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

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**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 soil-borne 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...
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 nonpathogenic 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. australis*, *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. australis*, *G. bickii*, *G. nelsonii*, *G. sturtianum*) in 2001–2002 in the eastern and central parts of Australia (Table 1). At each site, 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 km² 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,
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 $2.5 \times 10^5$ to $8.5 \times 10^7$ 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 necrosis; 2 = necrosis on 50% but <100% of the foliage; 3 ≥ 50% but <100% foliar necrosis; 4 = 100% foliar necrosis).

### 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.
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 ± 0.2 × 10⁶ 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, Australia). DNA concentrations were determined using a GeneQuant II spectrophotometer (Pharmacia Biotech, Australia) 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 nonspecific 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 32P-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 MgCl₂, 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
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* and reference *Fov* strains 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 chloride. 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

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**Table 2.** Primers used in this study.

| Locus                        | Primer sequence (5¢ to 3¢) | Length | Tm (°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 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.|

Tm, annealing temperature; Amp., amplification; Seq., sequencing.
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. *vasinfectum* (*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 | – | – |
| 71120 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 | DQ435346 | DQ435364 | – | – |
| 6543 Nonpathogenic A A-I/A13 Refuge soil | DQ435347 | DQ435365 | – | – |
| 7081 Nonpathogenic A A-II/A19 Field soil | DQ435348 | DQ435366 | – | – |
| 7108 Nonpathogenic B B-26 Field soil | DQ435349 | DQ435367 | – | – |
| 7070 Nonpathogenic E E-32 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 Nonpathogenic A – Wild Gossypium soil | EU246541 | EU246588 | EU246624 | EU246658 |
| 3547 Nonpathogenic 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-1338122† | *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).
Table 4. Translation elongation factor (EF-1\(\alpha\)), 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-1\(\alpha\) GenBank acc no. | mtSSU GenBank acc no. | NIR GenBank acc no. | PP GenBank acc no. |
|----------------------|-------------------|------------------|---------------------------------|-----------------------|---------------------|-------------------|
| NRRL25300            | *Fusarium*        | begoniae         | AF160293                        | (*)                   | (*)                 | (*)               |
| NRRL31238            | *Fusarium*        | brasiliicum      | AY452963                        | (*)                   | (*)                 | (*)               |
| NRRL31281            | *Fusarium*        | brasiliicum      | AY452964                        | (*)                   | (*)                 | (*)               |
| NRRL22678            | *Fusarium*        | brasilense       | AY320144                        | (*)                   | (*)                 | (*)               |
| NRRL22743            | *Fusarium*        | brasilense       | AY320145                        | (*)                   | (*)                 | (*)               |
| NRRL13618            | *Fusarium*        | bulbicola        | AF160294                        | (*)                   | (*)                 | (*)               |
| NRRL13721            | *Fusarium*        | cerealis         | AF212464                        | (*)                   | (*)                 | (*)               |
| NRRL25491            | *Fusarium*        | cerealis         | AF212465                        | (*)                   | (*)                 | (*)               |
| NRRL28387            | *Fusarium*        | commune          | AF246832                        | (*)                   | (*)                 | (*)               |
| NRRL26434            | *Fusarium*        | concentricum     | AF339333                        | (*)                   | (*)                 | (*)               |
| NRRL31171            | *Fusarium*        | cortaderiae      | AY452961                        | (*)                   | (*)                 | (*)               |
| NRRL31205            | *Fusarium*        | cortaderiae      | AY452960                        | (*)                   | (*)                 | (*)               |
| NRRL25475            | *Fusarium*        | culmorum         | AF212463                        | (*)                   | (*)                 | (*)               |
| NRRL32888            | *Fusarium*        | culmorum         | AF212462                        | (*)                   | (*)                 | (*)               |
| NRRL22275            | *Fusarium*        | cuneirostrum     | AY320158                        | (*)                   | (*)                 | (*)               |
| NRRL31104            | *Fusarium*        | cuneirostrum     | AY320159                        | (*)                   | (*)                 | (*)               |
| NRRL31044            | *Fusarium*        | foetens          | AY320072                        | (*)                   | (*)                 | (*)               |
| NRRL31045            | *Fusarium*        | foetens          | AY320073                        | (*)                   | (*)                 | (*)               |
| NRRL28854            | *Fusarium*        | fractiflexum     | AF339302                        | (*)                   | (*)                 | (*)               |
| NRRL22945            | *Fusarium*        | guttiflora       | AF160297                        | (*)                   | (*)                 | (*)               |
| NRRL29642            | *Fusarium*        | hostae           | AF324322                        | (*)                   | (*)                 | (*)               |
| NRRL29643            | *Fusarium*        | hostae           | AF324323                        | (*)                   | (*)                 | (*)               |
| NRRL25200            | *Fusarium*        | lactis           | AF160272                        | (*)                   | (*)                 | (*)               |
| V01268               | *Fusarium*        | langsethiae      | AJ420822                        | (*)                   | (*)                 | (*)               |
| V01271               | *Fusarium*        | langsethiae      | AJ420823                        | (*)                   | (*)                 | (*)               |
| NRRL26231            | *Fusarium*        | miscanthi        | AF324321                        | (*)                   | (*)                 | (*)               |
| NRRL26239            | *Fusarium*        | miscanthi        | AF324332                        | (*)                   | (*)                 | (*)               |
| NRRL13604            | *Fusarium*        | napiforme        | AF160266                        | (*)                   | (*)                 | (*)               |
| NRRL25179            | *Fusarium*        | nisikadoi        | AF324329                        | (*)                   | (*)                 | (*)               |
| NRRL25183            | *Fusarium*        | nisikadoi        | AF324330                        | (*)                   | (*)                 | (*)               |
| BBAA65634            | *Fusarium*        | oxysporum f. sp. vasisinfestum (race 1) | AF362145 | AF362178 | AF362145 | AF362178 |
| BBAA64495            | *Fusarium*        | oxysporum f. sp. vasisinfestum (race 2) | AF362144 | AF362177 | AF362144 | AF362177 |
| BBAA65633            | *Fusarium*        | oxysporum f. sp. vasisinfestum (race 2) | AF362146 | AF362179 | AF362146 | AF362179 |
| BBAA65635            | *Fusarium*        | oxysporum f. sp. vasisinfestum (race 2) | AF362147 | AF362180 | AF362147 | AF362180 |
| BBAA65636            | *Fusarium*        | oxysporum f. sp. vasisinfestum (race 2) | AF362148 | AF362181 | AF362148 | AF362181 |
| BBAA65653            | *Fusarium*        | oxysporum f. sp. vasisinfestum (race 2) | AF362141 | AF362174 | AF362141 | AF362174 |
| BBAA65655            | *Fusarium*        | oxysporum f. sp. vasisinfestum (race 2) | AF362149 | AF362182 | AF362149 | AF362182 |
| Accession identifier | Genus | Species | EF-1a GenBank acc no. | mtSSU GenBank acc no. | NR 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 |
| BBA64496             | Fusarium | oxysporum f. sp. vasinfectum (race 3) | AF362143 | AF362176 | AF362143 | AF362176 |
| BBA66845             | Fusarium | oxysporum f. sp. vasinfectum (race 3) | AF362153 | AF362186 | AF362153 | AF362186 |
| BBA69712             | Fusarium | oxysporum f. sp. vasinfectum (race 3) | AF362162 | AF362195 | AF362162 | AF362195 |
| BBA66846             | Fusarium | oxysporum f. sp. vasinfectum (race 4) | AF362146 | 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 | AF107391 | – | – | – |
| NRRL22400             | Fusarium | solani f.sp. batas | AF178343 | – | – | – |
| NRRL22402             | Fusarium | solani f.sp. batas | AF178344 | – | – | – |
| VI01313               | Fusarium | sporotrichoides | AJ20818 | – | – | – |
| VI01319               | Fusarium | sporotrichoides | AJ20819 | – | – | – |
| NRRL13613             | Fusarium | succisae | AF160291 | – | – | – |
| NRRL31085             | Fusarium | tucumaniae | AY220170 | – | – | – |
| NRRL31086             | Fusarium | tucumaniae | AY220171 | – | – | – |
| NRRL22292             | Fusarium | virgiliforme | AY220188 | – | – | – |
| NRRL22489             | Fusarium | virgiliforme | AY220189 | – | – | – |
| NRRL26432             | Gibberella | circinata | AF333929 | – | – | – |
| NRRL28894             | Gibberella | moniliformis | AF273313 | – | – | – |
| NRRL29169             | Gibberella | zeae | AF212461 | – | – | – |
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 Springs-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...
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).
The incidence of wild *Fusarium* 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 *Fusarium* 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. *Fusarium*) 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 *Fusarium* 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 *Fusarium* 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 non-pathogenic lineage A isolates probably represented local Australian relatives of *Fusarium* (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 *Fusarium* and nonpathogenic *Fusarium 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 topology. The pathogenicity of isolates on cotton (before slash; Fov, F. oxysporum f. sp. vasinfectum; WFov = 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.

The Australian Fov that included nonpathogenic and weakly pathogenic wild isolates occurred in two distinct and equally well supported clades (bootstrap value 100%), while lineage B and E strains of lineage A were clustered in a well supported group (Fig. 5). Thus, all pathogenic and nonpathogenic isolates shared 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 VCG 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-Arkarooola, while the remaining isolates were incompatible with each other.
Our results provide strong support for the hypothesis that VCG 01111 and VCG 01112 of \( F_{ov} \) evolved locally in Australia (Davis et al. 1996). Not only are the Australian \( F_{ov} \) strains morphologically and genetically distinct from the eight races of \( F_{ov} \) found in other countries (Bayaa et al. 1995; Davis et al. 1996; Kim et al. 2005), but each VCG of the Australian \( F_{ov} \) 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 \( F_{ov} \) as sister to native lineage A strains rather than to representatives of the eight races of \( F_{ov} \) 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 \( F_{ov} \) 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
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,
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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 asymptotically 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.

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