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Phylogeny and origin of *Fusarium oxysporum* f. sp. *vanillae* in Indonesia

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Vanilla stem rot, caused by *Fusarium oxysporum* f. sp. *vanillae* (Fov), is the main constraint to increasing vanilla production in the major vanilla-producing countries, including Indonesia. The current study investigated the origin of Fov in Indonesia using a multigene phylogenetic approach. Nineteen Fov isolates were selected to represent Indonesia, the Comoros, Mexico and Réunion Island. The translation elongation factor 1 alpha gene and the mitochondrial small subunit ribosomal RNA gene phylogenies resolved the Fov isolates into three distinct clades in both phylogenetic species of the *F. oxysporum* species complex, indicating a polyphyletic pattern of evolution. In addition, Fov isolates from Indonesia were also polyphyletic. These results suggest that the vanilla stem rot pathogen in Indonesia has a complex origin. The implications for disease management are discussed.

Keywords: introduction, pathogen evolution, spread, vanilla stem rot, wilt

Introduction

Vanilla stem rot, caused by *Fusarium oxysporum* f. sp. *vanillae* (Fov; Tombe *et al.*, 1994), is the main production constraint in major vanilla-producing countries, including Indonesia (Thomas *et al.*, 2002). The pathogen has been recovered from all production areas in Indonesia (Pinaria *et al.*, 2010), causing devastating losses to smallholder farmers whose livelihood relies heavily on income generated from this high value crop. Management of diseases caused by *F. oxysporum* relies predominantly on host resistance (Fravel *et al.*, 2003), but to date all of the limited *Vanilla planifolia* cultivars available in Indonesia are highly susceptible to this pathogen. Although Fov is prevalent in most production regions worldwide, there is no information on the origins and evolutionary patterns of this pathogen.

No sexual structure has ever been observed in the *F. oxysporum* species complex (FOSC; Booth, 1971). This fungus is hence believed to transmit its whole genome as a unit from one generation to the next, with different parts of the genome sharing the same evolutionary history (Taylor *et al.*, 1999b). Isolates within a *forma specialis* were traditionally predicted to be genetically similar and separate from those with different host specificities. It was therefore thought that gene genealogies could be used to trace the evolution of phenotypic characters such as host specificity within this species complex (O'Donnell *et al.*, 1998; Taylor *et al.*, 1999a). However, O'Donnell *et al.* (1998) revealed that *F. oxysporum* f. sp. *cubense* evolved from multiple independent origins, i.e. the host specificity to banana has a polyphyletic origin. There are now numerous evolutionary studies on pathogenic strains within the FOSC. Although there are examples of monophyletic *formae speciales*, e.g. *lillii*, *tulipae* and *ciceris* (Baayen *et al.*, 2000; Jimenez-Gasco *et al.*, 2002) the majority are not, e.g. *asparagi*, *dianthi*, *gladioli*, *lini* (Baayen *et al.*, 2000), *vasinfectum* (Skovgaard *et al.*, 2001), *canariensis* (Laurence *et al.*, 2015) and *cucumerinum* (Lievens *et al.*, 2007).

Previous Fov population studies in Indonesia revealed the presence of multiple vegetative compatibility groups (VCG; Tombe *et al.*, 1994; Pinaria, 2010) and a number of distinct fingerprinting haplotypes. As VCG analysis can be used to infer evolutionary origin (Elias *et al.*, 1993), these findings suggest that the origin of Fov in Indonesia may be complex, with the possibility of a non-monophyletic pattern of evolution.

The knowledge of whether Fov was introduced into Indonesia and/or evolved locally has practical disease management implications. At present most planting materials in vanilla-producing areas in Indonesia are based on clonal propagation sourced from established farms or nurseries, many of which have varying levels of disease incidence, hence facilitating the spread of the pathogen over large distances. However, these production areas are often geographically disparate island provinces, a scenario in which the control of pathogen spread can potentially be achieved to a great extent.

With an aim of elucidating the origin of Fov in Indonesia, this study was designed to test two hypotheses: (i) that Fov isolates obtained from Indonesia and overseas

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including Mexico, where the host vanilla originated, form a monophyletic evolutionary lineage; and (ii) that the Indonesian Fov isolates form a monophyletic lineage. Evolutionary inferences were made by constructing gene genealogies from partial DNA sequences of the translation elongation factor (*EF-1α*) and the mitochondrial small subunit ribosomal RNA genes (*mtSSU*).

Materials and methods

Fungal isolates

Nineteen Fov isolates, representative of different geographical regions, were used in this study (Table 1). These isolates were isolated from vanilla stems showing typical stem rot symptoms, including water-soaking, chlorosis, internal discolouration and necrosis. The confirmation and identification of isolates were undertaken as described by Burgess *et al.* (1994). The overseas isolates were recovered from diseased stems from the Comoros and La Réunion, kindly provided by Dr Michel Grisoni (CIRAD, La Réunion, France), and from Mexico, supplied by Dr Araceli Perez Silva (Instituto Tecnologico de Tuxtepec, Mexico). Cultures of the 19 isolates are maintained in the culture collection of the Royal Botanic Gardens, Sydney, Australia.

Pathogenicity test

The pathogenicity of the 19 Fov isolates was tested on vanilla stems to rule out the possibility of endophytic and nonpathogenic isolates. The glasshouse tests were conducted with three replications for each isolate. The inoculum was generated by incorporating colonized millet seed into sterile potting mix. A control mix was made by adding 10 mL sterile deionized water only to the millet seed. Details of the pathogenicity test and disease assessment were as previously described by Pinaria *et al.* (2010).

DNA extraction, PCR amplification and DNA sequencing

The Fov cultures were grown for 10 days on potato dextrose agar at 25°C under a combination of fluorescent white and ultraviolet light, and DNA was extracted as described by Pinaria *et al.* (2010).

Two gene regions, the translation *EF-1α* and the *mtSSU*, were sequenced. The *EF-1α* was amplified using primers EF-1 and EF-2 (Carbone & Kohn, 1999). PCR amplification, purification of PCR product and DNA sequencing of the *EF-1α* gene were carried out as described by Pinaria *et al.* (2010).

The *mtSSU* gene region was amplified using primers MS 1 and MS 2 (White *et al.*, 1990). PCR amplification, product purification and DNA sequencing of the *mtSSU* were conducted as per the *EF-1α* gene region. The purified sequencing product was sent to the DNA sequencing facility at the Ramaciotti Centre for gene function analysis at the University of New South Wales, Sydney, Australia.

Phylogenetic analysis

The *EF-1α* and the *mtSSU* sequences were aligned with reference sequences of the FOSC obtained from GenBank using the multiple alignment program CLUSTALW (v. 1.83) plug-in (Thompson *et al.*, 1997) in the software GENEIOUS v. 5.3.6 (Kearse *et al.*, 2012). The alignment was edited manually using GENEIOUS v. 5.3.6 and all polymorphisms were confirmed by re-examining the electropherograms. Twenty-four reference sequences downloaded from GenBank were selected based on previous published phylogenetic trees by Lievien *et al.* (2000) and O'Donnell *et al.* (1998) and are listed in Table 1. Sequences generated in this study were deposited in GenBank with accession numbers also listed in Table 1.

Unweighted parsimony and neighbour-joining analyses were conducted using PAUP v. 4.0b10 (Swofford, 2002) on the individual and combined *EF-1α* and *mtSSU* data sets. Heuristic searches for maximum parsimony trees were conducted with 1000 random addition sequences and tree bisection reconnection branch swapping algorithm. Gaps were treated as missing data. Trees were rooted using the out-group method. Two *Fusarium* spp. (NRRL 22903 and 25184) used by O'Donnell *et al.* (1998) were used as the out-group for rooting gene trees. The partition-homogeneity test (PHT) implemented within PAUP was used to evaluate concordance of the two gene data sets. The consistency index (CI) and retention index (RI) were calculated for each tree to evaluate the amount of homoplasy present. Bootstrap analysis implemented with PAUP was assessed using 1000 random replications for all trees, with bootstrap values of ≥50 indicating clade stability.

The Shimodaira–Hasewaga (SH) test was used for constraint testing under maximum likelihood criteria on the combined *EF-1α* and *mtSSU* data set. Monophyly of the global and the Indonesian population of Fov were used as the SH test constraints. Monophyly was rejected if the constrained tree (monophyletic) log likelihood score was significantly different from unconstrained topology with 95% confidence level ($P < 0{\cdot}05$). All SH testing was implemented in PAUP v. 4.0b10.

Results

Pathogenicity test

The first sign of discolouration was observed 7 days after inoculation. All 19 Fov isolates induced necrosis and discolouration symptoms. Five isolates (2, 155, 176, 424 and 397) caused symptoms of discolouration in two replicates, whereas the remaining isolates produced symptoms in all three replicates. No symptoms were detected in the negative control at the completion of the pathogenicity test (60 days). The pathogen was successfully re-isolated from each isolate tested.

Phylogenetic analysis

The *EF-1α* gene data set consisted of 656 nucleotide characters, of which 36 (5·5%) were parsimony informative. Parsimony analysis generated 26 most parsimonious trees (MPT) of 83 steps with three distinct clades (CI = 0·96, RI = 0·98; Fig. 1). Clade I included one isolate of Fov (from Indonesia) and five isolates from other *formae speciales* (NRRL 22550, 25603, 26029, 26035 and 26038) with strong bootstrap support (90%). Clade II consisted of two isolates of Fov (both from Indonesia) and 10 isolates from other *formae speciales* (NRRL 25420, 25598, 25607, 25609, 26178, 26961, 28356, 28401, 28923 and 28928) with bootstrap support of

Table 1 Sources and accessions of *Fusarium oxysporum* f. sp. *vanillae* isolates used in the study and GenBank and fungal herbarium accessions of reference strains

F. oxysporum forma specialis	Fungal herbarium accession[a]	Study accession	GenBank accession		Country of origin
			mtSSU rDNA	EF-1α	
F. oxysporum f. sp. *batatas*	NRRL 26409[b]		AF008450	AF008484	
F. oxysporum f. sp. *canariensis*	NRRL 26035[b]		AF008451	AF008485	
F. oxysporum f. sp. *cubense*	NRRL 25603[b]		AF008453	AF008487	
	NRRL 25607[b]		AF008455	AF008489	
	NRRL 25609[b]		AF008456	AF008490	
	NRRL 26029[b]		AF008459	AF008493	
	NRRL 26038[b]		AF008460	AF008494	
F. oxysporum f. sp. *dianthi*	NRRL 26961[c]		AF250568	AF246840	
	NRRL 28356[c]		AF250582	AF246854	
	NRRL 28401[c]		AF250587	AF246859	
F. oxysporum f. sp. *gladioli*	NRRL 26989[c]		AF250575	AF246847	
	NRRL 26992[c]		AF250573	AF246845	
F. oxysporum f. sp. *glycines*	NRRL 25598[b]		AF008462	AF008496	
F. oxysporum f. sp. *lini*	NRRL 28923[c]		AF250604	AF246876	
	NRRL 28928[c]		AF250605	AF246877	
F. oxysporum f. sp. *melonis*	NRRL 26178[b]		AF008469	AF008503	
F. oxysporum f. sp. *opuntiarum*	NRRL 28368[c]		AF250599	AF246871	
	NRRL 28279[c]		AF250564	AF246836	
F. oxysporum f. sp. *passiflorae*	NRRL 22549[b]		AF008471	AF008505	
F. oxysporum f. sp. *perniciosum*	NRRL 22550[b]		AF008472	AF008506	
F. oxysporum f. sp. *tulipae*	NRRL 26954[b]		AF250566	AF246838	
F. oxysporum f. sp. *vanillae*	RBG 5370	2	KM102470	KM115168	Indonesia (North Sulawesi)
	RBG 5391	57	KM102475	KM115183	Indonesia (North Sulawesi)
	RBG 5371	77	KM102477	KM115184	Indonesia (West Java)
	RBG 5373	102	KM102486	KM115171	Indonesia (West Java)
	RBG 5392	104	KM102488	KM115173	Indonesia (West Java)
	RBG 5393	149	KM102484	KM115174	Indonesia (Jogjakarta)
	RBG 5375	155	KM102471	KM115169	Indonesia (West Nusatenggara)
	RBG 5394	173	KM102483	KM115181	Indonesia (West Nusatenggara)
	RBG 5376	176	KM102472	KM115170	Indonesia (West Nusatenggara)
	RBG 5382	331	KM102474	KM115182	Indonesia (Lampung)
	RBG 5395	397	KM102481	KM115186	Réunion
	RBG 5396	401	KM102478	KM115175	Réunion
	RBG 5397	408	KM102473	KM115177	Réunion
	RBG 5398	410	KM102480	KM115178	Réunion
	RBG 5399	424	KM102479	KM115172	Comoros
	RBG 5400	434	KM102476	KM115179	Mexico
	RBG 5401	439	KM102487	KM115180	Mexico
	RBG 5402	441	KM102482	KM115185	Mexico
	RBG 5403	442	KM102485	KM115176	Mexico
F. oxysporum f. sp. *vasinvectum*	NRRL 22550[b]		AF008478	AF008512	
Fusarium sp.	NRRL 22903[b]		U34509	AF008513	
Fusarium sp.	NRRL 25184[b]		U61608	AF008514	

[a]NRRL: United States Department of Agriculture Northern Regional Research Laboratory (also American Research Service, ARS) culture collection; RBG: Royal Botanic Gardens Sydney culture collection.
[b]O'Donnell *et al.* (1998).
[c]Baayen *et al.* (2000).

61%. Clade III grouped together 16 isolates of Fov (all overseas and remaining Indonesian isolates) and seven from other *formae speciales* (NRRL 22549, 26409, 26954, 26989, 26992, 28279 and 28368) with bootstrap support of 62%.

Analysis of the *mtSSU* gene data set resulted in 695 nucleotide characters, of which 23 (3·3%) were parsimony informative. Parsimony analysis generated 100 MPTs of 52 steps, also with three distinct clades (CI = 0·87, RI = 0·95; Fig. 2). Again, the three clades had relatively high bootstrap support with clustering of taxa as per the *EF-1α* tree.

Results of the PHT ($P = 0·15$) suggested that the *EF-1α* gene tree and the *mtSSU* gene tree generated the same underlying phylogeny. The two gene regions were then combined for further phylogenetic analysis. No major topological variations were found between trees generated by neighbour-joining and unweighted

Figure 1 One of 26 most parsimonious trees generated from the *EF-1α* gene rooted by out-group method. NRRL 25184 and 22903 *Fusarium* sp. were used as the out-group. Bootstrap values are indicated above nodes. Isolates beginning with NRRL are reference strains obtained from GenBank. *Fusarium oxysporum* f. sp. *vanillae* isolates are marked with numbers followed by f.sp. *vanillae*, and capital letters indicate the origin of the isolates (C, Comoros; I, Indonesia; R, Réunion Island; M, Mexico). Clade designation is according to O'Donnell *et al.* (1998). The Phylogenetic Species boundary *sensu* Laurence *et al.* (2014) is indicated (PS1 and PS2).

parsimony on the combined gene data set (data not shown). For the parsimony analysis, the combined *EF-1α* and *mtSSU* gene data set consisted of 1351 nucleotide characters, 57 (4·2%) of which were parsimony informative. Parsimony analysis of the combined data set yielded 99 MPT of 134 steps (CI = 0·93, RI = 0·97) with three distinct clades (Fig. 3).

Overall bootstrap support increased when characters from the two gene regions were combined, in particular values on the nodes separating the three distinct clades (Fig. 3). The combined gene data set tree shows that isolates of Fov from Indonesia are scattered across the different clades. In Clade III, the Indonesian isolates are clustered with isolates from the Comoros, Mexico and Réunion Island, whereas Clade I and II consist of Indonesian isolates alone. The combined gene tree also reveals that Fov isolates from Indonesia in Clade III (102, 104 and 149) are identical with one isolate from Réunion Island (401) and one from Mexico (442). The SH test confirmed the topology observation, rejecting the monophyly of both the global and Indonesian Fov populations ($P < 0·05$).

Discussion

Phylogenies inferred from the *EF-1α*, *mtSSU* and combined data sets clearly showed that isolates of Fov resolved into three distinct clades in both phylogenetic species (*sensu* Laurence *et al.*, 2014), suggesting the pathogen of vanilla stem rot has not evolved from a recent common ancestor. This finding revealed that Fov is polyphyletic, consisting of at least three distinct lineages. The first hypothesis that isolates of Fov obtained from Indonesia and overseas form a monophyletic evolutionary lineage is rejected.

The three clades within the FOSC were first documented by O'Donnell *et al.* (1998). It was assumed, without clear scientific justification, that isolates within a

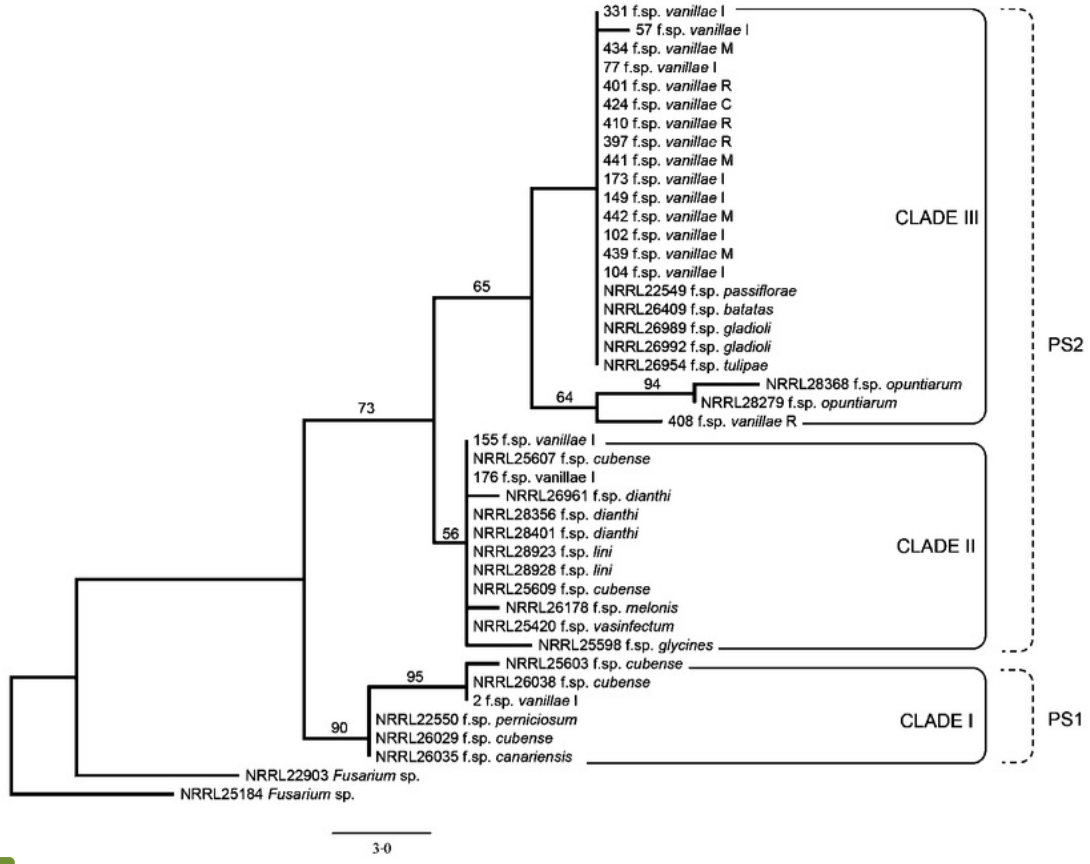

Figure 2 One of 100 most parsimonious trees generated from the *mtSSU* gene rooted by out-group method. NRRL 25184 and 22903 *Fusarium* sp. were used as the out-group. Bootstrap values are indicated above nodes. Isolates beginning with NRRL are reference strains obtained from GenBank. *Fusarium oxysporum* f. sp. *vanillae* isolates are marked with numbers followed by f.sp. *vanillae*, and capital letters indicate the origin of the isolates (C, Comoros; I, Indonesia; R, Réunion Island; M, Mexico). Clade designation is according to O'Donnell *et al.* (1998). The Phylogenetic Species boundary *sensu* Laurence *et al.* (2014) is indicated (PS1 and PS2).

forma specialis were genetically more similar than isolates with different host specificities, and likely to have a common origin (Kistler, 1997). Many *formae speciales* have now been shown not to be so (e.g. O'Donnell *et al.*, 1998; Baayen *et al.*, 2000; Skovgaard *et al.*, 2001; Jimenez-Gasco *et al.*, 2002; Mbofung *et al.*, 2007; Wunsch *et al.*, 2009). *Formae speciales* with a polyphyletic origin tend to have at least two or more VCGs (Baayen *et al.*, 2000). Similar to this finding, at least four different VCGs were found within the Indonesian Fov isolates (Pinaria, 2010).

On the basis of the evidence that Fov isolates are scattered across the three different lineages, the issue as to the origin of the pathogen in Indonesia is not a simple one, raising the question of how pathogenicity to the same host evolved. Covert (1998) proposed that horizontal (vegetative) spread of dispensable chromosomes carrying pathogenicity genes or gene clusters across evolutionary distinct lineage could shed light on that question. Indeed, Ma *et al.* (2010) demonstrated horizontal gene transfer (HGT) *in vitro* of one such chromosome from a pathogenic strain, *F. oxysporum* f. sp. *lycopersici*, to a nonpathogenic strain, giving the latter the ability to cause the same symptoms on tomato as the pathogenic strain. This chromosome is now understood to contain effector genes that confer cysteine-rich proteins found in xylem sap during infection, designated Secreted In Xylem (*SIX*; Rep *et al.*, 2002, 2004; Rep, 2005; De Wit *et al.*, 2009). Furthermore, HGT is the most probable explanation of the incongruence between the *EF-1α* and *SIX* gene phylogenies in the *forma specialis canariensis* at the population level and is probably widespread within the FOSC (Laurence *et al.*, 2015).

The 16 isolates of Fov in Clade III represent not only isolates from Indonesia but all other geographic origins included in the investigation, suggesting a common ancestor for these isolates. Clades I and II contain the remaining three Indonesian isolates with no overseas

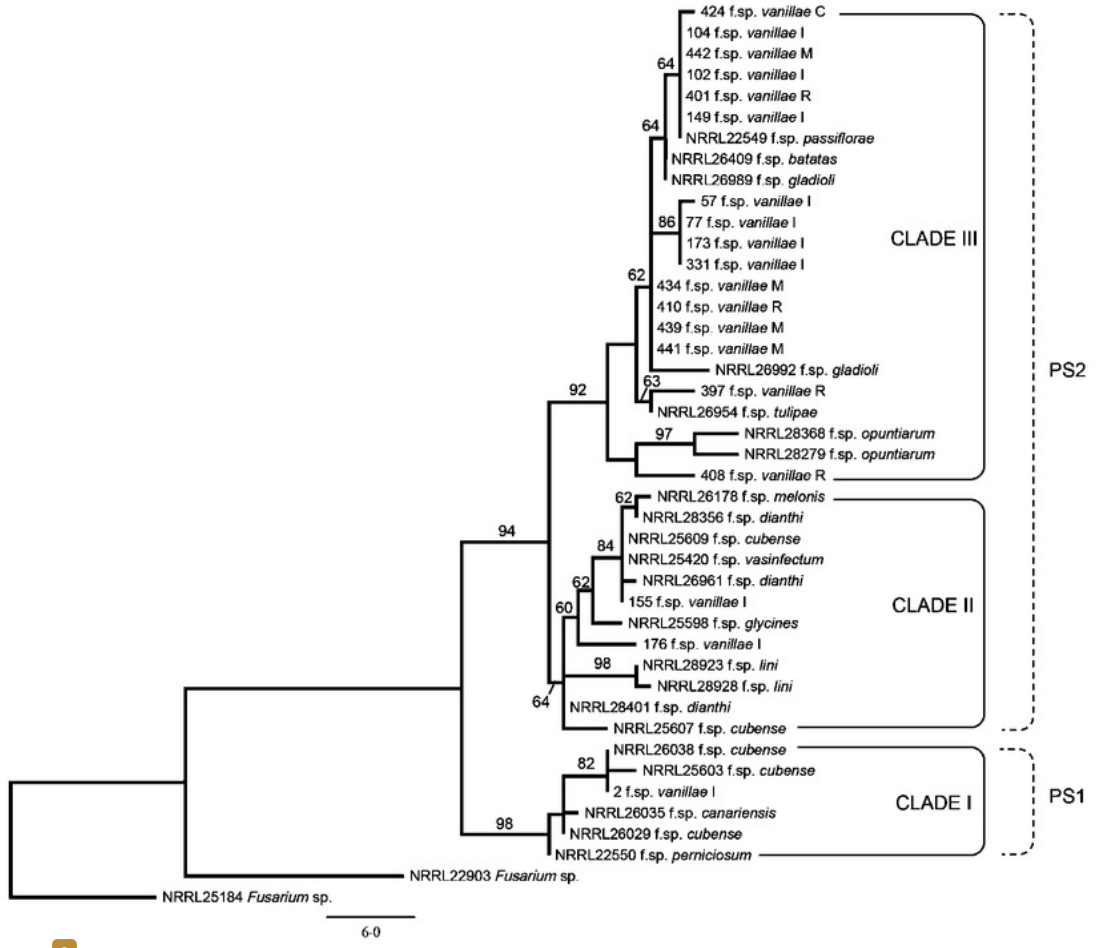

Figure 3 One of 99 most parsimonious trees generated from combined *EF-1α* and *mtSSU* data sets and rooted by out-group method. NRRL 25184 and 22903 *Fusarium* sp. were used as the out-group. Bootstrap values are indicated above nodes. Isolates beginning with NRRL are reference strains obtained from GenBank. *Fusarium oxysporum* f. sp. *vanillae* isolates are marked with numbers followed by f. sp. *vanillae*, and capital letters indicate the origin of the isolates (C, Comoros; I, Indonesia; R, Réunion Island; M, Mexico). Clade designation is according to O'Donnell *et al.* (1998). The Phylogenetic Species boundary *sensu* Laurence *et al.* (2014) is indicated (PS1 and PS2).

isolates. These findings suggest that there are multiple possibilities for the origin of Fov in Indonesia. One origin is most likely introduction from overseas, while the second origin is either a separate introduction or local evolution, combined with perhaps the horizontal transfer of pathogenicity elements.

The host *V. planifolia* is thought to have originated in Central America. Given that many important plant pathogens are believed to have co-evolved with their host (Fetch *et al.*, 2003), Central America is a possible source of Fov in Indonesia. It is also well understood that human activities play a significant role in contributing to the global migration of pathogens through commerce, especially via the movement of host germplasm. Vanilla was introduced from Mexico into Europe around 1510 by Dr Fransisco Hernandez (Webster, 1995), which was then sent to Indonesia from Antwerp to Buitenzorg (now Bogor), Java in 1819 (Weis, 2002). Although vanilla is a relatively new crop in Indonesia, the high global demand since its introduction has been a driving force for the rapid expansion of vanilla cultivation throughout the country via vegetative propagation. In the absence of enforceable regulations on plant movement and inspections, such dissemination of planting material could potentially be spreading plant pathogens including Fov. There have been many reports of *F. oxysporum* being an endophytic fungus (e.g. Leslie *et al.*, 1990; Kuldau & Yates, 2000; Vu *et al.*, 2006; Athman *et al.*, 2007; Wang *et al.*, 2007) and Fov is no exception. It has been isolated as an endophyte from vanilla stems without any external or internal symptoms, yet such an endophytic isolate was shown to be pathogenic in greenhouse trials (Liew

et al., 2008) and genetically identical to pathogenic isolates (authors' unpublished data).

An interesting finding in this investigation is the polyphyly of Fov isolates obtained from Indonesia and that some of these isolates could have evolved from indigenous sources. These isolates are not opportunistic endophytes co-isolated from diseased tissue but rather have been shown to be true pathogens of vanilla. It is speculated that the pathogen may be derived from indigenous nonpathogenic strains having acquired the pathogenicity elements from an introduced pathogenic strain via HGT. The concept of the pathogen of an introduced crop having an indigenous origin is not novel. It has been shown in the cotton wilt pathogen, *F. oxysporum* f. sp. *vasinfectum*, in Australia (Davis, 1996; Wang *et al.*, 2004; Kim *et al.*, 2005). More recently Laurence *et al.* (2014) demonstrated evidence for the HGT of *SIX* genes in *F. oxysporum* f. sp. *canariensis*, which have also been shown to be polyphyletic.

In summary, the vanilla stem rot pathogen in Indonesia appears to have been derived from multiple origins, whereby introduction from overseas is one probable scenario, although a separate indigenous derivation or introduction from the surrounding region cannot be ruled out. This has important implications for further investigations as well as disease management. While work is on-going in obtaining a more representative and comprehensive collection of Fov isolates throughout the various vanilla-growing regions in Indonesia to further understand the diversity within this polyphyletic population, quarantine measures should be in place. These measures should not only include screening of germplasm introduced from overseas but should also consider inter-regional transfer of planting materials. Acknowledging the challenge of implementing internal quarantine, education on hygiene measures in disease management should be stepped up. Current and future work on screening for host resistance should include representatives of genetically disparate isolates. Investigations into the presence and nature of *SIX* genes should also be conducted.

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