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Investigation of the diversity of effector genes in the banana pathogen, *Fusarium oxysporum* f. sp. *cubense*, reveals evidence of horizontal gene transfer

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SUMMARY

It is hypothesized that the virulence of phytopathogenic fungi is mediated through the secretion of small effector proteins that interfere with the defence responses of the host plant. In *Fusarium oxysporum*, one family of effectors, the *Secreted In Xylem* (*SIX*) genes, has been identified. We sought to characterize the diversity and evolution of the *SIX* genes in the banana-infecting lineages of *F. oxysporum* f. sp. *cubense* (*Foc*). Whole-genome sequencing data were generated for the 23 genetic lineages of *Foc*, which were subsequently queried for the 14 known *SIX* genes (*SIX1–SIX14*). The sequences of the identified *SIX* genes were confirmed in a larger collection of *Foc* isolates. Genealogies were generated for each of the *SIX* genes identified in *Foc* to further investigate the evolution of the *SIX* genes in *Foc*. Within *Foc*, variation of the *SIX* gene profile, including the presence of specific *SIX* homologues, correlated with the pathogenic race structure of *Foc*. Furthermore, the topologies of the *SIX* gene trees were discordant with the topology of an infraspecies phylogeny inferred from *EF-1α*/*RPB1*/*RPB2* (translation elongation factor-1α/RNA polymerase II subunit I/RNA polymerase II subunit II). A series of topological constraint models provided strong evidence for the horizontal transmission of *SIX* genes in *Foc*. The horizontal inheritance of pathogenicity genes in *Foc* counters previous assumptions that convergent evolution has driven the polyphyletic phylogeny of *Foc*. This work has significant implications for the management of *Foc*, including the improvement of diagnostics and breeding programmes.

Keywords: effectors, *Fusarium oxysporum* f. sp. *cubense*, horizontal gene transfer.

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INTRODUCTION

The collective host range of the *Fusarium oxysporum* species complex (FOSC) is remarkably diverse, encompassing not only plant species, but also animals as distantly related as arthropods and humans (Nucci and Annaisie, 2007; Ortoneda *et al.*, 2004; Snyder and Hansen, 1940; Teetor-Barsch and Roberts, 1983). In contrast with the broad host range of the species complex as a whole, each individual pathogenic isolate possesses the ability to infect just one or a few host species (Snyder and Hansen, 1940). Pathogenic isolates are conventionally grouped into infraspecific assemblages, known as *formae speciales* (ff. spp.), on the basis of the host species affected. However, this classification, which is based solely on the host-specific pathogenicity of an isolate, is an informal taxonomic grouping and is often not phylogenetically informative (O'Donnell *et al.*, 2013; Snyder and Hansen, 1940). *Formae speciales* are further divided into 'races' in which pathogenic variation exists in relation to different cultivars of the same host species. In this study, we have focused on the *forma specialis* (f. sp.) *cubense*, which includes all FOSC isolates pathogenic to *Musa* spp. (Snyder and Hansen, 1940). Significant pathogenic variation exists across this *forma specialis* with respect to various commercial cultivars, and it is therefore divided into four races (Stover, 1990; Stover and Buddenhagen, 1986; Stover and Simmonds, 1987). Race 1 is pathogenic to Gros Michel (AAA genome), Pisang Awak (ABB) and a range of other cultivars, primarily with the AAB genome (Stover, 1990; Stover and Buddenhagen, 1986; Stover and Simmonds, 1987); race 2 is pathogenic to race 1-susceptible cultivars, as well as Bluggoe and other cultivars with the ABB genome (Stover, 1990; Stover and Buddenhagen, 1986; Stover and Simmonds, 1987); race 3 affects *Heliconia* species, not banana, and is therefore no longer considered to be part of the *cubense* race structure (Ploetz and Pegg, 2000; Waite, 1963); and race 4 is pathogenic to all race 1- and 2-susceptible cultivars plus the Cavendish subgroup (AAA) (Stover and Simmonds, 1987; Su *et al.*, 1986). The isolates within race 4 are commonly divided into two groups: 'tropical race 4' (TR4) and

'subtropical race 4' (STR4). TR4 is defined as those isolates which cause disease on Cavendish in tropical conditions, whereas STR4 is defined as those isolates which, in the absence of predisposing factors, are unable to infect Cavendish in the tropics, but do cause disease in subtropical conditions (Moore *et al.*, 1993).

As an alternative to the race classification system, FOSC isolates can be characterized according to vegetative compatibility, which is the ability of an isolate to anastomose and form a stable heterokaryon (Moore *et al.*, 1993; Ploetz and Correll, 1988; Puhalla, 1985). Isolates that are vegetatively compatible with one another are said to comprise a vegetative compatibility group (VCG) and typically share common biological, physiological and pathological traits (Caten and Jinks, 1966). Within *F. oxysporum* f. sp. *cubense* (*Foc*), 25 VCGs (0120–01224) have thus far been described (Bentley *et al.*, 1995; Gerlach *et al.*, 2000; Katan and Di Primo, 1999; Moore *et al.*, 1993; Ploetz and Correll, 1988). However, VCG 0127 originally reported by Ploetz and Correll (1988) is no longer considered to be valid (Ploetz, 1994). Associations between VCGs and race are summarized in Table 1. Phylogenetic analysis of the various genetic lineages of *Foc* have repeatedly demonstrated that this *forma specialis* has a polyphyletic evolutionary history (Baayen *et al.*, 2000; Fourie *et al.*, 2009; Groenewald *et al.*, 2006).

The molecular mechanisms underpinning the virulence of *Foc* are of great scientific interest as they relate to the applied management of Fusarium wilt and, more generally, to our understanding of the evolution of the host–pathogen interaction. Presently, the 'zig–zag model' is the accepted paradigm for the molecular interactions between phytopathogens and their hosts (Jones and Dangl, 2006). Briefly, under this model, conserved molecular patterns associated with microbial pathogens, such as flagellin or chitin, elicit an initial defence response from the host plant. Pathogens have evolved mechanisms to subvert this initial defence response, including the secretion of small effector proteins at the infection interface. Effector proteins are known to have a range of functions, including masking of the presence of the pathogen, suppression of host defence responses and transcriptional reprogramming of the host cell. Analogous to an arms race, plants have evolved classes of resistance genes that associate with and recognize these effectors and trigger a second wave of defence responses. Pathogens that are able to mitigate or suppress this secondary level of defence have a distinct selective advantage. Consequently, the genes coding for effectors are often under strong selection pressure to gain mutations in these genomic regions that result in a loss of recognition by the corresponding resistance proteins produced by the host. It has been demonstrated in multiple plant pathogens, including *F. oxysporum*, that the host range and specificity of an isolate are affected by the effectors in its molecular arsenal (Gawehns *et al.*, 2014; Houterman *et al.*, 2008, 2009; Ma *et al.*, 2015; Rep *et al.*, 2004, 2005).

Table 1 Race structure of *Fusarium oxysporum* f. sp. *cubense* by vegetative compatibility group (VCG) (Bentley *et al.*, 1995, 1998; Gerlach *et al.*, 2000; Groenewald *et al.*, 2006; Jones, 2000; Katan and Di Primo, 1999; Moore *et al.*, 1993; Ploetz, 1994; Ploetz and Correll, 1988).

VCG	VCG complex	Race
0120	0120–01215	STR4
0121	None	R4*
0122	None	R4*
0123	None	1
0124	0124–0125–0128–01220	1, 2
0125	0124–0125–0128–01220	1, 2
0126	None	R1/R4†
0127	N/A	No longer valid‡
0128	0124–0125–0128–01220	1, 2
0129	0129–01211	STR4
01210	None	1
01211	0129–01211	STR4
01212	None	Undetermined
01213	01213–01216	TR4§
01214	None	2
01215	0120–01215	STR4
01216	01213–01216	TR4§
01217	None	1
01218	None	1
01219	None	Undetermined
01220	0124–0125–0128–01220	1
01221	None	Undetermined
01222	None	Undetermined
01223	None	Undetermined
01224	None	Undetermined

*The subclassification of VCGs 0121 and 0122 to either TR4 or STR4 is ambiguous as there are reports that these VCGs cause disease on Cavendish in tropical conditions; however, it has been observed that these VCGs are less aggressive than isolates of VCG 01213/16 (Buddenhagen, 2009; Moore *et al.*, 1993; O'Neill *et al.*, 2011).

†The race of VCG 0126 is ambiguous as isolates from VCG 0126 demonstrate many traits associated with race 4, such as the production of odoratum on medium and a close genetic relationship to other VCGs of race 4. However, there is limited evidence to suggest that it is capable of infecting Cavendish and it is commonly referred to as race 1 based on its host range (Pegg *et al.*, 1994).

‡VCG 0127, originally reported by Ploetz and Correll (1988), is no longer considered to be valid (Ploetz, 1994).

§VCG 01213 and 01216 are now considered to be the same VCG complex (VCG 01213/16) (Bentley *et al.*, 1998).

In the phytopathogenic isolates of *F. oxysporum*, the *Secreted In Xylem* (*SIX*) genes form the only family of effectors that has been identified to date. In the tomato-infecting *forma specialis*, *F. oxysporum* f. sp. *lycopersici* (*Fol*), 14 *SIX* genes (*SIX1–SIX14*) have been experimentally confirmed (Houterman *et al.*, 2007; Lievens *et al.*, 2009; Rep *et al.*, 2004; Schmidt *et al.*, 2013; Takken and Rep, 2010). Of these 14 genes, five *Fol-SIX* genes (*SIX1*, *SIX3*, *SIX4*, *SIX5* and *SIX6*) have been validated to encode genuine effector proteins *in planta* (Gawehns *et al.*, 2014; Houterman *et al.*, 2007; Lievens *et al.*, 2009; Ma *et al.*, 2015; Rep *et al.*,

2004; Schmidt *et al.*, 2013; Takken and Rep, 2010). The protein products of *SIX1*, *SIX3/SIX5* and *SIX4* are also avirulence factors as they are recognized by host defence or immune receptors (I-3, I-2 and I-1, respectively) that have been introgressed into commercial cultivars of tomato (Houterman *et al.*, 2008, 2009; Ma *et al.*, 2015; Rep *et al.*, 2004, 2005). Consistent with the zig–zag model, the emergence of new races in *Fol* is associated with either mutations in the coding region of the effector gene or complete effector gene loss events, which both result in the loss of recognition by the host immune receptors. Although the specific functions of the SIX proteins are unknown, some evidence indicates that the SIX proteins promote virulence through the manipulation of the hormone pathways and defence responses of the host (Gawehns *et al.*, 2014; Ma *et al.*, 2013, 2015; Thatcher *et al.*, 2012).

Initially, the *SIX* genes were thought to be unique to *Fol*; however, numerous homologues have since been identified in other *formae speciales*, including *betae*, *canariensis*, *cepae*, *ciceris*, *conglutinans*, *cubense*, *fragariae*, *lilii*, *lycopersici*, *medicaginis*, *melonis*, *niveum*, *passiflorae*, *pisi*, *radicis-cucumerinum*, *radicis-lycopersici*, *raphani*, *vasinfectum* and *zingiberi* (Chakrabarti *et al.*, 2011; Covey *et al.*, 2014; Fraser-Smith *et al.*, 2014; Guo *et al.*, 2014, Laurence *et al.*, 2015; Lievens *et al.*, 2009; Meldrum *et al.*, 2012; Taylor *et al.*, 2016; Thatcher *et al.*, 2012; Williams *et al.*, 2016). In *Foc*, homologues of *SIX1*, *SIX2*, *SIX6*, *SIX7*, *SIX8*, *SIX9* and *SIX13* have been identified (Fraser-Smith *et al.*, 2014; Guo *et al.*, 2014; Meldrum *et al.*, 2012; Taylor *et al.*, 2016; Van Dam *et al.*, 2016). However, research thus far has been limited to a narrow sample of the phylogenetic diversity of *Foc*.

In this study, we used a whole-genome sequencing approach to screen isolates from *Foc* VCGs 0120 to 01223 for initial identification of homologues to *SIX1* to *SIX14*. Subsequent polymerase chain reaction (PCR) analyses confirmed the distribution and diversity of the *SIX* genes established by the whole-genome dataset. Gene tree analysis revealed that the *SIX* genes in *Foc* had an evolutionary history that was incongruent with the phylogeny of *Foc* established through nuclear genes conventionally used for phylogenetic reconstruction. We hypothesize that this incongruence is reflective of historical horizontal transfer of *SIX* genes between genetically distinct lineages of *Foc*.

RESULTS

Variation in distribution and nucleotide sequence of *SIX* genes in VCGs of *Foc*

To initially identify *SIX* gene homologues in *Foc*, whole-genome datasets were generated for 23 VCGs of *Foc* ($n = 28$); three *F. oxysporum* isolates from asymptomatic banana plants and five other *formae speciales* were queried for homologues of *Fol-SIX1* to *Fol-SIX14* (Table 2). In addition, the genomes of other *F. oxysporum* isolates available through public databases were also queried for homologues of *Fol-SIX1* to *Fol-SIX14* (Table S2, see Supporting Information). Seven *SIX* genes were identified in the VCGs of *Foc*: *SIX1*, *SIX2*, *SIX6*, *SIX8*, *SIX9*, *SIX10* and *SIX13*. An orthologue of *SIX4* was also identified in the VCGs of *Foc*; however, the start codon of *SIX4* was non-functional and is predicted to be a pseudogene. To further confirm the presence and sequence of the *SIX* gene homologues identified in the *Foc* whole-genome datasets, a larger collection of *Foc* isolates was screened using PCR. The results from the analysis of the whole-genome data and PCR screens are summarized in Table 3.

The *SIX* genes identified in the VCGs of races 1 and 2 were generally conserved both within a VCG and between the different VCGs (Table 3). The variation within the VCGs of races 1 and 2 was primarily observed within the *SIX1* homologues. The VCGs of races 1 and 2 were distinguishable from the VCGs of race 4 by both the presence/absence of several *SIX* genes, such as *SIX8*, as well as differences in the *Foc-SIX* gene homologues they carried. The VCGs 01212, 01221, 01222 and 01223 (all of an undetermined race) exhibited *Foc-SIX* gene profiles that were most similar to VCGs known to be races 1 and 2 (Table 3).

In contrast with the conserved combination of *Foc-SIX* genes identified in the VCGs of races 1 and 2, those *Foc* VCGs that were assigned to race 4 exhibited a more variable *Foc-SIX* gene profile. Despite their shared host range, the VCGs of STR4 (VCGs 0120, 0126, 0129, 01211 and 01215), VCG 0121, VCG 0122 and TR4 (VCG 01213, 01213/16, 01216) were each shown to carry a unique combination of *Foc-SIX* genes.

Of the seven *SIX* genes detected in the VCGs of *Foc*, only *SIX1* and *SIX9* were identified in all isolates of *Foc*. Interestingly, these two genes demonstrated different degrees of genetic diversity; nine distinct *Foc-SIX1* homologues were identified across the VCGs of *Foc*, whereas a single identical *SIX9a* homologue was identified in all isolates of *Foc* (Table 3).

It was also shown that the other *formae speciales* screened in this study did not share the same combination of *SIX* genes as any of the *Foc* VCGs (Table 4), nor were any of the sequences identified in other *formae speciales* identical to the *Foc-SIX* gene homologues. Of particular interest was the lack of any *SIX* genes in any of the isolates of *F. oxysporum* isolated from asymptomatic banana plants (Table 4).

Discordance between the evolutionary history of the *Foc-SIX* genes and housekeeping genes

In order to determine whether the evolutionary history of *SIX* genes in *Foc* was consistent with the infraspecies evolution of *Foc*, we initially established the infraspecies relationships of the *F. oxysporum* isolates in this study for which whole-genome sequence information was available. A combined gene dataset comprising the translation elongation factor (*EF-1α*) and two of

Table 2 *Fusarium oxysporum* isolates used in this study, obtained as monoconidial cultures from the Department of Agriculture and Fisheries, Queensland Government, Australia; the Department of Primary Industry and Fisheries, Northern Territory Government, Australia; and the Agricultural Research Service Culture Collection, US Department of Agriculture.

Forma specialis	VCG*	Accession†	Host	Geographical origin
cubense	0120	44012	*Musa* AAA 'Cavendish'	Australia
cubense	0120	40182	*Musa* AAA 'Cavendish'	Australia
cubense	0120	58620	*Musa* sp. (unidentified)	Indonesia
cubense	0120	59164	*Musa* AAA 'Gros Michel'	South Africa
cubense	0120	58614	*Musa* sp. (unidentified)	Canary Islands
cubense	0121	62962	*Musa* AA 'Sucrier'	Taiwan
cubense	0121	62969	*Musa* AAA 'Cavendish'	Taiwan
cubense	0121	59104	*Musa* sp. (unidentified)	Indonesia
cubense	0121	58741	*Musa* AAA 'Cavendish'	Malaysia
cubense	0121	59084	*Musa* AAA 'Gros Michel'	Indonesia
cubense	0122	62892	*Musa* AAA 'Cavendish'	Philippines
cubense	0122	62901	*Musa* AA 'Pisang Lilin'	Philippines
cubense	0122	62894	*Musa* AAA 'Cavendish'	Philippines
cubense	0122	59154	*Musa* AAA 'Cavendish'	Philippines
cubense	0122	62893	*Musa* AAA 'Cavendish'	Philippines
cubense	0123	62895	*Musa* AAB 'Latundan'	Philippines
cubense	0123	59051	*Musa* sp. (unidentified)	Indonesia
cubense	0123	58737	*Musa* sp. (unidentified)	Malaysia
cubense	0123	58807	*Musa* sp. (unidentified)	Thailand
cubense	0123	58780	*Musa* AAB 'Latundan'	Philippines
cubense	0124	62933	*Musa* sp. (unidentified)	Honduras
cubense	0124	43997	*Musa* AAB 'Lady Finger'	Australia
cubense	0124	62911	*Musa* sp. (unidentified)	India
cubense	0124	58802	*Musa* sp. (unidentified)	Thailand
cubense	0124	62953	*Musa* ABB 'Bluggoe'	Brazil
cubense	0125	62957	*Musa* sp. (unidentified)	India
cubense	0125	44010	*Musa* AAB 'Lady Finger'	Australia
cubense	0125	62952	*Musa* ABB 'Bluggoe'	Brazil
cubense	0125	58692	*Musa* ABB 'Pisang Awak'	Malaysia
cubense	0125	58788	*Musa* sp. (unidentified)	Thailand
cubense	0126	59161	*Musa* sp. (unidentified)	Papua New Guinea
cubense	0126	59152	*Musa* AAB 'Latundan'	Philippines
cubense	0126	59046	*Musa* sp. (unidentified)	Indonesia
cubense	0126	58639	*Musa* sp. (unidentified)	Indonesia
cubense	0126	59060	*Musa* sp. (unidentified)	Indonesia
cubense	0128	22887	*Musa* ABB 'Bluggoe'	Australia
cubense	0128	44013	*Musa* ABB 'Bluggoe'	Australia
cubense	0128	44479	*Musa* sp. (unidentified)	Australia
cubense	0128	44616	*Musa* sp. (unidentified)	Australia
cubense	0128	44016	*Musa* ABB 'Bluggoe'	Australia
cubense	0129	40255	*Musa* AAB 'Lady Finger'	Australia
cubense	0129	42186	*Musa* AAB 'Lady Finger'	Australia
cubense	0129	63615	*Musa* AAB 'Lady Finger'	Australia
cubense	0129	44466	*Musa* AAB 'Lady Finger'	Australia
cubense	0129	63330	*Musa* sp. (unidentified)	Australia
cubense	01211	44073	*Musa* sp. (unidentified)	Australia
cubense	01211	39259	*Musa* AAB 'Lady Finger'	Australia
cubense	01210	26029‡	*Musa* AAB 'Silk'	USA
cubense	01212	62955	*Musa* sp. (unidentified)	India
cubense	01212	59037	*Musa* sp. (unidentified)	India
cubense	01213	40340	*Musa* AAA 'Cavendish'	Australia
cubense	01213	58734	*Musa* AAA 'Cavendish'	Malaysia
cubense	01213	58651	*Musa* AAA 'Cavendish'	Indonesia
cubense	01213	62560	*Musa* AAA 'Cavendish'	Indonesia
cubense	01214	25609‡	*Musa* ABB 'Harare'	Malawi
cubense	01214	36113‡	*Musa* ABB 'Bluggoe'	Malawi
cubense	01215	36112‡	*Musa* AAA 'Cavendish'	South Africa

Table 2 *Continued*

Forma specialis	VCG*	Accession†	Host	Geographical origin
cubense	01216	62779	*Musa* AAA 'Cavendish'	Indonesia
cubense	01216	59049	*Musa* AAA 'Cavendish'	Indonesia
cubense	01216	58697	*Musa* AAA 'Cavendish'	Malaysia
cubense	01216	58725	*Musa* AAA 'Cavendish'	Malaysia
cubense	01216	58746	*Musa* AAA 'Cavendish'	Malaysia
cubense	01217	58698	*Musa* sp. (unidentified)	Malaysia
cubense	01217	59147	*Musa* sp. (unidentified)	Malaysia
cubense	01217	58681	*Musa* sp. (unidentified)	Malaysia
cubense	01217	58683	*Musa* sp. (unidentified)	Malaysia
cubense	01217	58723	*Musa* sp. (unidentified)	Malaysia
cubense	01218	63259	*Musa* sp. (unidentified)	Indonesia
cubense	01218	58645	*Musa* sp. (unidentified)	Indonesia
cubense	01218	58700	*Musa* sp. (unidentified)	Malaysia
cubense	01218	58619	*Musa* sp. (unidentified)	Indonesia
cubense	01218	59041	*Musa* sp. (unidentified)	Indonesia
cubense	01219	58634	*Musa* sp. (unidentified)	Indonesia
cubense	01219	63261	*Musa* sp. (unidentified)	Indonesia
cubense	01219	58624	*Musa* sp. (unidentified)	Indonesia
cubense	01219	59115	*Musa* BB 'Pisang Kepok'	Indonesia
cubense	01219	58636	*Musa* sp. (unidentified)	Indonesia
cubense	01220	58803	*Musa* sp. (unidentified)	Thailand
cubense	01220	42103	*Musa* AAA 'Cavendish'	Australia
cubense	01221	36118†	*Musa* ABB 'Pisang Awak'	Thailand
cubense	01222	59170	*Musa* sp. (unidentified)	Uganda
cubense	01223	36116‡	*Musa* AAB 'Mysore'	Malaysia
cubense	0120/15	59028	*Musa* AAA 'Cavendish'	Canary Islands
cubense	0120/15	59052	*Musa* sp. (unidentified)	Indonesia
cubense	0120/15	62942	*Musa* AAA 'Cavendish'	South Africa
cubense	01213/16	59785	*Musa* AAA 'Cavendish'	Australia
cubense	01213/16	58821	*Musa* AAA 'Cavendish'	Malaysia
cubense	01213/16	59130	*Musa* AAA 'Cavendish'	Indonesia
cubense	0124/22	58813	*Musa* sp. (unidentified)	Uganda
cubense	0124/5	62950	*Musa* sp. (unidentified)	China
cubense	0124/5	58774	*Musa* AAA 'Gros Michel'	Mexico
cubense	0124/5	44080	*Musa* AAB 'Lady Finger'	Australia
fragariae	N/A	53860	*Fragaria* × *ananassa*	Australia
medicaginis	N/A	5189	*Medicago sativa*	Australia
niveum	N/A	36955§	*Citrullus* sp.	Australia
passiflorae	N/A	28044	*Passiflora edulis*	Australia
zingiberi	N/A	39299	*Zingiber officinale*	Australia
Non-path¶	N/A	29093	*Musa* sp. (unidentified)	Australia
Non-path¶	N/A	29094	*Musa* sp. (unidentified)	Australia
Non-path¶	N/A	45952	*Musa* sp. (unidentified)	Australia

*VCG, vegetative compatibility group.

†BRIP accession code assigned by the Department of Agriculture and Fisheries, Queensland Government, Australia unless otherwise indicated.

‡NRRL accession code assigned by the Agricultural Research Service Culture Collection, US Department of Agriculture.

§Accession code assigned by the Department of Primary Industry and Fisheries, Northern Territory Government, Australia.

¶Putative non-pathogen of banana (Forsyth *et al.*, 2006).

the large RNA polymerase II subunits (*RPB1* and *RPB2*) was employed to generate phylogenetic trees using maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP) as tree building methods. The resulting trees produced by the methods all recovered very similar topologies with similar clade support. The VCGs of *Foc* demonstrated polyphyletic relationships across the phylogenetic tree (Fig. 1). The VCGs of races 1 and 2, the VCGs 01212, 01221, 01222 and 01223 of *Foc* (all of undetermined race) as well as all other isolates of *F. oxysporum* were placed in clade A of the phylogenetic tree. The VCGs of TR4, STR4, VCG 01219 (undetermined race) and VCG 01210 were placed in clade B (Fig. 1). The placement of VCG 01210 with the VCGs of race 4, rather than the VCGs of race 1/race 2, was particularly intriguing, given that the *SIX* genes identified in VCG 01210

Table 3 Homologues of *Secreted In Xylem* (*SIX*) genes detected in isolates of *Fusarium oxysporum* f. sp. *cubense*. Single-letter code indicates the sequence variant of a *Foc-SIX* gene homologue detected in an associated isolate. A '–' has been used to denote the absence of a *SIX* gene in the corresponding isolate.

			SIX gene													
Race	VCG*	Accession†	1	2	3	4**	5	6	7	8	9	10	11	12	13	14
1	0123	62895	d, f§	–	–	b	–	b	–	–	a	–	–	–	a	–
1	0123	59051	f	–		b		b	–	–	a	–			a	
1	0123	58737	d, f	–		b		b	–	–	a	–			a	
1	0123	58807	d, f	–		b		b	–	–	a	–			a	
1	01210	26029‡	d, f	b¶	–	b	–	b	–	–	a	–	–	–	a	–
1	01217	58698	d, f	–	–	b	–	b	–	–	a	–	–	–	a	–
1	01217	59147	d, f	–	–	b	–	b	–	–	a	–	–	–	a	–
1	01217	58681	d	–		b		b	–	–	a	–			a	
1	01217	58683	d, f	–		b		b	–	–	a	–			d	
1	01217	58723	d, f	–		b		b	–	–	a	–			–	
1	01218	63259	d, f	–	–	b	–	b	–	–	a, b	–	–	–	a	–
1	01218	58645	d, f	–		b		b	–	–	a, b	–			a	
1	01218	58700	d, f	–		b		b	–	–	a, b	–			–	
1	01218	58619	d, f	–		b		b	–	–	a, b	–			a	
1	01218	59041	d, f	–		b		b	–		a, b	–			a	
1	Unknown	N2†† ‡‡	d, f	–	–	b	–	b	–	–	a	–	–	–	a	–
1, 2	0124	62933	f	–	–	b	–	b	–	–	a	–	–	–	a	–
1, 2	0124	43997	f	–		b		b	–	–	a	–			a	
1, 2	0124	62911	d, f	–		b		b	–	–	a	–			a, b	
1, 2	0124	58802	d, f	–		b		b	–	–	a	–			a	
1, 2	0124	62953	f	–		b		b	–	–	a	–			a	
1, 2	0124/5	62950	d, f	–		b		b	–	–	a	–			a	
1, 2	0124/5	44080	d, f	–		b		b	–	–	a	–			a	
1, 2	0124/22	58813	d, f	–		b		b	–	–	a	–			a	
1, 2	0125	62957	f	–	–	b	–	b	–	–	a	–	–	–	a	–
1, 2	0125	44010	f	–		b		b	–	–	a	–			a	
1, 2	0125	62952	d, f	–		b		b	–	–	a	–			a	
1, 2	0125	58692	d, f	–		b		b	–	–	a	–			a	
1, 2	0125	58788	d, f	–		b		b	–	–	a	–			a	
1, 2	0128	22887	f	–	–	b	–	b	–	–	a	–	–	–	–	–
1, 2	0128	44013	f	–		b		b	–	–	a	–			–	
1, 2	0128	44479	f	–		b		b	–	–	a	–			–	
1, 2	0128	44616	f	–		b		b	–	–	a	–			–	
1, 2	0128	44016	f	–		b		b	–	–	a	–			–	
1, 2	01220	58803	d, f	–	–	b	–	b	–	–	a	–	–	–	a	–
1, 2	01220	42103	f	–		b		b	–	–	a	–			a	
2	01214	25609‡	f	–	–	–	–	–	–	–	a, c	–	–	–	a	–
2	01214	36113‡	f	–	–	–	–	–	–	–	a, c	–	–	–	a	–
STR4	0120	44012	g	d	–	a	–	–	a	a3, b	a	–	–	–	–	–
STR4	0120	40182	g	d		a		–	a	a3, b	a	–			–	
STR4	0120	58620	g	d		a		–	a	a3, b	a	–			–	
STR4	0120	59164	g	d		a		–	a	a3, b	a	–			–	
STR4	0120	58614	g	d		a		–	a	a3, b	a	–			–	
STR4	0120/15	59028	g	d		a		–	a	a3, b	a	–			–	
STR4	0120/15	62942	g	d		a		–	a	a3, b	a	–			–	
STR4	0126	59161	g	c	–	a	–	–	a	a3, b	a	–	–	–	–	–
STR4	0126	59152	g	c		a		–	a	a3, b	a	–			–	
STR4	0126	59046	g	c		a		–	a	a3, b	a	–			–	
STR4	0126	58639	g	c		a		–	a	a3, b	a	–			–	
STR4	0126	59060	g	c		a		–	a	a3, b	a	–			–	
STR4	0129	40255	g	c	–	a	–	–	a	a3, b	a	–	–	–	–	–
STR4	0129	42186	g	c	–	a	–	–	a	a3, b	a	–	–	–	–	–
STR4	0129	63615	g	c		a		–	a	a3, b	a	–			–	
STR4	0129	44466	g	c		a		–	a	a3, b	a	–			–	
STR4	0129	63330	g	c		a		–	a	a3, b	a	–			–	
STR4	01211	39259	g	c	–	a	–	–	a	a3, b	a	–	–	–	–	–

Table 3 *Continued*

			SIX gene													
Race	VCG*	Accession†	1	2	3	4**	5	6	7	8	9	10	11	12	13	14
STR4	01211	44073	g	c		a		–	a	a3, b	a	–			–	
STR4	01215	36112‡	g	d	–	c	–	–	a	a3, b	a	–	–	–	–	–
TR4	01213	40340	a, h, i	a	–	c	–	a	–	a1, a2	a	–	–	–	a, e	–
TR4	01213	62560	a, h, i	a	–	c	–	a	–	a1, a2	a	–	–	–	a, e	–
TR4	01213	58734	a, h, i	a		c		a	–	a1, a2	a	–			a, e	
TR4	01213	58651	a, h, i	a		c		a	–	a1, a2	a	–			a, e	
TR4	01213/16	59785	a, h, i	a		c		a	–	a1, a2	a	–			a, e	
TR4	01213/16	58821	a, h, i	a		c		a	–	a1, a2	a	–			a, e	
TR4	01213/16	59130	a, h, i	a		c		a	–	a1, a2	a	–			a, e	
TR4	01213/16	54006‡,‡‡	a, h, i	a	–	c	–	a	–	a1	a	–	–	–	a	–
TR4	01216	62779	a, h, i	a	–	c	–	a	–	a1, a2	a	–	–	–	a, e	–
TR4	01216	59049	a, h, i	a		c		a	–	a1, a2	a	–			e	
TR4	01216	58697	a, h, i	a		c		a	–	a1	a	–			a	
TR4	01216	58725	a, h, i	a		c		a	–	a1, a2	a	–			a, e	
TR4	01216	58746	a, h, i	a		c		a	–	a1, a2	a	–			a, e	
TR4	Unknown	B2††,‡‡	a, h, i	a	–	c	–	a	–	a1	a	–	–	–	a, e	–
R4	0121	62962	b, h, i	a	–	c	–	a	b	a1, a2	a	a	–	–	e	–
R4	0121	62969	b, h, i	a	–	c	–	a	b	a1, a2	a	a	–	–	e	–
R4	0121	59104	b, h, i	a		c		a	b	a1, a2	a	a			e	
R4	0121	58741	b, h, i	a		c		a	b	a1, a2	a	a			e	
R4	0121	59084	b, h, i	a		c		a	b	a1, a2	a	a			e	
R4	0122	62892	c, i	–	–	–	–	–	–	a3	a	–	–	–	c	–
R4	0122	62901	c, i	–		–		–	–	a3	a	–			c	
R4	0122	62894	c, i	–		–		–	–	a3	a	–			c	
R4	0122	59154	c, i	–		–		–	–	a3	a	–			c	
R4	0122	62893	c, i	–		–		–	–	a3	a	–			c	
UD	01212	62955	f	–	–	b	–	b	–	–	a	–	–	–	a	–
UD	01212	59037	f	–		b		b	–	–	a	–			a	
UD	01219	58634	g	c	–	a	–	–	a	a3, b	a	–	–	–	–	–
UD	01219	63261	g	c		a		–	a	a3, b	a	–			–	
UD	01219	58624	g	c		a		–	a	a3, b	a	–			–	
UD	01219	59115	g	c		a		–	a	a3, b	a	–			–	
UD	01219	58636	g	c		a		–	a	a3, b	a	–			–	
UD	01221	36118‡	e	–	–	–	–	–	–	–	a	–	–	–	a, d	–
UD	01222	59170	d, f	–	–	–	–	–	–	–	a	–	–	–	a	–
UD	01223	36116‡	d	–	–	b	–	b	–	–	a	–	–	–	a	–

Whole-genome datasets were initially analysed for *Foc-SIX* gene homologues in the accessions that are shaded. Subsequent PCR analysis confirmed the presence and sequence of the *Foc-SIX* gene homologues in all accessions.

*VCG, vegetative compatibility group.

†BRIP accession code assigned by the Department of Agriculture and Fisheries, Queensland Government, Australia unless otherwise indicated.

‡NRRL accession code assigned by the Agricultural Research Service Culture Collection, US Department of Agriculture.

§Coding sequence has been interrupted by a transposon with homology to an NHT-2 retrotransposon (GenBank no. KP213325.1).

¶Predicted pseudogene as a result of a frameshift mutation.

**Predicted pseudogene as a result of a non-function start codon.

††Chinese race 1 (N2) and race 4 isolate (B2) originally described by Guo *et al.* (2014).

‡‡Whole-genome data were downloaded from the National Center for Biotechnology Information (NCBI) for analysis. The *Foc-SIX* gene homologues in these accessions were not confirmed by polymerase chain reaction (PCR).

were almost identical to those of other VCGs of race 1 (Table 3; Fig. 1).

To further investigate the evolution of the *SIX* genes in *Foc*, we generated gene trees for each of the *SIX* genes present in *Foc* (Fig. 2). Each of the phylogenetic methods used in this process generated similar topologies for each of the *SIX* genes, with the exception of *SIX1*. The *SIX* gene sequences identified within the VCGs of *Foc* generally formed close sister clades to one another, although exceptions to this trend were observed in the gene trees of *SIX1*, *SIX9* and *SIX13*. In the *SIX1* gene tree, all tree-building methods generated a tree in which the *Foc-SIX1a–g* homologues formed a monophyletic group, whereas *Foc-SIX1i* was placed in a

Table 4 *Fusarium oxysporum Secreted In Xylem* (*SIX*) gene profile by *forma specialis*.

		SIX gene													
Forma specialis	Accession	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Arabidopsis-infecting (Arabidopsis Fo5176)	5176*	a	–	–	a, b[§]	–	–	–	a	a	–	–	–	–	–
conglutinans (Focg)	54008[†]	a	–	–	a, b[§]	–	–	–	a	–	–	–	–	–	–
fragariae (Fof)	53860*	a, b, c	–	–	–	–	–	–	–	–	–	–	–	a	–
Human-infecting (Fosc 3a)	32931[†]	–	–	–	–	–	–	–	a[¶]	–	–	–	–	–	–
lycopersici (4287) (Fol 4287)	34936[†]	a	a	a	–	a	a	a	a, b	a	a	a	a	a	a
lycopersici (Fol MN25)	54003[†]	a	a	a	–	a	a	a	a	a	a	a	a	a	a
medicaginis (Fomg)	5189*	a, b, c**	–	–	–	–	–	–	a	–	–	–	–	a, b	–
melonis (Fom)	26406[†]	a	–	–	–	–	a	–	–	–	–	a	–	a	–
niveum (Fon)	36955[‡]	a[††]	–	–	a	–	a	–	a, b	a	–	a	–	a, b	–
passiflorae (Fopf)	28044*	–	–	–	–	–	a	–	a	a, b[‡‡]	–	a	–	–	–
pisi (Fop)	37622[†]	a, b	–	–	–	–	–	–	–	–	–	–	–	a	a
radicis-lycopersici (Forl)	26381[†]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
raphani (For)	54005[†]	–	–	–	–	–	–	–	a	a, b, c, d	–	–	–	–	–
Soil biocontrol (Fo 47)	54002[†]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
vasinfectum (Fov)	25433[†]	–	–	–	–	–	–	–	–	a, b	–	–	–	a	–
zingiberi (Foz)	39299*	–	–	–	–	–	–	a	–	a	a		a		–
Non-pathogen (Fo 29093)	29093*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Non-pathogen (Fo 29094)	29094*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Non-pathogen (Fo 45952)	45952*	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Single-letter code indicates the *SIX* gene homologue(s) detected in a particular isolate. *SIX* gene homologue designations correspond to the homologues identified within an individual *forma specialis*. A '–' has been used to denote the absence of a *SIX* gene in the corresponding isolate. Abbreviations of the isolates are shown in parentheses.

*Accession codes are BRIP codes assigned by the Department of Agriculture and Fisheries, Queensland Government, Australia.

[†]Accession codes are NRRL codes assigned by the Agricultural Research Service Culture Collection, US Department of Agriculture.

[‡]Accession code assigned by the Department of Primary Industry and Fisheries, Northern Government, Australia.

[§]Pseudogene as a result of an incomplete coding sequence.

[¶]Incomplete coding sequence. Gene located at the end of a contig and no overlapping contigs could be found.

**Incomplete coding sequence.

[††]Incomplete coding sequence interrupted by a sequence with homology to a long terminal repeat (LTR) retrotransposon.

[‡‡]Incomplete coding sequence.

separate sister lineage with the *SIX1* homologues of some other *formae speciales*, most notably the *SIX1* sequences identified in *F. oxysporum* f. sp. *fragariae* (Fig. 2a). The best tree recovered from the ML search method placed the *Foc-SIX1h* homologue in close association with *SIX1* homologues identified in other *formae speciales* (Fig. 2a). However, the best trees recovered using MP and BI methods recovered a tree that placed *Foc-SIX1h* with the *Foc-SIX1a–g* homologues. The placement of the *Foc-SIX1h* clade remains unresolved, as all three phylogenetic methods did not recover high support values for the placement of the *Foc-SIX1h* clade. The *Foc-SIX9* sequences also demonstrated very close relationships; however, a *SIX9* sequence identified in *raphanin* was shown to be highly similar to *Foc-SIX9a* and was found to be clustered in the same clade (Fig. 2f). The *Foc-SIX13a* and *Foc-SIX13b* homologues formed a monophyletic group, whereas *Foc-SIX13d* and *Foc-SIX13e* clustered into a distinct clade with the *SIX13* homologues identified in other *formae speciales* (Fig. 2h). The polyphyletic distribution of the VCGs of *Foc*, as determined by housekeeping genes, contrasted strongly with the topologies of the *SIX* gene trees, in which most of the *Foc-SIX* gene homologues formed close sister clades to one another.

Evidence for the horizontal inheritance of *SIX* genes in the VCGs of *Foc*

As a result of the discordance between the infraspecies phylogeny and the genealogies of the *SIX* genes, we further investigated the evolutionary history of the *SIX* genes in *Foc* through a series of topological constraint tests. The incongruence between the infraspecies phylogeny and the *SIX* genes primarily occurred for the datasets in which *Foc-SIX* gene homologues were identified in isolates occurring in both clade A and clade B of the infraspecies phylogeny. Therefore, topological constraint analyses were conducted only with the *SIX* genes identified in the VCGs of *Foc* from both clade A and clade B of the infraspecies phylogeny. To model vertical inheritance of the *SIX* genes in *Foc*, an 'infraspecies constraint' was implemented to force all taxa in the included *SIX* gene datasets to resemble the backbone of the infraspecies phylogeny, as determined previously using the combined *EF-1α/RPB1/RPB2* (Fig. 3a). With the exception of *SIX9*, all infraspecies constrained trees were found to have significantly worse tree scores when compared with the unconstrained gene trees ($P < 0.05$) (Table 5). Thus, this constraint analysis rejected the hypothesis of vertical inheritance of

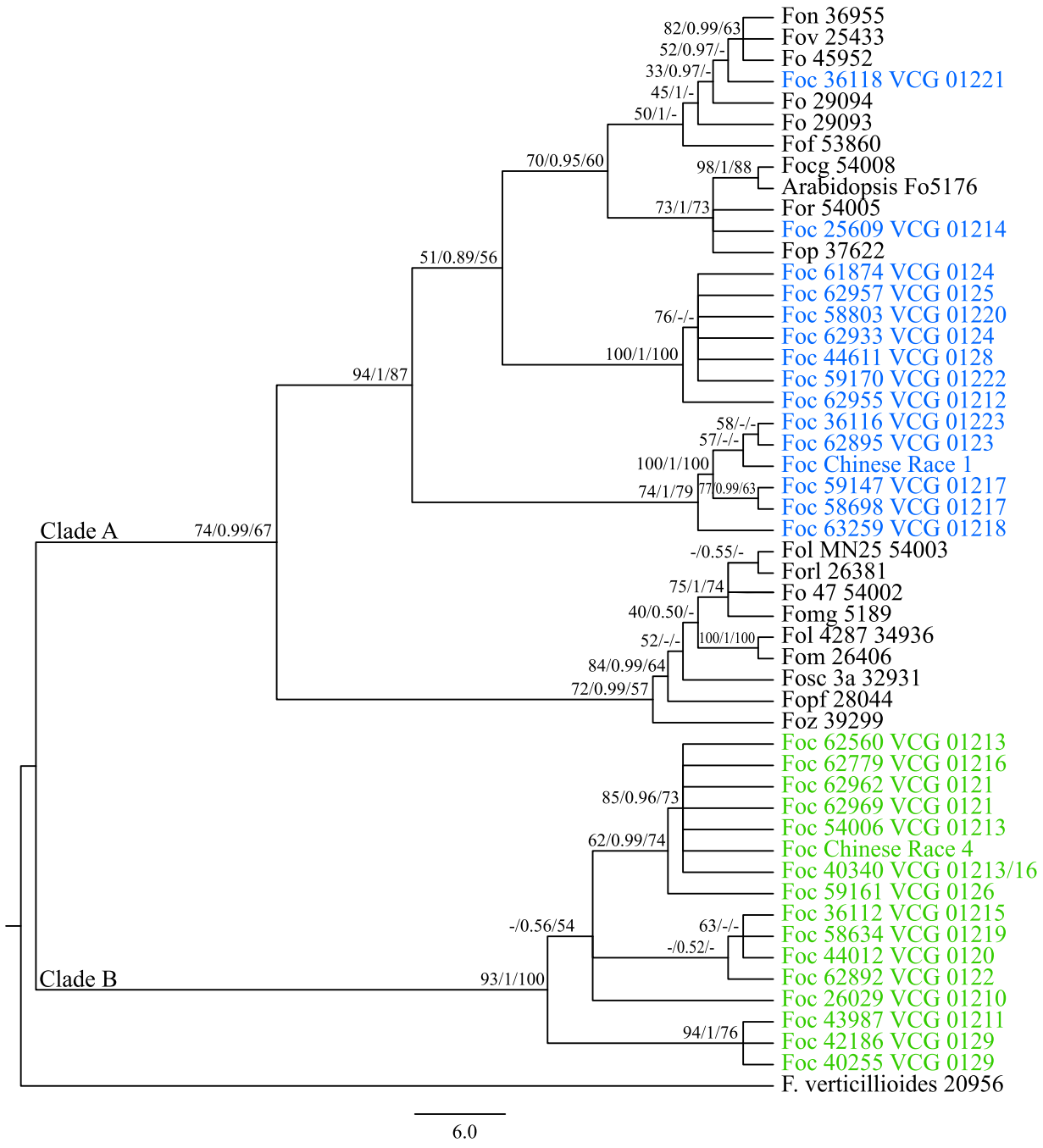

Fig. 1 Best infraspecies phylogenetic tree inferred using maximum likelihood (ML) from the concatenated datasets of translation elongation factor-1α (*EF-1α*), RNA polymerase II subunit I (*RPB1*) and RNA polymerase II subunit II (*RPB2*) identified in isolates of *Fusarium oxysporum* used in this study. Trees with similar topologies were also inferred using maximum parsimony (MP) and Bayesian interference (BI) methods. Internal node support is indicated as ML bootstrap proportions/Bayesian probabilities/MP bootstrap proportions. The two major clades are as indicated. For each isolate, the abbreviated *forma specialis* and accession code as defined in Tables 3 and 4 are indicated. The isolates of *F. oxysporum* f. sp. *cubense* (*Foc*) are also labelled with their respective vegetative compatibility group (VCG). The isolates of *Foc* in clade A are shown in blue, whereas the isolates of *Foc* in clade B are shown in green. The other *formae speciales* and *F. verticillioides* NRRL 20956 are shown in black. Nucleotide sequences from *F. verticillioides* served as an outgroup to root the tree.

SIX1, *SIX6* and *SIX13* in *Foc*, whereas, surprisingly, the constraint analysis did not find a significant difference between the constrained and unconstrained trees for *SIX9*. A closer inspection of the best-performing ML tree produced for *SIX9* under the infraspecies constraint revealed that the gene tree had placed the *SIX9* sequences identified in TR4, STR4, VCG 0126, VCG 01210 and

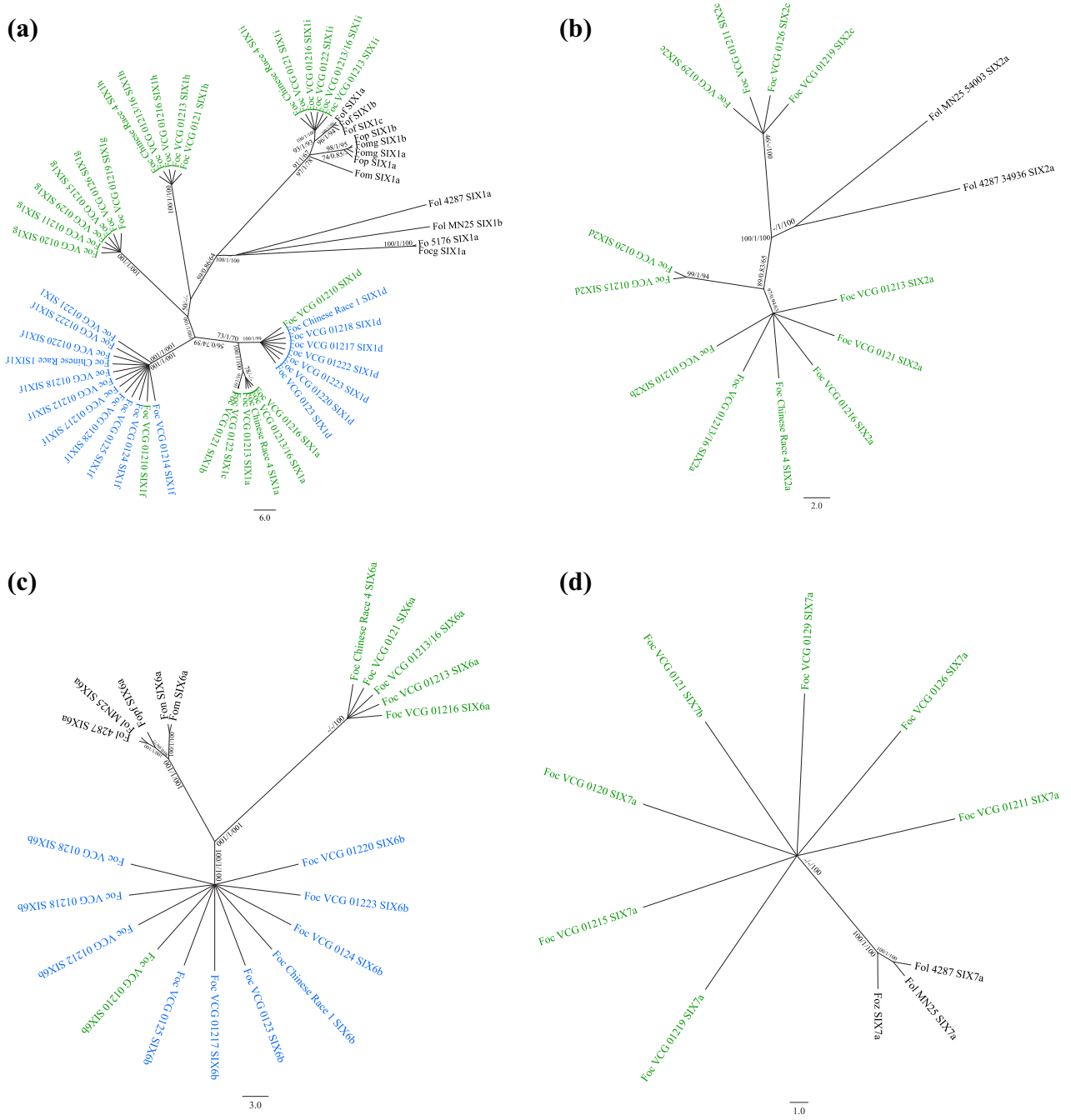

Fig. 2 Best genealogies of the *Secreted In Xylem* (*SIX*) genes *SIX1* (a), *SIX2* (b), *SIX6* (c), *SIX7* (d), *SIX8* (e), *SIX9* (f), *SIX10* (g) and *SIX13* (h) generated using the maximum likelihood (ML) method. Trees with similar topologies were also inferred using maximum parsimony (MP) and Bayesian interference (BI) methods. Internal node support is indicated as ML bootstrap proportions/Bayesian probabilities/MP bootstrap proportions. For each external node, the abbreviated *forma specialis* as defined in Tables 3 and 4 in which the sequence was identified and the *SIX* gene homologue variant are indicated. For *Fusarium oxysporum* f. sp. *cubense* (*Foc*), the vegetative compatibility group (VCG) in which the *SIX* gene homologue was identified is also indicated. The VCGs of *Foc* that clustered in clade A of the infraspecies phylogeny are shown in blue, whereas the VCGs of *Foc* that clustered in clade B of the infraspecies phylogeny are shown in green. The sequences from other *formae speciales* are shown in black.

VCG 01219 into a monophyletic ingroup to the other *SIX9* sequences identified in *Foc*. Therefore, even under the infraspecies constraint, the best-performing tree produced placed the *SIX9* sequences of *Foc* into a close, monophyletic relationship, resulting in a constrained gene tree with a topology that was not significantly different from the unconstrained gene tree of *SIX9* (Table 5).

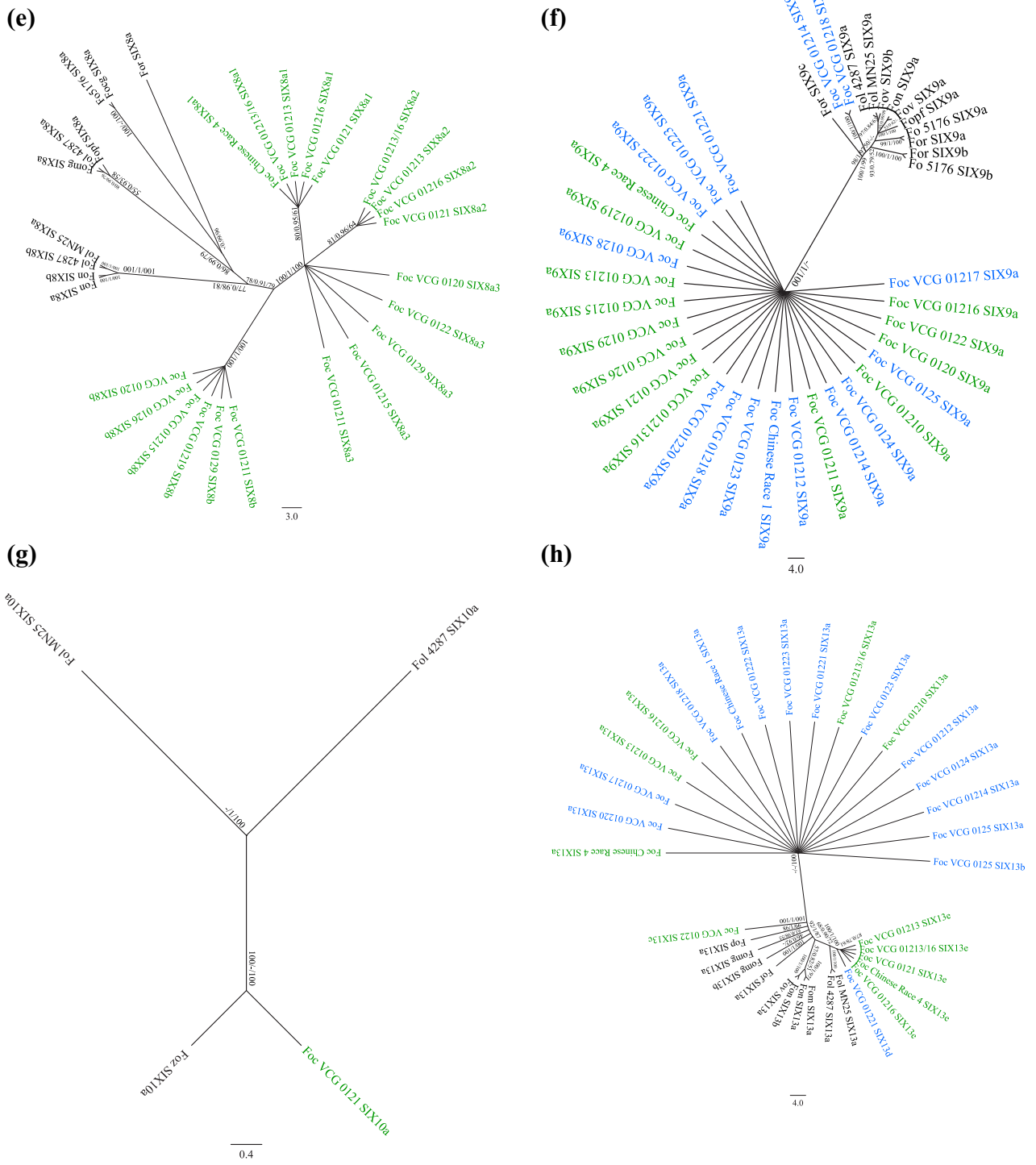

Fig. 2 (Continued).

To model horizontal inheritance of the *SIX* genes in *Foc*, a 'strict *Foc-SIX* monophyly constraint' forced the homologues of all *Foc-SIX* sequences into a monophyletic clade, whereas the *SIX* homologues from other *formae speciales* formed a monophyletic sister clade (Fig. 3b). Under the strict clade constraint, the tree score for *SIX6* was not significantly different from the tree scores of the respective unconstrained trees ($P > 0.05$) (Table 5). However, the tree scores of *SIX1*, *SIX9* and *SIX13* were significantly

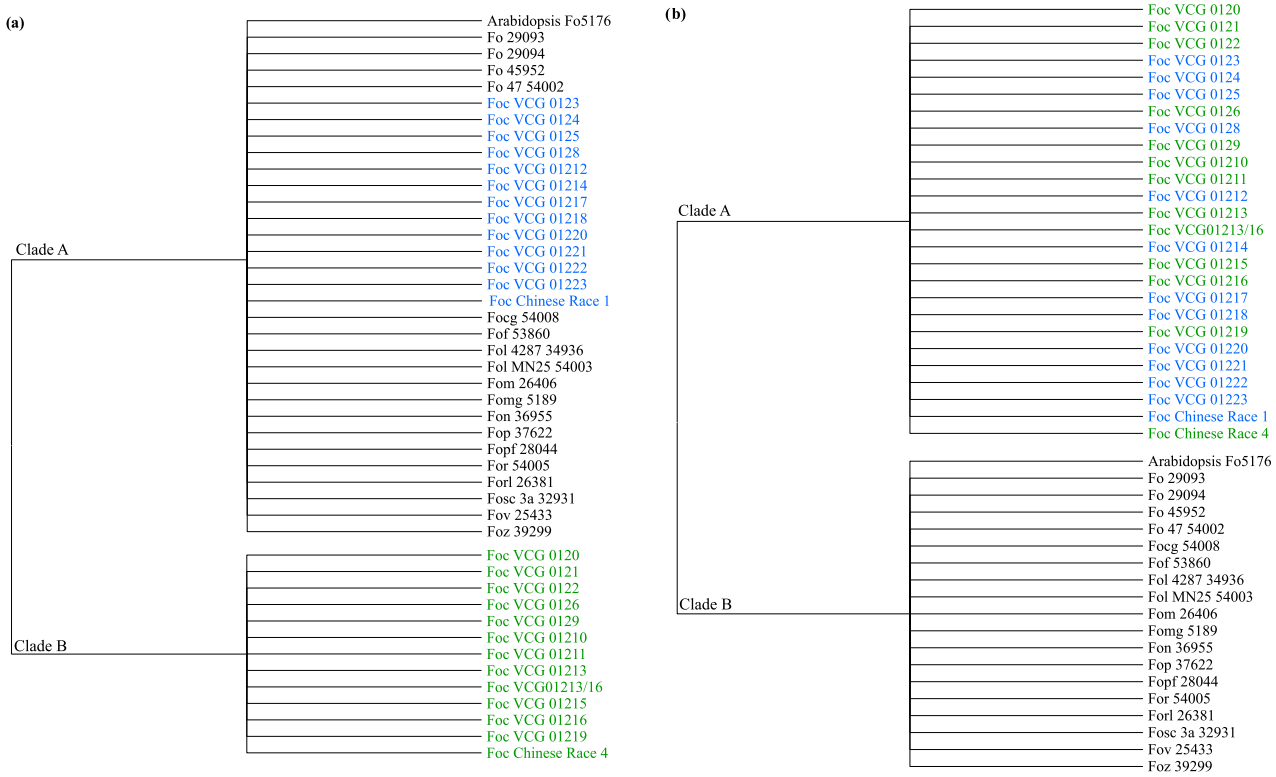

Fig. 3 A visual representation of the models implemented for the topological constraint testing of the *Secreted In Xylem* (*SIX*) gene trees using an infraspecies backbone constraint model (a) and a *Fusarium oxysporum* f. sp. *cubense* (*Foc*) monophyletic constraint model (b). The infraspecies model constrained the *SIX* gene sequences identified in the vegetative compatibility groups (VCGs) of *Foc* and other *formae speciales* into the two major clades identified in the infraspecies phylogeny (Fig. 1). For each isolate, the abbreviated *forma specialis* and accession code as defined in Tables 3 and 4 are indicated. Under the *Foc* monophyletic model, the *SIX* gene sequences identified in the VCGs of *Foc* were constrained into a monophyletic clade, whereas the *SIX* gene sequences identified in other *formae speciales* were constrained into a reciprocating sister clade.

worse than their respective unconstrained trees ($P < 0.05$). The unconstrained trees of *SIX1*, *SIX9* and *SIX13* demonstrated that a subset of the *Foc-SIX* gene homologues formed well-supported ingroups with *SIX* homologues from other *formae speciales* (Fig. 2a,f,h). Specifically, *Foc-SIX1i* was shown to cluster with the sequences of *Fof-SIX1*, whereas *Foc-SIX13d* and *Foc-SIX13e* clustered with *SIX13* homologues identified in *Fof*, *Fol*, *Fom*, *Fomg*, *Fon*, *Fop* and *Fov*. Furthermore, the unconstrained tree of *SIX9* showed that *Foc-SIX9a* was more similar to the *For-SIX9c* homologue than it was to *Foc-SIX9b* and *Foc-SIX9c*. We hypothesized that the strict monophyly clade constraint imposed on the *SIX1*, *SIX9* and *SIX13* gene trees significantly affected the tree scores as a result of the placement of *Foc-SIX1i*, *For-SIX9c*, *Foc-SIX13c*, *Foc-SIX13d* and *Foc-SIX13e*, respectively. We tested this hypothesis through a 'relaxed *Foc-SIX* monophyly constraint' in which most of the *Foc-SIX* homologues and the *SIX* homologues from other *formae speciales* were again placed into reciprocating sister polytomies. However, the relaxed *Foc-SIX* clade constraint permitted the *Foc-SIX1i*, *For-SIX9c*, *Foc-SIX13d* and *Foc-SIX13e* homologues to freely sort across the constrained tree topology. Under the relaxed clade constraint, the tree scores of *SIX1*, *SIX9* and *SIX13* were not significantly different from the respective unconstrained gene trees ($P > 0.05$) (Table 5). The *Foc-SIX* clade constraint analyses of the *Foc-SIX* genes support the hypothesis of horizontal gene transfer of the *SIX* genes in *Foc*. Our results indicate that the role of horizontal gene transfer in the evolution of the *SIX* genes in *Foc* is complicated and dynamic.

DISCUSSION

In the *Fol*–tomato pathosystem, changes in the cultivar-specific pathogenicity of *Fol* are associated with mutations in the *SIX* genes that are recognized by host resistance genes (Houterman *et al.*, 2008, 2009; Rep *et al.*, 2004). These mutations in the corresponding *SIX* genes lead to a loss of recognition by the host and thus a gain of virulence phenotype in *Fol*. Consequently, the hypothesis of the current study was that the differences in pathogenicity of the races of *Foc* to banana cultivars may be reflected in the presence of effectors carried by each race. We have demonstrated that, within the *forma specialis* of *Foc*, there is variation in the distribution and diversity of the *SIX* genes.

Table 5 Results of topological constraint testing and tree score analysis using the maximum likelihood criterion.

Constraint analysis	Difference in PSLL scores	AU	SH
Species constraint			
SIX1	240.5	<0.01*	<0.01*
SIX6	14.3	0.05*	0.01*
SIX9	0.1	0.231	0.222
SIX13	207.3	<0.01*	<0.01*
Strict monophyly constraint			
SIX1	41.8	<0.01*	<0.01*
SIX6	0.1	0.194	0.173
SIX9	13.9	<0.05*	<0.05*
SIX13	47.5	<0.01*	<0.01*
Relaxed monophyly constraint			
SIX1	0.1	0.313	0.302
SIX9	0.1	0.241	0.216
SIX13	0.1	0.299	0.294

The *Secreted In Xylem* (*SIX*) gene datasets included in the analysis were constrained according to a species or strict monophyly topological constraint. The resulting tree scores from the best trees were compared for significant differences from the tree score of the unconstrained trees using the Approximately Unbiased (AU) and Shimodaira–Hasegawa (SH) tests. PSLL, per site log likelihood score.

*Significant at $P < 0.05$.

The gene tree analysis revealed that the *SIX* genes in *Foc* have been both vertically and horizontally inherited in the lineages of *Foc*. The VCGs of *Foc* with the same host range, particularly the VCGs of races 1 and 2, share a highly conserved combination of *SIX* genes in demographically diverse lineages. In contrast, the *SIX* gene haplotypes for the VCGs of race 4 are distinct from the VCGs of races 1 and 2. We hypothesize that the highly conserved *SIX* gene haplotypes within a VCG and/or race is a result of the vertical inheritance of the *SIX* genes in these genetic lineages of *Foc*. A particularly interesting case of gene conservation was observed for the pseudogene, *Foc-SIX4*. Despite not being predicted to code a functional protein, *Foc-SIX4* was identified in many lineages of *Foc* and the nucleotide sequence of *SIX4* within the VCGs of *Foc* was highly conserved (percentage sequence similarity). In three of the genome assemblies of *Foc*, *SIX4* and *SIX6* are in close proximity to one another (~500 bp). It is possible that the retention and conservation of *SIX4* in *Foc* are a result of a linkage effect from a functional copy of *SIX6*. Another interesting observation was the identification of the same *Foc-SIX9a* homologue in all VCGs of *Foc* screened in this study. Currently, the role of SIX9 in *F. oxysporum* during the infection of a host has not been investigated. This study makes *SIX9* an excellent candidate for further functional validation.

The gene trees also revealed that several *SIX* genes common to the VCGs of races 1, 2 and 4 shared a common ancestor. Many studies have repeatedly demonstrated that the genes conventionally used to infer the genetic relationships of *Foc* are poorly correlated with the host-specific pathogenicity of this pathogen and, as a result, the genetic lineages of *Foc* exhibit polyphyletic relationships (Baayen *et al.*, 2000; Bentley *et al.*, 1995, 1998; Boehm *et al.*, 1994; Fourie *et al.*, 2009; Koenig *et al.*, 1997; O'Donnell *et al.*, 1998, 2013). It has been hypothesized previously that the polyphyletic phylogeny of *Foc* is a result of the independent evolution of pathogenicity to banana in multiple lineages of *F. oxysporum* (Fourie *et al.*, 2009; O'Donnell *et al.*, 1998). However, we now hypothesize that the horizontal transfer of pathogenicity-determining factors, such as the *SIX* genes, may have driven the polyphyletic evolution of *Foc*. The modelling of different inheritance patterns supported the horizontal acquisition of these genes in the genetically distinct lineages of *Foc*. Currently, we propose that a horizontal transfer event of a subset of *SIX* genes occurred between a race 1 or 2 donor, and the recipient was an ancestor of the race 4 lineages. We also hypothesize that the ancestor of the race 4 lineages either already harboured *SIX* genes prior to this transfer or was perhaps the recipient in further horizontal transfer events of *SIX* genes, and thus acquired the race 4-specific homologues. Evidence to support this hypothesis was observed in the gene trees, which revealed that race 4-specific homologues, such as *Foc-SIX1i* and *Foc-SIX13e*, clustered with *SIX* homologues identified in other *formae speciales*. Alternatively, each of these genes was identified as being multicopy, especially in the TR4 VCG 01213/16. An alternative hypothesis to horizontal gene transfer is that gene duplication has allowed the sequences of these homologues to significantly diverge and produce homoplastic gene trees. Further studies, including additional whole-genome sequencing and assembly, will allow us to further investigate these hypotheses. Further evidence for horizontal gene transfer events with other *formae speciales* was observed for *Foc-SIX9a*, which formed a close sister polytomy to the *For-SIX9c* homologue. We hypothesize that these homologues have been involved in horizontal gene transfer; however, the directionality of the transfer is unknown, i.e. it is unclear whether *Foc* is the recipient or donor of these *SIX* genes. Evidence for the horizontal transfer of *SIX* genes has been reported in several *formae speciales*, including the Australian lineages of the cotton-infecting *F. oxysporum* f. sp. *vasinfectum*, *Arabidopsis*-infecting *Fo5176* isolate and *F. oxysporum* f. sp. *canariensis* [causal agent of wilting in the Canary Island date palm (*Phoenix canariensis*)] (Chakrabarti *et al.*, 2011; Laurence *et al.*, 2015; Thatcher *et al.*, 2012).

The underlying assumption for the hypothesis of horizontal transfer is that *Foc* also possesses small accessory chromosomes conferring pathogenicity that have been involved in horizontal transfer events, in a similar manner to *Fol*. Ma *et al.* (2010) demonstrated that a small accessory chromosome harbouring most of the *SIX* genes could be horizontally transferred from *Fol* to a genetically distinct isolate of *F. oxysporum* through the co-

incubation of the two isolates. This horizontal transfer event conferred a gain-of-pathogenicity towards tomato in the recipient strain. *Fol* is also a polyphyletic *forma specialis* and it is hypothesized that the polyphyletic relationships of the *Fol* isolates have arisen as a result of horizontal chromosome transfer events. Currently, the available genomes for *Foc* are highly fragmented and it is unclear whether the *Foc-SIX* genes reside on one or multiple chromosomes. It is also unclear whether *Foc* carries its own, unique accessory chromosomes as reported for *Fol*. An improved genome assembly for the small accessory chromosomes of *Foc* would greatly improve our understanding of the genomic organization and evolution of this pathogen.

The horizontal transfer of accessory chromosomes carrying pathogenicity genes between the lineages of *Foc* has important implications for the biosecurity and disease management of Fusarium wilt in bananas. The recent detection of TR4 (VCG 01213/16) in Africa, Australia, Jordan, Lebanon and Pakistan has demonstrated the importance of the rapid and reliable identification of the causal organism during the response to a disease incursion (ABGC, 2015; Garcia-Bastidas *et al.*, 2014; IPPC, 2013; Ordonez *et al.* 2016; Syed *et al.*, 2015). The horizontal movement of chromosomes harbouring the genes conferring the host- and cultivar-specific pathogenicity of *Foc* could produce novel pathotypes of *Foc* that would not be identified with a conventional molecular diagnostic based on vertically inherited housekeeping genes. As a consequence, the molecular identification of *Foc* should include genes that can be inherited vertically through asexual reproduction, as well as genes inherited through horizontal chromosome transfer events, such as the *SIX* genes.

Currently, it is not known whether the *Foc* SIX proteins also facilitate the infection of banana plants as has been demonstrated in the tomato and *Arabidopsis* pathosystems (Gawehns *et al.*, 2014; Houterman *et al.*, 2008, 2009; Ma *et al.*, 2015; Rep *et al.*, 2004, 2005; Thatcher *et al.*, 2012). Further studies, such as gene silencing and complementation, are required to validate the SIX proteins as effectors in the *Foc*–banana pathosystem. Exciting new technology, such as genome editing with the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with Cas9 system, offers a new approach for genetic knock-outs and the incredibly accurate manipulation of target genes. Furthermore, our current understanding of effectors in *F. oxysporum* has been dictated by the initial studies completed in the *Fol*–tomato pathosystem. However, if we consider the evolutionary distance separating tomato and banana, and also hypothesize that fungal effector proteins facilitate the host-specific pathogenicity of *F. oxysporum*, it should be expected that novel and undiscovered effectors remain to be identified in the lineages of *Foc*. The ongoing development of innovative solutions to this significant pathogen of banana is contingent on developing an improved understanding of pathogen evolution and functional molecular biology.

EXPERIMENTAL PROCEDURES

Fusarium isolates and culture

Monoconidial isolates of *F. oxysporum* f. sp. *cubense* (VCGs 0120–01223), *fragariae*, *medicaginis*, *niveum*, *passiflorae* and *zingiberi*, and three putatively non-pathogenic endophytes of banana were analysed in this study (Table 2). Isolates were cultured on half-strength potato dextrose agar Difco (Becton, Dickinson and Co., Sparks, MD, USA) for 7 days at 25 °C, after which the mycelium was harvested and stored at −80 °C. Total cellular DNA was extracted using the BioSprint 15 DNA Plant Kit (Qiagen Pty. Ltd., Hilden, Germany) on a BioSprint 15 workstation (Qiagen Pty. Ltd., Hilden, Germany).

Next-generation sequencing (NGS)

DNA libraries with insert sizes of *c.* 400 bp were created using the Nextera XT DNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The Illumina MiSeq platform was used to generate paired-end, 150-bp sequence reads which were subsequently imported into Geneious v. 6.1.8 (http://www.geneious.com; Kearse *et al.*, 2012) for analysis.

Identification of *SIX* genes in the genomes of *F. oxysporum*

Sequence reads from each isolate were mapped to reference sequences for *SIX1–SIX14* within Geneious v. 6.1.8 (Table S1, see Supporting Information) using the default parameters associated with medium sensitivity mapping. Under these parameters, the mapping was allowed to iteratively search the datasets up to five times. Reads that mapped equally well to multiple sites in the reference were excluded from the mapping analysis. These constructs were manually inspected and used to generate consensus sequences, which were deposited into GenBank under the accessions KX434886–KX435052. When multiple homologues were detected in a single isolate, we manually curated a consensus sequence for each individual homologue, using the single nucleotide polymorphism (SNP) variants and paired reads to distinguish between each homologue. Multiple sequence alignments were then created for each *SIX* gene using the ClustalW interface within Geneious v. 6.1.8. In order to provide context for the diversity of *SIX* genes in *F. oxysporum*, *SIX* gene sequence data from the whole genomes of *F. oxysporum* publically available on GenBank were incorporated into the multiple sequence alignments. The details of the genomes included in the analysis are provided in Table S2.

PCR analysis of *SIX* genes in *Foc*

A larger collection of 89 *Foc* isolates representing all VCGs and including the isolates in the NGS panel were screened for the *Foc-SIX* genes using PCR (Table S3, see Supporting Information). Primers were designed both manually and using Primer3 to amplify each of the *Foc-SIX* genes identified from whole-genome sequencing analysis. The primers and respective thermocycling conditions for *SIX* gene amplification are given in Table S3. PCR amplifications were performed in a Applied Biosystems Thermo Fisher Scientific (Singapore) thermocycler in 25 μL reactions consisting of 12.5 μL of GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 μL of

forward primer (10 μM; Integrated DNA Technologies Inc., Singapore), 1 μL of reverse primer (10 μM; Integrated DNA Technologies, Inc.), 1 μL of template DNA and 9.5 μL of nuclease-free water. The results of the PCR amplifications were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide on a UV transilluminator. Amplicons were purified using the Wizard SV PCR and Gel Clean-Up kit (Promega), and sequenced in the forward and reverse directions using the PCR amplification primers in a single-pass Sanger sequencing reaction by Macrogen (Seoul, South Korea). In the instances in which multiple amplicons were identified from initial sequencing for *SIX1* and could not be selectively amplified using PCR, the amplicons were cloned using the pCR2.1 vector with the TOPO TA kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and transformed into *Escherichia coli* Top10 competent cells (Invitrogen), according to the manufacturer's instructions. The vectors from 10 positive colonies were purified using the PureYield Plasmid Mini Prep kit (Promega) and sequenced using the M13-FP universal primer at Macrogen. The resulting chromatograms were analysed within Geneious v. 6.1.8 and aligned with the consensus sequences generated by the whole-genome sequencing analysis using the ClustalW plugin within Geneious v. 6.1.8.

Phylogenetic analysis

EF-1α, *RPB1* and *RPB2* were selected as phylogenetic loci from which to infer the phylogenetic relationships of the isolates used in this study. For each locus, the sequence reads from each isolate were mapped to a reference sequence from *F. oxysporum* originally identified by O'Donnell *et al.* (1998, 2013) (Table S1). Consensus sequences were generated for each gene and deposited at GenBank. The resulting sequences were employed to generate multiple sequence alignments using the ClustalW interface within Geneious v. 6.1.8 (Biomatters Ltd.). The multiple sequence alignments were manually inspected and gaps were removed prior to phylogenetic analysis. The concatenated and gap-free alignment resulted in a sequence alignment of 4045 base pairs (*EF-1α*, 621 bp; *RPB1*, 1607 bp; *RPB2*, 1817 bp).

The phylogenetic analysis of the concatenated *EF-1α/RPB1/RPB2* dataset was performed using MP, ML and BI methods. For each of the phylogenetic reconstruction methods, *F. verticillioides* was used as an outgroup in the analysis. The MP analysis was conducted within PAUP* v4.0α (Swofford, 2003), using a heuristic search and 1000 random sequence additions with tree bisection–reconnection on the partitioned dataset. Branch support was assessed using 1000 bootstraps. The ML analysis was performed using the software RAxML v8.1 (Stamatakis, 2014). The concatenated alignment was partitioned into the three genes and the ML tree was recovered from 1000 ML searches using the GTR + Γ substitution model for each of the partitioned gene sets. Branch support was determined from 1000 bootstrap replicates. The BI analysis was performed in MrBayes v3.2 (Huelsenbeck and Ronquist, 2001), in which the concatenated alignment was partitioned into the three genes and the GTR + G model of substitution was applied to each gene. Two independent analyses were conducted across four Markov chain Monte Carlo (MCMC) chains for 3 000 000 generations. The trees were sampled every 2000 generations and the first 750 000 trees were used as burn-in. Convergence was inferred when the average split standard deviation reached <0.01 and when the log likelihood scores stabilized. The resulting trees were viewed and edited in the software FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Gene trees were also constructed for the *SIX* genes that were identified in the isolates screened in this study, including *SIX1*, *SIX2*, *SIX6*, *SIX7*, *SIX8*, *SIX9*, *SIX10* and *SIX13*. Nucleotide sequence alignments were generated from the consensus sequences of the *SIX* genes identified in the isolates of *Foc* and other *formae speciales* screened in this study. In addition, *SIX* gene sequence data from the publically available genomes of *F. oxysporum* were included in the analyses to provide context. Nucleotide sequence alignments were performed using ClustalW within Geneious v. 6.1.8 and manually inspected. Gene trees were generated using the methods described above for the *EF-1α/RPB1/RPB2* dataset.

Phylogenetic hypothesis testing

A series of topological constraint tests was conducted to investigate the discordance between the *SIX* gene trees and the infraspecies phylogeny of *F. oxysporum*. The topological constraint analyses were only conducted with the datasets in which *Foc-SIX* gene homologues were identified in isolates from both clades A and B of the infraspecies phylogeny. First, an 'infraspecies constraint' forced the taxa in the included *SIX* gene datasets to resemble the backbone of the understood infraspecies phylogeny of the *F. oxysporum* isolates included in this study. A 'strict *Foc-SIX* gene monophyly constraint' forced the homologues of *Foc-SIX* into a monophyletic clade, whereas the *SIX* homologues from other *formae speciales* were placed in a reciprocating sister polytomy. The 'relaxed monophyly constraint' was very similar to the strict monophyly constraint in that it forced most of the *Foc-SIX* homologues and the *SIX* homologues from other *formae speciales* into monophyletic, reciprocating sister polytomies. However, unlike the strict monophyly constraint, the conflicting *Foc-SIX1i*, *Foc-SIX9c*, *Foc-SIX13d* and *Foc-SIX13e* homologues were allowed to freely sort across the constrained tree topology.

Each of the manually generated constraint trees was used as input in an ML analysis conducted in RAxML with 1000 bootstrap replicates. The resulting best-site log likelihood scores from the ML analyses were compared for significant differences ($P < 0.05$) with those of the unconstrained trees using the Shimodaira–Hasegawa (SH) and Approximately Unbiased (AU) tests in CONSEL (http://www.sigmath.es.osaka-u.ac.jp/shimo-lab/prog/consel/; Shimodaira and Hasegawa, 2001).

AUTHOR CONTRIBUTIONS

E.C., S.F.-S., J.B. and E.A.B.A. planned and designed the research. E.C., S.F.-S., M.Z., W.T.O.N., R.A.M. and L.T.T.T.-N. performed the experiments. E.C. and S.F.-S. analysed the data. E.C. and S.F.-S. prepared the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 The coding DNA sequences (CDSs) of the translation elongation factor (*EF-1α*, 630 bp), RNA polymerase II subunit I (*RPB1*, 1607 bp) and subunit II (*RPB2*, 1847 bp) used to query the *Fusarium oxysporum* datasets analysed in this study for the purpose of constructing phylogenetic relationships.

Table S2 Accession details for the whole-genome sequencing projects for the isolates of *Fusarium oxysporum* analysed in this study.

Table S3 Polymerase chain reaction (PCR) primers and thermocycling conditions for the amplification of *Foc-SIX* gene homologues.