains (Fig. 2). The two wild *Fov strains* caused only slight disease symptoms on cotton cultivar

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Table 5. Number of isolates of *Fusarium oxysporum* that are nonpathogenic on cotton (NP) and isolates of *F. oxysporum* f. sp. *vasinfectum* (*Fov*) recovered from *Gossypium* soil (summarized by *Gossypium* species and geographic regions respectively), uncultivated refuge soil, cultivated field soil, and diseased cotton plants, by lineage.

		Lineage						
		A	B	C	D	E	Others	Total
Source	Path*	*N*† (%)‡	*N* (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)
Gossypium soil by *Gossypium* species								
G. australe	NP	0 (0)	82 (59)	36 (26)	0 (0)	2 (1)	5 (4)	125 (89)
	Fov§	0 (0)	10 (7)	2 (1)	0 (0)	1 (1)	2 (1)	15 (11)
G. bickii	NP	0 (0)	36 (73)	0 (0)	0 (0)	0 (0)	6 (12)	42 (86)
	Fov	0 (0)	6 (12)	0 (0)	0 (0)	0 (0)	1 (2)	7 (14)
G. nelsonii	NP	0 (0)	47 (77)	5 (8)	2 (3)	0 (0)	1 (2)	55 (90)
	Fov	0 (0)	6 (10)	0 (0)	0 (0)	0 (0)	0 (0)	6 (10)
G. sturtianum	NP	18 (6)	144 (46)	52 (17)	3 (1)	25 (8)	14 (4)	256 (82)
	Fov	14 (4)	19 (6)	0 (0)	2 (1)	17 (5)	4 (1)	56 (18)
Gossypium soil by geographic regions								
Mount Isa (QLD)	NP	0 (0)	18 (16)	87 (76)	2 (2)	0 (0)	2 (2)	109 (95)
	Fov	0 (0)	4 (3)	2 (2)	0 (0)	0 (0)	0 (0)	6 (5)
Longreach (QLD)¶	NP	1 (1)	102 (82)	0 (0)	0 (0)	1 (1)	4 (3)	108 (86)
	Fov	1 (1)	11 (9)	0 (0)	0 (0)	4 (3)	1 (1)	17 (14)
Alice Springs (NT)**	NP	1 (1)	146 (74)	6 (3)	3 (2)	4 (2)	11 (6)	171 (86)
	Fov	1 (1)	20 (10)	0 (0)	2 (1)	1 (1)	3 (2)	27 (14)
Leigh Creek (SA)††	NP	16 (13)	43 (35)	0 (0)	0 (0)	22 (18)	9 (7)	90 (73)
	Fov	12 (10)	6 (5)	0 (0)	0 (0)	13 (10)	3 (2)	34 (27)
Gossypium soil	NP	18 (3)	309 (55)	93 (17)	5 (1)	27 (5)	26 (5)	478 (85)
	Fov	14 (2)	41 (7)	2 (0)	2 (0)	18 (3)	7 (1)	84 (15)
Refuge soil	NP	2 (6)	32 (91)	0 (0)	0 (0)	1 (3)	0 (0)	35 (100)
	Fov	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Field soil	NP	11 (6)	4 (2)	0 (0)	0 (0)	145 (81)	0 (0)	160 (90)
	Fov	18 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	18 (10)
Diseased plants	NP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Fov	81 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	81 (100)

*Pathogenicity of the isolates against cotton (*G. hirsutum*).
†Number of isolates.
‡Percentage of the isolates in the total recovered.
§Weakly pathogenic wild *Fov* for those isolates from *Gossypium* soils.
¶Longreach-Theodore (QLD).
**Alice Springs-Tennant Creek (NT).
††Leigh Creek-Arkaroola (SA).

Sicot 189 with severity ranging from 0.1 to 0.5, whereas the two reference *Fov strains* (derived from diseased cotton plants) caused significantly more severe disease symptoms (range: 1.3–2.9). Plants of *G. sturtianum Gos*-5250 were susceptible to both the wild and reference *Fov* strains (severity range: 1.3–3.1). While no significant difference in disease severity was found between wild *Fov strain* 3556 and the two reference *Fov* strains on *Gos*-5250, wild *Fov* strain 2613 caused significantly more severe disease symptoms (Fig. 2).

AFLP analysis

Of the 562 isolates of native *F. oxysporum* from soil associated with wild *Gossypium*, 94% (529) were grouped into five genetic lineages designated A, B, C, D, and E (Table 5). The lineage groupings were supported by the results of both an UPGMA (similarities between any two lineages <50%), and a bootstrap analysis, in which the bootstrap values based on data from four representatives per lineage ranged from 95 to 100 (Fig. 3).

The distribution of lineages in isolates from soil associated with wild *Gossypium* varied among species and region (Table 5). Lineage B predominated in four of the five regions irrespective of host species while lineages A and E were similarly distributed but were concentrated in the Leigh Creek-Arkaroola region. In contrast, lineage C was restricted to the Mount Isa region where it predominated; and only lineage B was found in association with *G. bickii* populations (Table 5).

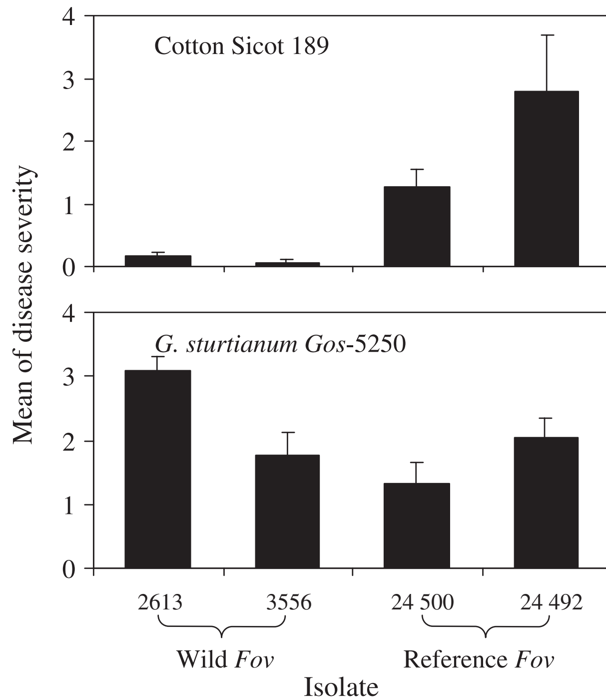

Figure 2 Mean disease severity on plants of cotton (Sicot 189) and *Gossypium sturtianum* (*Gos* 5250) caused by wild *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*) from soil associated with wild *Gossypium* (strains 2613 and 3556) and the *Fov* found in cotton fields (VCG 01111 strain 24500 and VCG 011112 strain 24492). Bars on top of columns represent standard deviations.

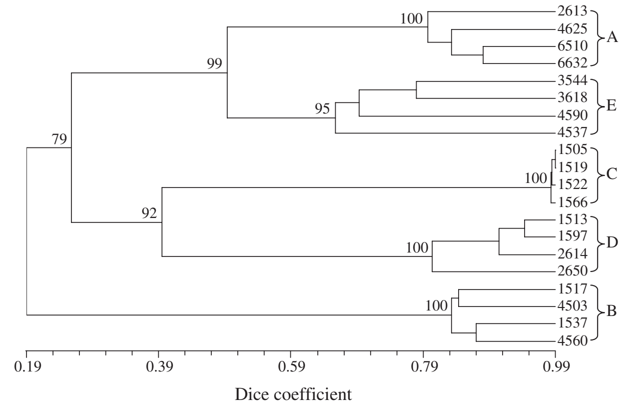

Figure 3 Unweighted pair-group with arithmetic average dendrogram constructed from AFLP fingerprints of four representative strains of each of the five genetic lineages (A, B, C, D and E) of Australian *Fusarium oxysporum* identified in this study. A total of 137 polymorphic bands revealed by the primer combination of *Eco*RI + AC and *Mse*I + A were used. Numbers at the nodes of major clusters represent bootstrap values (%) generated by 1000 replicates.

The incidence of wild *Fov* in soils associated with wild *Gossypium* also varied among the five lineages, ranging from *c.* 40% in lineages A and E, down to 11% and 2% in lineages B and C respectively. The incidence of wild *Fov* in lineage D (29%) is based on the occurrence of only two individuals in a sample size of seven (Table 5).

Lineages A, B, and E were also found in Boggabilla soil and plant samples (Table 5; Fig. 4). Ninety-five genetically distinct haplotypes clustering into three well-supported lineages (bootstrap values 100%) were identified among the 294 isolates from this area. Nineteen, 26, and 50 haplotypes were detected in lineages A, B and E, respectively. The level of genetic similarity among haplotypes within lineages was relatively high (72–75%), while genetic similarities between isolates from different lineages was considerably lower – 50% between lineages A and E, and only 13% between isolates in lineage B and those in lineages A or E. Lineage A could be further divided into two subgroups (A-I and A-II), but no clear subdivision was distinguishable in the other lineages (Fig. 4).

All pathogenic isolates (i.e. *Fov*) from Boggabilla belonged to lineage A, regardless of origin (Table 5; Fig. 4). They were distributed among eight haplotypes with five (A01-A05) in subgroup A-I and three (A14-A16) in subgroup A-II. Both reference *Fov* strains of VCG 01111 fell within subgroup A-I and both reference strains of VCG 01112 were placed in subgroup A-II. Of the eight *Fov* haplotypes, four (A02, A03, A05, A14) were recovered from both diseased plants and cultivated field soil, two (A04, A15) were found only in diseased plants, and two (A01, A16) only in the soil (Fig. 4). Thirteen nonpathogenic strains (fields: 11; refuge: 2) clustered with the pathogenic lineage A isolates, with eight in subgroup A-I and three in subgroup A-II. They were highly variable and represented a range of different haplotypes. These nonpathogenic lineage A isolates probably represented local Australian relatives of *Fov* (Fig. 4).

Ninety-three percent (182) of the nonpathogenic isolates from Boggabilla belonged to lineages B and E but the spatial distribution of these was almost mutually exclusive. Lineage E accounted for 82% of isolates from cultivated fields while 91% of isolates from the refuge soil were attributable to lineage B (Table 5). None of lineage A, B, or E nonpathogenic haplotypes were common to both the cultivated field and refuge soils (Fig. 4).

Sequence analysis

In the initial phylogenetic analysis concatenated sequences of two genes (EF-1α, mtSSU) from 18 isolates representing lineage A (12), B (3), and E (3) were used to explore relationships among the Australian *Fov* and nonpathogenic *F. oxysporum* (Fig. 5). The combined sequence alignment comprised 1404 base pairs (EF-1α: 738 bp;

Figure 4 A UPGMA dendrogram illustrating genetic relationships among 95 haplotypes of nonpathogenic *Fusarium oxysporum* (against cotton) and *Fov* based on pairwise Dice estimates of genetic similarity revealed using AFLPs. Bootstrap values ≥58% (10 000 replicates) are shown above nodes. Haplotypes in shaded boxes are pathogenic, i.e. *Fov*. The number of isolates per haplotype from diseased plants, cultivated fields, and refuge soil are listed to the right of the dendrogram. Brackets at the extreme right of the figure denote the lineages and subgroups discussed in the text. Isolates numbers followed by a superscript 'a', 'b', and 'c' refer to representative strains from soil associated with wild *Gossypium*, VCG 01111 and VCG 01112 of the Australian *Fov*, respectively.

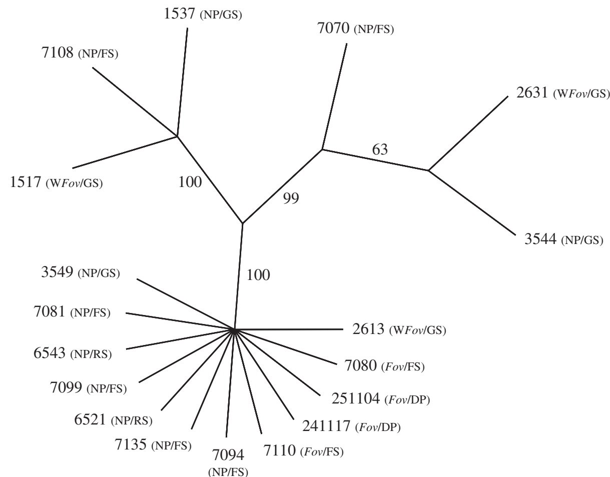

Figure 5 The single most parsimonious unrooted topology (length = 75 steps; consistency index = 0.9867; retention index = 0.9939) obtained from a heuristic parsimony optimized analysis of a concatenated matrix of the translation elongation factor-1α (EF-1α) gene and mitochondrial small subunit (mtSSU) rDNA sequences from four *Fov,* eight lineage A, three lineage B, and three lineage E isolates. Bootstrap values (10 000 replicates) are placed beside each branch of the typology. The pathogenicity of isolates on cotton (before slash; *Fov* = *F. oxysporum* f. sp. *vasinfectum*; W*Fov* = wild *F. oxysporum* f. sp. *vasinfectum*; NP = nonpathogenic) and their origin (behind slash; GS = *Gossypium* soil; RS = uncultivated refuge soil; FS = cultivated field soil; DP = diseased plant) are given in brackets.

mtSSU: 666 bp), of which 66 were phylogenetically informative. No signal incongruence between the EF-1α and mtSSU genes was detected using the partition homogeneity test ($P = 1.00$).

Unweighted maximum parsimony analysis yielded a single most parsimonious tree of 75 steps in length (consistency index = 0.987; retention index = 0.994; Fig. 5). The branching pattern of the tree was congruent with the UPGMA dendrograms of AFLP fingerprints (Figs 3 and 4), and further demonstrated that strains cluster based on genetic similarity rather than level of pathogenicity on cotton. Thus, all pathogenic and nonpathogenic isolates of lineage A were clustered in a well supported group (bootstrap value 100%), while lineage B and E strains occurred in two distinct and equally well supported clades that included nonpathogenic and weakly pathogenic wild *Fov* strains (Fig. 5).

In the second phylogenetic analysis, the relationships of the Australian *Fov* (including VCG 01111 and VCG 01112) and nonpathogenic lineage A isolates to representatives of *Fov* race 1–8 from other regions of the world was explored using concatenated sequences from four genes (EF-1α, mtSSU, NIR, PP). Data for races 1–8 was obtained from GenBank (Table 3). The concatenated alignment was 2399 bp in length, and four indels, encoded as binary characters, were appended to the end (EF-1α: 653 bp + two indels; mtSSU: 677 bp + one indel; NIR: 483 bp + one indel; PP: 586 bp). Unweighted maximum parsimony analysis yielded five equally parsimonious trees. There was no significant evidence of signal incongruity in the mtSSU sequence relative to the EF-1α, NIR, and PP sequences, and hence a parsimony optimized topology was derived from the unmodified alignment. To accommodate signal variation, the F81 substitution model was applied to the mtSSU partition, while the HKY model was applied to the EF-1α, NIR, and PP partitions in the Bayesian estimation of posterior probabilities (consistency index = 0.929; retention index = 0.992; Fig. 6). This topology demonstrates that the Australian *Fov* strains (VCG 01111 and VCG 01112) share a more recent common ancestor with both nonpathogenic and weakly pathogenic lineage A isolates of native *F. oxysporum* from soils associated with wild *Gossypium* than they do with *Fov* race 1–8 from overseas.

In the final phylogenetic analysis, the relationships between characteristic Australian *F. oxysporum*-like isolates and representatives of key *Fusarium* lineages in the order *Hypocreales* were assessed using EF-1α sequences. This analysis was limited to a single gene to maximize the ability to incorporate a wider diversity of taxa. The alignment comprised 709 bp of EF-1α sequence appended by 51 indels encoded as binary characters for a composite length of 760 characters. The topology illustrated in Fig. 7 is an unrooted consensus of 24 equally parsimonious trees (consistency index = 0.642; retention index = 0.948). This topology (i) confirms the close phylogenetic relationships among the pathogenic (VCG 01111 and 01112) and nonpathogenic lineage A isolates evident in Figs 5 and 6; (ii) illustrates the sister relationships between the lineage A isolates and *F. oxysporum* f. sp. *vasinfectum* race 1–8 relative to *F. foetens*; (iii) reaffirms the close phylogenetic relationships among the Australian lineage E isolates and *F. oxysporum* f. sp. *vasinfectum* race 1–8 (see Fig. 6); (iv) suggests the lineage B is a component of the widespread *F. fujikuroi* complex; and (v) suggests that lineage C and D represent fungi that heretofore have not been sequenced possibly representing new taxa.

VCG tests

None of the 32 lineage A isolates derived from soil associated with wild *Gossypium* (Table 5) were compatible with either of the VCG associated with the four reference Australian *Fov* strains. Three successful pairings were observed among six isolates from Leigh Creek-Arkaroola, while the remaining isolates were incompatible with each other.

Figure 6 Consensus parsimony optimized phylogenetic tree based on concatenated alignment (2399 bp + four indels encoded as binary characters) of the translation elongation factor-1α (653 bp + two indels), mitochondrial small subunit rDNA (677 bp + one indel), nitrate reductase (483 bp + one indel), and phosphate permease (586 bp) gene sequences. This unrooted topology is the consensus of five equally parsimonious trees (consistency index = 0.9286; retention index = 0.9915). Posterior probabilities, estimated in a separate Bayesian analysis, are indicated for each node; bootstrap values (1000 replicates) are indicated parenthetically beneath each posterior probability.

Discussion

Our results provide strong support for the hypothesis that VCG 01111 and VCG 01112 of *Fov* evolved locally in Australia (Davis et al. 1996). Not only are the Australian *Fov* strains morphologically and genetically distinct from the eight races of *Fov* found in other countries (Bayaa et al. 1995; Davis et al. 1996; Kim et al. 2005), but each VCG of the Australian *Fov* is grouped with a cluster of local nonpathogenic *F. oxysporum* strains in a single discrete lineage (lineage A; Fig. 4). This is further supported by multiple phylogenetic analyses that consistently place Australian *Fov* as sister to native lineage A strains rather than to representatives of the eight races of *Fov* that occur elsewhere in the world (Figs 5–7). That these putative precursors are endemic to Australia is well supported by their presence in both cultivated and uncultivated soils in cotton growing areas, and in a wide variety of soils associated with wild *Gossypium* located away from agricultural regions. The observation that some nonpathogenic lineage A strains are more related to the VCG 01111 strains than they are to the VCG 01112 strains, and the converse (Fig. 4), suggests that VCG 01111 and VCG 01112 arose independently.

The hypothesis that VCG 01111 and VCG 01112 of *Fov* evolved within Australia would be further strengthened by detection of their nonpathogenic progenitors, that is, native lineage A strains that are vegetatively compatible with the two known VCGs. However, the nonpathogenic lineage A strains found in soils associated with wild *Gossypium* in this study are highly unlikely to be direct progenitors of either VCG 01111 or VCG 01112 as none of them were vegetatively compatible. This is consistent with the observation of genetic similarity among the six compatible lineage A isolates. The minimum genetic similarity among these isolates is 94%, while the greatest genetic similarity between nonpathogenic isolates and pathogenic isolates obtained in this study was only 86% (Fig. 4).

The complexity of establishing the origins of pathogenic *F. oxysporum* is evident in a number of Fusarium wilt disease complexes (Gordon and Okamoto 1992a; Appel and Gordon 1994, 1996; Skovgaard et al. 2002). As

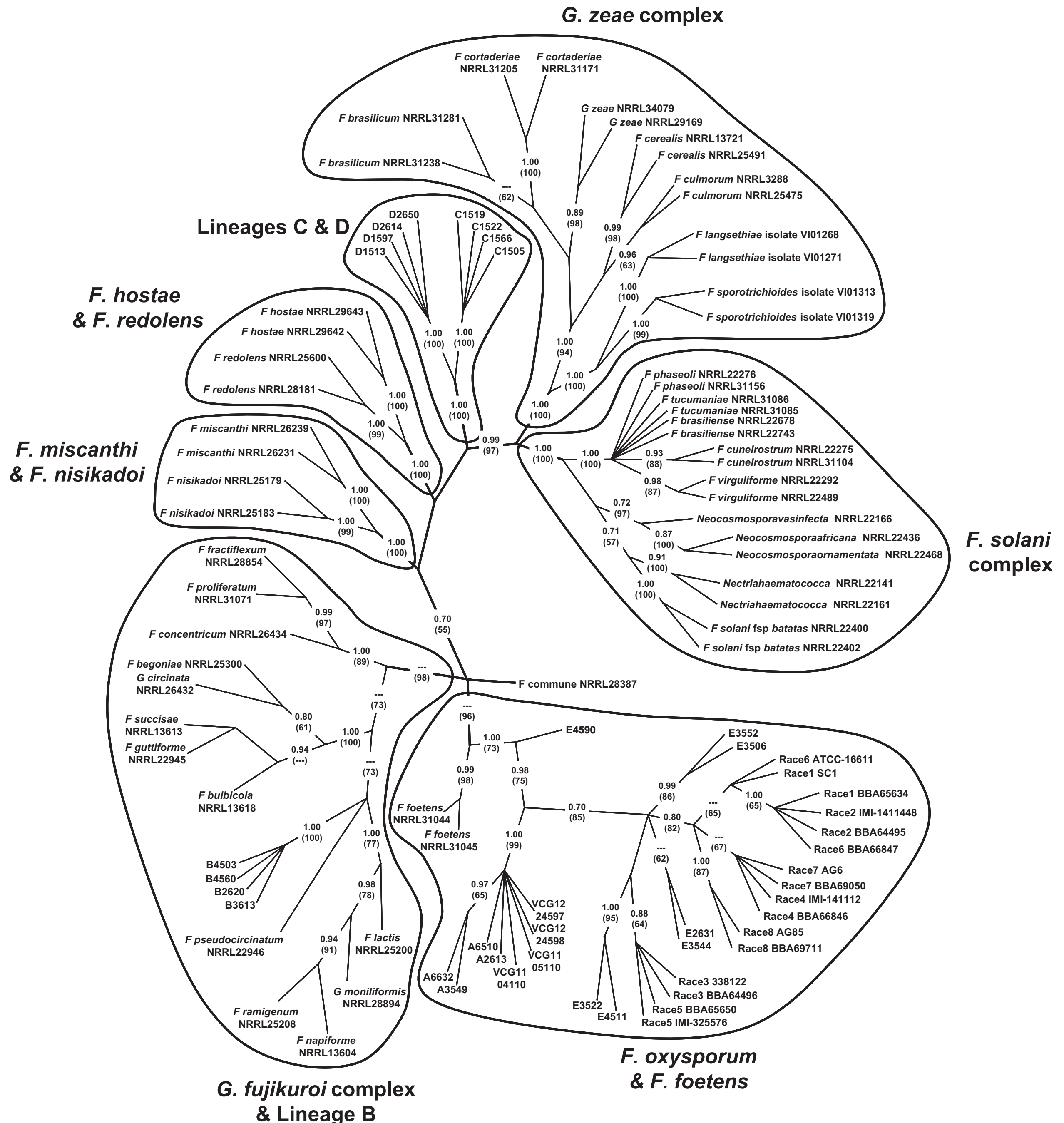

Figure 7 Consensus parsimony optimized phylogenetic tree based on the translation elongation factor-1α gene sequences (709 bp + 51 indels encoded as binary characters) from the Australian *Fusarium oxysporum*-like isolates and representatives of other key *Fusarium* lineages in the order *Hypocreales*. This unrooted topology is the consensus of 24 equally parsimonious trees (consistency index = 0.6416; retention index = 0.9480). Posterior probabilities, estimated in a separate Bayesian analysis, are indicated for each node; bootstrap values (1000 replicates) are indicated parenthetically beneath each posterior probability.

is the case here, conclusions regarding the origin of newly emergent pathogens are often complicated by difficulty in discriminating between nonpathogenic progenitors and avirulent mutants of pathogenic strains. Some of the ambiguity could arise from different expectations regarding genetic relationships and vegetative compatibilities

between pathogenic *F. oxysporum* that have arisen locally and co-occurring nonpathogenic populations. A reasonable expectation is that a pathogen may still be identical or similar to its ancestral strains, and consequently, most searches for the ancestors of pathogens focus on the same VCG (Gordon and Okamoto 1992b; Appel and Gordon 1994; Katan et al. 1994; Woudt et al. 1995). This is biologically realistic because individuals within a VCG are probably clonally derived, and genetic variation arises from mutation or other nonsexual means. Following this reasoning, *F. oxysporum* f. sp. *vasinfectum* and f. sp. *lycopersici* were regarded as exotic to Israel and California, respectively, because nonpathogenic forms that were vegetatively compatible with the pathogens were not observed in the soil community (Katan and Katan 1988; Elias et al. 1991). Conversely, the recovery of local, nonpathogenic *F. oxysporum* strains that were vegetatively compatible with races 1 and 2 of *F. oxysporum* f. sp. *melonis* in VCG 0134 in Maryland suggested a local origin for that pathogen (Appel and Gordon 1994).

However, while it is expected that recently emerged pathogens will be vegetatively compatible with their nonpathogenic progenitors, this expectation may never be realized when attempting to address older derivative–progenitor relationships. It cannot be assumed that the evolution of pathogenicity is temporally linked with the appearance of disease epidemics in an agricultural crop. For example, the ability of wild *Fov* strain 2613 (from soils associated with wild *Gossypium*) to cause mild Fusarium wilt symptoms on cotton suggests that some lineage A isolates could be characterized as 'aggressive endophytes', i.e. they can colonize the vasculature of cotton plants to the extent that some mild but typical disease symptoms are evident. If this is the case, then among a genetically diverse pool of native *F. oxysporum* genotypes, it is reasonable to expect variation in the levels of endophytic aggression. Therefore, it is possible that the progenitors of VCG 01111 and VCG 01112 progenitors were predisposed to be pathogenic on cotton, and have only increased in frequency and aggressiveness as cotton has been grown extensively in Australia. Thus strictly nonpathogenic VCG 01111 and VCG 01112 progenitors may never have been present. The potential for the evolution of increased virulence in weakly pathogenic lineage A strains from native cotton hosts has recently been demonstrated experimentally (Wang et al. 2008).

Regardless of evolutionary origins, the observation that *Fov* strains in cultivated field soils were overwhelmingly outnumbered by nonpathogenic (on cotton) lineage E isolates was surprising for two reasons. Only one lineage E isolate was recovered from the refuge soil and this lineage accounted for only 8% of the isolates from soils associated with wild *Gossypium* (Table 5; Fig. 4), and based on the phylogenetic analyses, lineage E isolates are more closely related to *Fov* race 1–8 than are any of the pathogenic or nonpathogenic lineage A isolates (Figs 6 and 7). So despite the fact that lineage E related genotypes have become pathogenic on cotton elsewhere in the world and can increase in frequency under cultivation, in Australia the phylogenetically distinct lineage A has given rise to a new group of cotton pathogens.

Why lineage E isolates are over represented in cultivated fields is not clear, but it would appear some selective mechanism is operating. One possibility is that some lineage E isolates are pathogenic on rotation crops. Wheat has been grown in these fields in rotation with cotton and the cultivation of wheat can select for certain fungal genotypes (Edel et al. 1997). Previous studies have also demonstrated that the composition of *F. oxysporum* populations is affected by the application of certain fertilizers (Wang et al. 1999), and it is possible that lineage E isolates may have a fitness advantage under cultivated conditions, i.e. the application of fertilizers and the incorporation of crop debris.

The genetic structure of *F. oxysporum* populations in uncultivated soils and how immigration from cultivated fields impinges on these native populations and *vice-versa*, is largely unexplored, as is generally the case for host–pathogen interactions across the agro-ecological interface (Burdon and Thrall 2008). Gordon et al. (1992) found no spatial structure among isolates from adjacent cultivated and native California soils, with most mtDNA haplotypes occurring in both soils, indicating a high level of gene flow. In contrast, our results showed that the composition of nonpathogenic (on cotton) *F. oxysporum* populations from agricultural fields and uncultivated refuges differed dramatically (Table 5; Fig. 4). A better knowledge of the ecological processes underlying this dramatic shift in the composition of *F. oxysporum* populations will improve our understanding of the emergence of the Fusarium complex in cotton growing areas in Australia, and ultimately be useful in the development of novel control strategies and improved disease management protocols (Burdon and Thrall 2008).

This study extends our knowledge of indigenous *F. oxysporum* populations in Australia, but also raises interesting questions regarding the relationship of lineage A to E to other *F. oxysporum* and *Fusarium* species. The EF-1α gene sequences were compared with those in the public database using BLAST searches. The results showed that VCG 01111 and VCG 01112 of *Fov* found in the cotton fields as well as lineages A and E from soil associated with wild *Gossypium* in Australia are clearly included in the *F. oxysporum* clade; lineage B belongs in the *Gibberella fujikuroi* complex; while lineages C and D are distinct from known sequences (Fig. 7). These results reflect: (i) a lack of a one-to-one correlation between morphological,

biological species, and phylogenetic species as shown in many other studies (O'Donnell et al. 1998a; Leslie et al. 2001); (ii) morphological and/or biological species show global geographic ranges, but phylogenetic species usually harbour several to many endemic species (Taylor et al. 2006); and (iii) limitations in morphological identification of *F. oxysporum*. The unique phylogenetic status of the two Australian VCGs of *F. oxysporum* f. sp. *vasinfectum* and lineage A in the *Fusarium* species complex has been recently confirmed by O'Donnell et al. (2009). Their work also showed that, within the *Fusarium oxysporum* species complex, only two from New Zealand showed some similarity to the Australian Fov isolates, suggesting that lineage A may be geographically restricted to the Pacific region.

Knowledge of *F. oxysporum* in natural ecosystems is limited although it is a common inhabitant of various native soils (McMullen and Stack 1983; Gordon et al. 1992; Summerell et al. 1993; Wang et al. 2004). However, even less is known about the extent to which pathogenic *F. oxysporum* strains are associated with wild relatives of a crop host and their pathogenicity towards the crop. The patterns of pathogenicity likely to be found in plant–pathogen associations are markedly affected by a range of life-history characters of the pathogen that can ultimately influence its transmission rate (Burdon 1987). It has been suggested that systems in which the pathogen is capable of saprophytic growth or is able to infect multiple host species may favour isolates that are less aggressive as transmission opportunities may be greater than for more specialized pathogens (Alexander 1981; May and Anderson 1983; Gordon and Martyn 1997). Factors such as host density and crop rotation, as well as pathogen saprophytic ability have been shown theoretically to influence the dynamics and persistence of soil-borne fungi (Thrall et al. 1997); the role of agronomic management in influencing the evolutionary trajectories of soil pathogen populations has not been widely explored.

In this study, although 15% of *F. oxysporum* isolates derived from soil associated with wild *Gossypium* showed pathogenicity on cotton (i.e. they were wild *Fov*) (Table 5), none killed any of the inoculated plants during the experimental period. Within the native Australian *Gossypium* populations, genotypes exist that are tolerant or resistant to the *Fov* occurring in cotton fields, while others are highly susceptible (Becerra Lopez-Lavalle et al. 2007). As a result, the selection pressures exerted by resistance differences in co-occurring wild *Fov* populations may have favoured the selective accumulation of isolates with enhanced pathogenicity – some of which may be pathogenic to cultivated cotton. This possibility is supported by evidence involving the occurrence of a distinct form of *F. oxysporum* f. sp. *cubense* that posed a serious economic risk to banana production in Sumatra only a few years after its establishment in the area. Tests showed that this form of *F. oxysporum* f. sp. *cubense* was asymptomatically associated with local wild bananas from which it presumably spread (Moore et al. 2001).

Wild relatives of cultivated crops have long been recognized as sources of valuable genes in resistance breeding (Kaiser et al. 1994; Bayaa et al. 1995; Huang and Lindhout 1997). However, relatively little is known about their importance either in the maintenance of the pathogens involved or in their epidemiology (Burdon and Thrall 2008). Pathogenic strains of *F. oxysporum* appear to gain or retain pathogenicity at the cost of losing some of their ecological breadth (Gordon and Martyn 1997). As a consequence, they risk being out-competed by non-pathogenic strains if the benefit of pathogenesis cannot be achieved regularly (Gordon and Martyn 1997). Wild *Fov* occurred in 61% of *G. sturtianum* populations, but in only a third of populations of the other three *Gossypium* species, suggesting a strong preference for *G. sturtianum* by wild *Fov* (Table 1). This suggests that some native *Gossypium* populations may not only be an inoculum reservoir for the pathogen but could also nurture the pathogen's evolutionary potential. Wild *Fov* occurring in all the five lineages identified in this study (Table 5) possesses significantly greater genetic diversity than does the *Fov* found in cotton fields that contains only two genotypes (Bentley et al. 2000). Given the proximity of cotton fields to some of these native *F. oxysporum* populations (e.g. in Theodore, Queensland some *G. sturtianum* populations occur within 200 meters of commercial cotton fields), there is little doubt that wild *Fov* could invade cotton fields as a result of clearing for new plantings or by dispersal in soil attached to stock or machinery.

Acknowledgements

This research was supported by the Cotton Research and Development Corporation of Australia. We thank S. Allen (Cotton Seed Distributors), D. Nehl (New South Wales Department of Primary Industries), W. Tate, B. Matheson, A. Becerra, F. Zich, J. Grace, M. Woods and J. Scown (CSIRO Plant Industry), and A. Fraser for their assistance in sample collection and experimental work.

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