

Phylogeny and genetic diversity of the banana *Fusarium* wilt pathogen *Fusarium oxysporum* f. sp. *cubense* in the Indonesian centre of origin

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Abstract: *Fusarium oxysporum* f. sp. *cubense* (Foc), the causal agent of Fusarium wilt or Panama disease on banana, is one of the major constraints in banana production worldwide. Indonesia is the centre of origin for wild and cultivated bananas, which likely co-evolved with Foc. This study explored the widest possible genetic diversity of Foc by sampling across Indonesia at 34 geographically and environmentally different locations in 15 provinces at six islands. This resulted in a comprehensive collection of ~200 isolates from 40 different local banana varieties. Isolates were identified and assessed using sequence analysis of the translation elongation factor-1alpha (*tef1*), the RNA polymerase II largest subunit (*rpb1*), and the RNA polymerase II second largest subunit (*rpb2*). Phylogenetic analyses of these genes allowed the identification of 180 isolates of *Fusarium oxysporum* f. sp. *cubense* (Foc), and 20 isolates of the *Fusarium fujikuroi* species complex (FFSC), the *Fusarium incarnatum-equiseti* species complex (FIESC), and the *Fusarium sambucinum* species complex (FSSC). Further analyses, incorporating a worldwide collection of Foc strains, revealed nine independent genetic lineages for Foc, and one novel clade in the *Fusarium oxysporum* species complex (FOSC). Selected isolates from each lineage were tested on the banana varieties Gros Michel and Cavendish to characterise their pathogenicity profiles. More than 65 % of the isolates were diagnosed as Tropical Race 4 (Foc-TR4) due to their pathogenicity to Cavendish banana, which supports the hypothesis that Foc-TR4 is of Indonesian origin. Nine independent genetic lineages for Foc are formally described in this study. This biodiversity has not been studied since the initial description of Foc in 1919. This study provides a detailed overview of the complexity of Fusarium wilt on banana and its diversity and distribution across Indonesia.

Key words: Morphology, New species, Panama disease, Pathogenicity, Tropical Race 4, 11 New taxa.

Taxonomic novelties: New species: *Fusarium cugenangense* N. Maryani, L. Lombard, Kema & Crous, *F. duoseptatum* N. Maryani, L. Lombard, Kema & Crous, *F. grosnichelii* N. Maryani, L. Lombard, Kema & Crous, *F. hexaseptatum* N. Maryani, L. Lombard, Kema & Crous, *F. kalimantanense* N. Maryani, L. Lombard, Kema & Crous, *F. odoratissimum* N. Maryani, L. Lombard, Kema & Crous, *F. phialophorum* N. Maryani, L. Lombard, Kema & Crous, *F. purpurascens* N. Maryani, L. Lombard, Kema & Crous, *F. sangayamense* N. Maryani, L. Lombard, Kema & Crous, *F. tardichlamydosporum* N. Maryani, L. Lombard, Kema & Crous, *F. tardicrescens* N. Maryani, L. Lombard, Kema & Crous.

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INTRODUCTION

Indonesia is one of the main centres of origin for banana in South-East Asia (Valmayor *et al.* 1999). Edible banana cultivars are descendants from two ancestral wild *Musa* species, *Musa acuminata* Colla (AA, 2n = 22) and *Musa balbisiana* Colla (BB, 2n = 22) (Simmonds 1962). These diversified into various edible varieties comprising diploids (AA, BB), triploids (AAA, AAB, ABB) and tetraploids (ABBB). Indonesia is the main contact area between species and subspecies of wild banana in sub-centres of diversity (Perrier *et al.* 2011) and, therefore, represents the primary gene centre for banana, resulting in a huge phenotypic and genotypic diversity. Indonesia is among the top 10 banana producing countries (FAOSTAT 2017) with over 200 varieties that are presently grown in almost every region of the Indonesian archipelago (Nasution 1993). The actual number of identified cultivated banana varieties could easily surpass 500. Banana is one of Indonesia's primary fruit commodities (BPS 2017), with most production supplying the domestic market.

Despite this great diversity and high popularity of bananas, there are some constraints on production. The most important of

these is fungal diseases, including Fusarium wilt, also known as Panama disease (Stover 1962a). Fusarium wilt is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), which first appeared in the 1900s in a banana plantation on Java (Stover 1962a) and thereafter disseminated to other banana production areas in Indonesia and beyond. This devastating agent of wilt on banana was first reported in the literature from samples collected in a Cuban banana plantation, and it subsequently gained notoriety as *Fusarium cubense* (Smith 1910).

The history of Fusarium wilt on banana goes back to the 20th century when this disease eliminated thousands of hectares of the favoured Gros Michel banana in Central America. The outbreak evolved into one of the worst plant epidemics of all times. The discovery of resistant Cavendish bananas eventually quenched the epidemic and the variety was so successful that it was disseminated around the world until it attained its current predominance in the global banana trade. The resistance of Cavendish bananas to the so-called Foc-Race1 strains, which caused the epidemic in Gros Michel is unique and durable. The risk of global monocultures is evident and problems surfaced again once other pathogenic *Fusarium oxysporum* strains

appeared that were able to cause *Fusarium* wilt in Cavendish plantations. A harmful strain was initially reported from Taiwan, from whence it spread further into South-East Asia, and recently to the Indian subcontinent, the Middle East and Africa (Ordóñez *et al.* 2015). The ongoing epidemic in Cavendish bananas is caused by a unique genotype, Vegetative Compatibility Group (VCG) 01213, of Foc and is called Tropical Race 4 (TR4). It has caused significant losses in commercial and subsistence production areas of Taiwan, Malaysia, and the northern territories of Australia (Su *et al.* 1986, Gerlach *et al.* 2000, Hermanto *et al.* 2009). In Indonesia, Nasir *et al.* (1999) reported that *Fusarium* wilt occurred from the Aceh province of Sumatra in the far west, to the far eastern Papua province. Losses in export Cavendish plantations in southern Sumatra have exceeded 70 %. In Northern Sumatra over 1 000 ha of plantations were destroyed within 3 yr after the appearance of the disease in this area (Nasir *et al.* 1999). Not only was Cavendish affected, but also many local popular varieties named in Bahasa Indonesia with 'Pisang' (= 'banana') variety names, such as Pisang Raja Bulu, P. Raja Sereh, P. Ambon, P. Mas and P. Barangan, were damaged. The affected varieties are very important for the local markets (Hermanto *et al.* 2009).

To date, no control method has yet been identified or successfully implemented to effectively manage TR4. This is further complicated by the soil-borne nature of Foc and its ability to produce persistent chlamydospores that contaminate soils for decades (Booth 1971). Essentially, there are presently no control methods, except prevention by using pathogen-free tissue culture plants planted in non-infested soil (Ploetz 1994), and the adoption of quarantine strategies. However, these practices are mostly applied in large commercial plantations, but not in smallholder settings. Evidently, the development of new resistant banana cultivars would be the most effective control strategy to follow, and therefore research on the diversity of this pathogen is essential, particularly since it has been shown to be polyphyletic (O'Donnell *et al.* 1998, 2009). It is therefore essential to acquire a better understanding of the differences between the genetic lineages for developing control strategies, and for effective resistance breeding.

In *Fusarium* systematics, Foc belongs to the *Fusarium oxysporum* species complex (FOSC). Four clades of FOSC have been identified using translation elongation factor 1- α (*tef1*) and mitochondrial subunit rDNA (*mtssu*), with Foc isolates clustering as basal lineage (O'Donnell *et al.* 2004). The incorporation of Foc isolates from native host populations, especially those from indigenous ecosystems, will be of great importance for diversity studies of this complex species.

Diversity studies on Foc isolates were conducted by using various physiological and molecular methods, which included VCGs (Moore *et al.* 1993), random amplified polymorphic DNA markers (RAPDs; Bentley *et al.* 1995), restriction fragment length polymorphisms (RFLPs; Koenig *et al.* 1997), amplified fragment length polymorphism (AFLP; Groenewald *et al.* 2006) and DNA sequence analyses (O'Donnell *et al.* 1998). These studies showed that the South-East Asian population of this fungus exhibits a high degree of variation, suggesting that Foc lineages co-evolved with their hosts in South-East Asia (Ploetz & Pegg 1997). However, these studies used Foc isolates from various disconnected geographical areas and lacked evidence on genetic diversity from the genetic centre of banana diversity, which is likely also the origin of the co-evolving Foc (Buddenhagen 2007). It has alternatively been suggested that Foc has

multiple independent evolutionary origins, both within and outside the *Musa* genetic centre (Bentley *et al.* 1998). Using the phylogenetic genealogical approach, O'Donnell *et al.* (1998) identified five independent genetic lineages of Foc in a global population. Using a similar approach and additional data, Fourie *et al.* (2009) found three additional lineages. However, neither of these studies included Indonesian populations, and hence only limited information is available on the diversity of Foc at the centre of origin of banana.

Here, we explore the genetic diversity among Indonesian Foc strains that were isolated from local banana varieties in various different ecosystems across the country. This overview of the complexity of *Fusarium* wilt of banana enables us to greatly improve our knowledge of the taxonomic and phylogenetic position of Foc in the FOSC.

MATERIALS AND METHODS

Isolates

A comprehensive survey of *Fusarium* wilt of banana was undertaken in Indonesia. In total, 34 locations in 15 provinces were visited, representing the main banana-producing regions in Java, Sumatra, Kalimantan, Sulawesi, Papua, and Nusa Tenggara (Table 1, Fig. 1). Sampling expeditions to the former three islands were undertaken in 2014, whereas the other islands were sampled in 2015. Sampling locations were identified in two to three different regions in each province. Diagnostic specimen were collected from diseased banana plants displaying typical *Fusarium* wilt symptoms: yellowing of older leaf margins, collapsed leaves at the petioles, and pseudostem discolouration and splitting. The pseudostems of the diseased plants were cut and discoloured vascular strands were sampled and placed on sterile filter paper to dry, and were eventually packed in a paper envelope. Global positioning coordinates were recorded and ecological parameters, including soil pH, light intensity and vegetation of the sampling area were collected at each site. For each banana plant sampled, the youngest (cigar) leaf was taken for ploidy identification of the germplasm by flow-cytometry analyses and morphological characterisation following Valmayor *et al.* (1999) and Simmonds & Shepherd (1955), as well as *in-situ* comparisons with local banana varieties in the *Musa* collection at the Indonesian Institute of Sciences (LIPI) Cibinong, Bogor, Indonesia.

Isolation

The dried pseudostem samples were cut into pieces of 2 × 3 cm and plated on Komada medium (Komada 1975). After approximately 2 d, fungal colonies resembling *Fusarium* were transferred to potato dextrose agar (PDA) plates (Leslie & Summerell 2006). Axenic cultures were derived by streaking a small amount of conidia, collected with the tip of an inoculation needle, on water agar (WA) plates, which allowed conidia to separate. After 24 h of incubation, plates were observed under a dissection microscope at 50× magnification and single germinating conidia were collected and transferred to PDA. Monospore isolates were either maintained on PDA or in 20 % (v/v) glycerol at -80 °C. All isolates were deposited in the Indonesian Culture Collection (InaCC) Cibinong, Indonesia. Twenty-four Foc isolates,

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