DIVERSITY AND DETECTION OF KENYAN AND NIGERIAN POPULATIONS OF *FUSARIUM OXYSPORUM* F. SP. *STRIGAE*

by

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DECLARATION

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SUMMARY

The Genus *Striga* contains some of the most destructive plant-parasitic weed species in the world. These include *S. hermonthica* and *S. asiatica*, which attack important staple food crops in sub-Saharan Africa such as maize, sorghum and millet. Of the two, S. *hermonthica* is more devastating and can cause yield losses on average of 50% and, ultimately, land abandonment. The parasite produces up to 200 000 seeds per plant, which causes a rapid build-up of a seed bank in the soil. Germination of seeds happens after a ripening period and when strigolactones, exuded by the roots of susceptible hosts, are detected. After a haustorium connects to the host, it parasitizes the host for water and nutrients, and in effect stunts the development of the host plant.

Striga infestations are difficult to manage due to its prolific nature and late emergence. The weed affects subsistence farmers most severely, as these growers have little access to, or the financial resources available, to control the pest with chemicals. Biological control, therefore, was proposed as a means of control, as this method can be integrated with existing farming practises, as its use would be safe to both the farmer and the environment. *Fusarium oxysporum* f. sp. *striga*e (Fos), a soil-borne fungal pathogen affecting *Striga* plants, was identified as a possible Biological Control Agent (BCA), as Fos was host specific and did not produce harmful secondary metabolites. Its population structure in Africa, and means to survive and disseminate in farmer fields, however, was unknown. The aims of this study, therefore, were to characterize Fos populations in two African countries, Kenya and Nigeria, and to develop molecular markers to rapidly and accurately identify the fungus.

The diversity of Fos in Kenya and Nigeria was investigated by means of vegetative compatibility group analysis (VCGs) and phylogenetic analysis. VCG analysis showed that the Kenyan isolates consisted of a single VCG, and that the Nigerian isolates were divided into seven VCGs and eight SMV's. A combined maximum likelihood tree of the translocation elongation factor (TEF) 1α and mitochondrial small sub-unit (MtSSU) gene areas revealed that Fos isolates from the two countries separated into two different clades. This suggested that there was two separate events of evolution, and that the Nigerian isolate group is older than the Kenyan group due to the greater number of VCGs present in Nigeria. Mating type analysis confirmed clonality within the Kenyan group, where all isolates in VCG 04708 contained only the *MAT1-1* gene. However, a larger diversity was found in the Nigerian group, where both mating type idiomorphs were present in the different VCGs.

Molecular markers that distinguish between the two Fos populations in Africa have been developed in this study. Primer-set Foxy2-F/R1 was developed form a single nucleotide polymorphism (SNP) in the TEF gene area, and primer set FK3-F/R from SNPs in the secreted-in-xylem 14 gene area. Both primer sets were tested against a collection of isolates that includes non-pathogenic F. *oxysporum* isolated from *Striga*, other *Fusarium* species associated with *Striga*, and other *formae speciales* of *F. oxysporum*. Sensitivity assays revealed that the Foxy2 primer only detected target Fos DNA at a concentration of 10 ng/µl in the presence of *S. asiatica* DNA. Primer-set FK3 on the other hand, could detect target Fos DNA at the low concentration of 0.1 ng/µl in the presence of *S. asiatica* DNA.

The findings in this study suggest that pathogenicity evolved at two separate events in Fos. The molecular markers designed could compliment the primer-set designed by Zimmerman *et al.* (2015) to aid in diagnostics and monitoring of Fos after application as a BCA.

OPSOMMING

Die Genus *Striga* word gesien as die mees verwoestende plant-parasietiese onkruid spesies in die wêreld. Daaronder val *S. hermonthica* en *S. asiatica*, wat belangrike stapel voedsel gewasse soos mielies, sorghum en millet aanval in sub-Sahara in Afrika. *Striga hermonthica* is die meer verwoestende plant parasiet uit die twee, en kan oeste verminder met ‘n gemiddeled van 50%, wat uiteindelik lei tot die opgee van die landbou grond. Hierdie plant-parasiet produseer tot en met 200 000 saad per plant, wat lei daartoe dat ‘n saad-bank baie vining op bou in die grond. Die saad ontkiem na ‘n ryp-wordings proses en wanneer strigolaktone, uit geskei deur die wortels, waar geneem word. ‘n Haustorium ontwikkel en verbind aan die gasheer plant, waar dit dan die gasheer plant parasiteer vir water en voedsel, wat lei tot vertraagde ontwikkeling van die gasheer.

Striga besmettings is moeilik om te bestuur as gevolg van die onkruid se produktiewe aard en laat opkoms. Die onkruid beïnvloed bestaansboere die ergste, aangesien hierdie boere minder toegang het tot, of oor die beskikbare finansiële hulpbronne beskik, om die parasiet chemies te beheer. Biologiese beheer is dus voorgestel as 'n manier van beheer, aangesien hierdie metode met bestaande boerderypraktyke geïntegreer kan word,en die gebruik daarvan vir beide die boer en die omgewing veilig sal wees. *Fusarium oxysporum* f. sp. *strigae* (Fos), ‘n grond gedraagde patogeniese fungus wat *Striga* plante aanval, was geidentifiseer as ‘n moontlike Biologiese Beheer Agent (BBA), aangesien dit bewys was dat Fos gasheer spesifiek is en nie enige skadlike sekondêre metaboliete produseer nie. Die bevolkings struktuur van hierdie fungus in Afrika, asook die oorlewing en verspreiding in die bewerkbare lande, is egter onbekend. Die doelstellings van hierdie studie was dus om die Fos-bevolkings in twee Afrika-lande, Kenia en Nigerië te karakteriseer en molekulêre merkers te ontwikkel om die swam vinnig en akkuraat te identifiseer.

Die diversiteit van Fos in Kenia en Nigerië was ondersoek deur om vegetatiewe verenigbaarheidsgroep (VVG) en filogenetiese analises te voltooi. Die VVG-analise het getoon dat die Keniaanse isolate bestaan uit 'n enkele VVG, en dat die Nigeriese isolate verdeel is in sewe VVG's en agt enkellid VVG's (EVVG’s). Die gekombineerde maksimum-waarskynlikheids filogenetiese analise van die translokasie-verlengings faktor (TEF) 1a en mitochondriale klein subeenheid (MtSSU) geengebiede het aan gedui dat die Fos populasie geskei kan word in twee verskillende afstammeling groepe. Hieruit kan afgelei word dat daar twee afsondelike evolusinêre ontstaans-gebeurtenisse was, en dat die Nigeriese isolaat-groep ouer is as die Keniaanse groepis, a.g.v. die groter aantal VVG's wat in Nigerië teenwoordig is. Die parings tipe

analise bevestig klonaliteit in die Keniaanse groep, waar alle isolate in VVG 04708 slegs die *MAT1-1* geen bevat. Daar is egter 'n groter diversiteit in die Nigeriese groep gevind, waar albei parings-tipe idiomorfe in die verskillende VVG's teenwoordig was.

Molekulêre merkers is in hierdie studie ontwikkel wat tussen die twee Fos-populasies in Afrika kan onderskei. Inleier stel, Foxy2-F / R1 is ontwikkel in die TEF geen-area van 'n enkel nukleotied polimorfisme (ENP), en die inleier stel FK3-F / R van ENP's in die afgeskei-in-xileem 14 geen-area. Albei inleier stelle is getoets teen 'n versameling isolate wat nie-patogene van *F. oxysporum* bevat, geïsoleer vanaf *Striga*, ander *Fusarium* spesies wat met *Striga* geassosieer word, en ander *formae spesiales* van *F. oxysporum*. Sensitiwiteits studies het aan gedui dat die Foxy2-inleier slegs Fos DNA op 'n konsentrasie van 10 ng/µl in die teenwoordigheid van *S. asiatica* DNA kon amplifiseer. Die FK3 inleier, kon egter Fos DNS op die lae konsentrasie van 0,1 ng/µl in die teenwoordigheid van *S. asiatica* DNS amplifiseer.

Hierdie studie dui daarop dat patogeniteit ontwikkel het tydens twee afsonderlike gebeurtenisse in Fos. Die molekulêre merkers wat ontwerp is in hierdie studie, kan die merker stel wat deur Zimmerman et al (2015) ontwerp is, komplimenteer, om te help met die diagnose en monitering van Fos in landbou grond na toediening.

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CONTENTS

CHAPTER 1

The significance and control of *Striga* spp. in Africa

INTRODUCTION

Striga is a parasitic weed of grain crops such as maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench) and millet (*Pennisetum americanum* (L.) Leeke) (Parker, 2009; Atera *et al*., 2013). Seed of *Striga* can survive for long periods in a wide range of soil types (Mourik, 2007), and infects the roots of host crops soon after germination. By the time that the crop emerges, the *Striga* plant has already established itself to access nutrients from its host. Its effect on the host can be devastating, resulting in little to no yield. For this reason *Striga*, which is often referred to as “witches weed” (Mohamed *et al.,* 2001), is regarded as the most important pest of agricultural crops on the continent.

Striga is endemic to most of the tropical countries in eastern and western Africa. Approximately 3.7 million ha of land in sub-Saharan Africa is infested with the parasite, where it causes crop losses of an average of 50% (De Groote, 2007). This, in turn, results in economic losses of up to US$ 7 billion annually (Atera *et al*., 2012). Most farmers affected by *Striga* are subsistence farmers in developing countries, rendering the parasite a significant threat to food security in tropical Africa (Parker, 2009; Atera *et al*., 2013). Due to the severity of the *Striga* problem, many ways to control or manage the weed have been investigated (Oswald, 2005), but no single technique or procedure has been successful (Franke *et al*., 2006; Hearne, 2009).

Biological control of *Striga* could form part of an integrated pest management (IPM) strategy of this pest (Hearne, 2009). The Fusarium wilt pathogen *Fusarium oxysporum* f. sp. *strigae* (Fos) has been found to be host specific towards several *Striga* species (Zarafi *et al*., 2014). Two isolates, Foxy 2 and FK3, are excellent candidates for controlling the parasite (Venne *et al*., 2009, Kangethe *et al*., 2016), and numerous efforts have been made to establish their biosafety (Elzein and Kroschel, 2004; Elzein and Kroschel, 2006; Elzein *et al*., 2008). Risk assessments for Fos isolates, including mycotoxin analysis, have been completed (Savard *et al*., 1997; Amalfitano *et al*., 2002), and the adaptability of the fungus to different environments should be considered (Louda *et al*., 2003).

This review on the importance and control of Striga spp. in Africa consists of three sections. Firstly, the *Striga* plant and its biology is described. Agricultural practices for the management of

Striga are then discussed, and finally the genetic diversity and development of molecular markers to monitor the distribution and spread of Fos when applied as a BCA is presented.

THE *STRIGA* PLANT

Origin and distribution

The genus *Striga* belongs to the family Orobanchaceae, which also includes species in the genera *Alectra* and *Orobanche*. All three genera are economically important parasitic weeds that attack their host's root-system to cause severe damage if left unattended (Sauerborn *et al*., 2007). *Striga* is believed to have originated in the Ethiopian–Sudanese region in Africa (Spallek *et al.,* 2013). It consists of 28 known species, of which 22 are described as abundant and widespread throughout the continent (Mohamed, *et al*., 2001). Other continents where *Striga spp*. are found include Asia, Australia and North America (Shaw *et al*., 1962). *Striga spp*. affect food crops of the Poaceae family such as maize, sorghum, rice and millet (Mohamed *et al.,* 2001). The exception is *S. gesnerioides* (Willd.), which does not affect Poaceae, but which has evolved to parasitize dicotyledonous plants (Spallek *et al.,* 2013). *Alectra* species are present in sub-Saharan Africa, where they attack legumes such as soybean and cowpea (Parker, 2012), while *Orobanche* spp. are found primarily in Europe and North Africa where they attack a wide range of crops (Parker, 2009).

Striga hermonthica (Del.), *S. asiatica* (L.) Kuntze and *S. gesnerioides* (Willd.) are the most economically important of all *Striga spp*. (Parker 2009) (Fig. 1). *Striga hermonthica*, known as the "giant witchweed" or "purple witchweed" because of the purple flowers it produces, is the most devastating *Striga spp*. (Parker 2009). It proliferates easily, spreads rapidly, and is largest in size (Mohamed *et al.,* 2001). It is found only in Africa, primarily in the Sahelian area as well as the tropical savannas of Africa (Spallek *et al.,* 2013), and is recorded to be a persistent problem in the Lake Victoria basin (Oswald, 2005). *Striga hermonthica* is often mistaken for *S. aspera* (Willd.) (known as "witchweed") (Spallek *et al.,* 2013), which also carries purple flowers but is smaller in size. *Striga aspera* occurs in the Sahelian and Sudanian areas of Africa, (Mohamed *et al.,* 2001), and is not found south of the equator. *Striga asiatica* is found throughout Africa, in some parts of Asia and in Australia (Spallek *et al.,* 2013). It was introduced into the USA as well (Shaw *et al*., 1962; Cochrane and Press, 1997), but extensive eradication programs limited its economic impact (Parker, 2009). *Striga asiatica*, known as the "red witchweed" or simply as "witchweed", is smaller than *S. hermonthica*, and produces distinct red flowers. It attacks important food crops that mostly includes sorghum, millet and maize (Mohamed, *et al.,* 2001). It

is the most widespread of all species, but is problematic in only some areas (Mohamed *et al.,* 2001).

Spread of *Striga* throughout Africa is influenced by the movement of grazing animals, running water and contaminated seed (Mourik, 2007; Atera *et al*., 2012). *Striga hermonthica* occurs on all soil types, while *S. asiatica*, *S. aspera* and *S. gesnerioides* occur predominantly on sandy soils. All *Striga* spp. are more predominant in nutrient poor soils (Spallek *et al.,* 2013). Crops affected by *Striga* spp. are important to subsistence farmers, as it provides food, animal feed, fuel and an income to households (Oswald, 2005).

Life cycle

The *Striga* plant has an intricate life cycle, that is dependand on its host to complete (Fig. 2) (Rich and Ejeta, 2007). Seeds germinate following exposure to host root exudates such as the strigolactones, dihydroquinones and sesquiterpene lactones (Bouwmeester *et al*., 2003; Yoneyama *et al*., 2010). These exudates also play a role in the establishment of arbuscular mycorrhizal (AM) fungi in plant roots (Dor *et al*., 2011). Of the exudates, strigolactone is most important as it stimulates germination of *Striga* seed at very low concentrations (Yoneyama *et al*., 2010). More than 14 different variants of strigolactones have been described from a variety of different plant species, and it is thought that more variants are still to be found (Yoneyama *et al*., 2010). A synthetic strigolactone, GR24, is known to cause both germination in *Striga* seed and the suppression of plant pathogenic fungi (Dor *et al*., 2011).

Striga seed is in a constant state of dormancy (Bouwmeester *et al*., 2003) and only germinates once all stages required for germination have been passed (Bouwmeester *et al*., 2003; Yoneyama *et al*., 2010). During the first or pre-conditioning stage, the seed has to be exposed to moisture at optimal temperatures (Bouwmeester *et al*., 2003) and must be incubated for at least 15 days to ensure adequate moisture uptake. When the seed is then exposed to root exudates it will germinate (stage two) and produce a radicle, which will grow towards a signal produced by root-exudates (Bouwmeester *et al*., 2003; Yoneyama *et al*., 2010). Since the seedling is so small its reserves may become exhausted before reaching the roots of the host plant (Bouwmeester *et al*., 2003). Therefore, the stronger the signal from the root-exudates, the more likely it is that germination will take place (Yoneyama *et al*., 2010). When the radicle reaches a host root, the third stage is initiated and a haustorium is produced (Spallek *et al.,* 2013).

The final stage of germination comprises of a complicated sequence of events. Haustorium formation is thought to be initiated through a system where 2,6-dimethoxy-p-benzoquinone is

released as a by-product of lignin oxidation, and reduced to an unbalanced semi-quinine intermediate (Spallek *et al.,* 2013). Once formed, the haustorium advances through the epidermis with mechanical force through distal cell development of the haustorium tip (Dörr, 1997; Hood *et al*., 1998). At the cortex the penetration action appears to change from mechanical to enzymatic (Dörr, 1997), with a dark discolouration (Hood *et al*., 1998) appearing in the cortex areas that were penetrated (Yoshikawa *et al*., 1978). After the endodermis is penetrated, the haustorial cells spread irregularly along the xylem cells and enter through the pit membranes. The haustorial cells then produce an osculum through which the invading parasitic cells lose their protoplasts and develop xylem elements (Rich and Ejeta, 2007) to acquire water and nutrients (Dörr, 1997). This causes severe stunting of the host, with crop losses between 20% and 80% (Spallek *et al.,* 2013), averaging at 50% (De Groote, 2007).

Once a primary haustorial connection has been established, secondary haustoria will form and connect to either the same or to another host plant (Westwood *et al*., 2010). After successful establishment, the first leaves of *Striga* will emerge from the seedling (Hood *et al*., 1998). Their growth, however, is slow, and the first leaves only emerge from the soil after six weeks (Rich and Ejeta, 2007). *Striga* has the ability to photosynthesize but is dependent on its host for nutrients and water. Flowers will start to form six weeks after emergence and produce fruit pods two weeks after pollination. *Striga hermonthica* can produce up to 200 000 seeds per plant (Rich and Ejeta, 2007; Hearne, 2009). The life cycle is completed after an average period of 13 weeks (Rich and Ejeta, 2007).

The high number of seeds released by each *Striga* plant results in a ‘seedbank’ (the build-up of viable seed in the soil over a certain period of time) forming quickly in the soil. *Striga* spp. have a long underground phase in their life cycle, which is undetectable to farmers (Spallek *et al.,* 2013). Newly released seed then goes through an after-ripening period (Rich and Ejeta, 2007), which prevents germination (Kust, 1963; Mourik, 2007). During this period the seeds will not respond to germination signals or root exudates (Rich and Ejeta, 2007).

INTEGRATED MANAGEMENT OF *STRIGA*

The practices or techniques applied to manage *Striga* can be divided into two groups; one having a prolonged or gradual effect over several seasons, and the other having an instant effect within the same growing season (Oswald, 2005). The first group of practices that focuses on a prolonged effect include soil fertility improvement, intercropping, crop rotation, adjusted planting times, and resistance breeding of the hosts. The second group focuses on an instant

effect, that includes herbicide application, push-pull technology, trap crops, host transplanting, hand weeding and biological control. Of these practices, soil fertility improvement, herbicide application, push-pull technology, trap crops, hand weeding and biological control all focus on seed bank reduction (Oswald, 2005; Hearne, 2009). The different management practices can also be combined to suit a farmer's needs in terms of practicality, watering needs, and overall cost and improved efficacy (Marley *et al*., 2004).

Soil fertility can be improved with organic fertilizers such as manure, compost or ash (Franke *et al*., 2006; Atera *et al*., 2013), which helps to improve soil texture and water-holding capacity, but also improve the organic matter in the soil. Increased soil organic matter has been shown to cause the decay of *Striga* seeds (Ayongwa *et al*., 2011). It could also increase beneficial organisms such as Arbuscular Mycorrhizal (AM) fungi, which have been shown to suppress germination of *S. hermonthica* seed (Gworgwor and Weber, 2003; Cardoso and Kuyper, 2006). Inorganic fertilizers, specifically fertilizers with a high nitrogen content, suppress the release of host root exudates that stimulate *Striga* germination (Cechin and Press, 1993). Enhancing soil fertility with inorganic fertilizers is expensive and not readily available to small growers (Hearne, 2009; Atera *et al*., 2013). Soil fertility improvement with manure or compost is a cheaper option and is also more readily available, but requires manual labour (Atera *et al*., 2013).

The 'Push-pull' technology is a combination of techniques to manage *Striga* that includes trap-cropping, intercropping and catch-cropping. It was originally developed for combatting stalk borers such as *Chilo partellus* (Swinhoe) and *Busseola fusca* (Fuller) (Khan *et al*., 2011). The push-pull technology uses Napier grass (*Pennisetum purpureum* (Schum.)) that acts as a trap crop for the stem borers (Khan *et al*., 2000), as well *Desmodium spp*., such as the *D. uncinatum* (Jacq.) (silver leaf *Desmodium*) and *D. intortum* (Mill.) (green leaf *Desmodium*); that act as repellent plant for stem borers (Demissie *et al*., 2011). *Desmodium spp*. also produce strigolactones, an exudate that causes suicidal germination of *Striga*. *Desmodium* is a non-host of *Striga*, and can therefore act as a trap-crop (Khan *et al*., 2011). Although the planting of push-pull crops requires no extra labour, the seed can be costly and not readily available. There is also little motivation for the farmer to use this technology if the plant cannot be used additionally for feeding livestock (Atera *et al*., 2013). Farmers would then rather inter-plant with a cash crops, such as soybean, which also can act as a trap crop (Carsky *et al*., 2000).

Resource-challenged farmers are reluctant to adopt practices or technologies if there is no immediate return (within the same season), or if the technology is too costly (Oswald, 2005; Franke *et al*., 2006). Imidazolinone-resistant (IR) maize combines herbicide application, as a

seed dressing, with *Striga*-resistant maize varieties (De Groote *et al*., 2008). The imidazolinone-coated seed and herbicides are costly and often not readily available (Oswald, 2005; Atera *et al*., 2013). Farmers are also reluctant to plant IR-maize because of their reduced yields and drought tolerance problems (Larsson, 2012). Hand weeding, another effective technology, is labour intensive and do not fit into the current farming practices (Atera *et al*., 2013). One of the main problems is that *Striga* appears late into the season when the weeding of these fields are already finished (Oswald, 2005).

Biological control as a strategy to manage *Striga* is an attractive option because it provides sustainable, long-term management without damaging the host-crop (Sauerborn *et al*., 2007). All biocontrol strategies focus primarily on reducing the amount of seed by either preventing seed-set and flowering, damaging the seeds, or stimulating suicidal germination to reduce the seed banks (Sauerborn *et al*., 2007). Various methods for biological control of *Striga* have been investigated. These include the use of insects, bacteria and fungi. *Smicronyx spp*. attacks *Striga* by laying their eggs in the seedpods, but this method was not very effective in controlling the weed (Sauerborn *et al*., 2007). Bacteria, such as *Pseudomonas spp*., were considered because of their ability to produce ethylene that stimulate suicidal germination (Berner *et al*., 1999), but were found not to be as effective as suicidal germination by GR24, a synthetic strigolactone (Babalola *et al*., 2007). Of the fungi evaluated, *Fusarium nygamai* (Burgess and Trimboli) and *Fusarium solani* (Mart.) Sacc. infected *Striga* seedlings and effectively arrested *Striga* development, but their use was abandoned due to the risk of mycotoxin production. Some *Fusarium equiseti* (Corda) Sacc. isolates also showed pathogenesis towards *Striga* seedlings, but it was *F. oxysporum f. sp striga* that was found most effective and appropriate for biological control of *Striga* spp. (Elzein *et al*., 2008; Yonli *et al*., 2010).

FUSARIUM OXYSPORUM AS A *STRIGA* BIOLOGICAL CONTROL AGENT

Fusarium oxysporum is an asexual fungus that is widely spread around the world. The species includes non-pathogenic and pathogenic strains that attack a wide range of hosts (Lievens *et al*., 2008). Based on the host it attacks, the species is sub-divided into *formae speciales* (Lievens *et al*., 2008). To determine a *formae speciales*, extensive pathogenicity testing needs to be conducted on a range of possible hosts as well as non-hosts (Elzein and Kroschel, 2006). Although laborious, pathogenicity testing is considered the most reliable and specific test, as molecular testing is only available for some *formae speciales* (Lievens *et al*., 2008). A *forma specialis* only attacks one host species or genus, such as *F. oxysporum* f. sp. *cubense* that

causes disease to banana (*Musa* sp.) (Gordon and Martyn, 1997). There are, however, exceptions where a *forma specialis* has a wider host range, such as *F. oxysporum* f. sp. *radices-lycopersici*, which can infect more than one host species (Manzies and Koch, 1990; Kistler, 1997). This host specification makes *F. oxysporum* attractive for biological control (Elzein *et al*., 2008). Fos was described after extensive testing of *F. oxysporum* isolates, including Foxy 2 and PSM 197, on non-host plants (Marley *et al*., 2005; Elzein and Kroschel, 2006), and the sequencing of the Internal Transcribed Spacer (ITS) region (Elzein *et al*., 2008). After this testing, isolate Foxy 2 was deposited as the reference isolate of Fos at the "Federal Biological Research Centre for Agriculture and Forestry", Berlin, Germany (Elzein and Kroschel, 2006; Elzein *et al*., 2008).

Biosafety of Fos

Concerns exist over the safety of BCA's to the environment, humans and animals (Louda *et al*., 2003). Environmental concerns include their ecological effect when released, their possible spread beyond the application site, and their host specificity. This includes possible effects on closely-related non-target species, as well as to other important non-related crop species (Louda *et al*., 2003). The concerns towards humans and animals primarily include possible toxicity, allergens (Butt and Copping, 2000) and opportunistic pathogenesis (Parke and Gurian-Sherman, 2001). It is, therefore, imperative to determine the risks associated with a possible BCA before their release.

A host range study conducted by Elzein and Kroschel (2006) indicated no risk presented by Fos isolate Foxy 2 to 25 plants tested, which included crops of solanaceous origins, tomatoes and eggplants. During a host specificity test by Zarafi *et al*. (2014), Fos isolates Foxy 2 and PSM 197 were tested against 26 potential economically important hosts, which included maize, millet, sorghum, *Desmodium* and other crops. Both isolates were pathogenic to *Striga* in Nigeria, but they also affected crops from solanaceous origins. This sparked a debate about the host specificity and purity of Foxy 2 (Avedi *et al.,* 2014).

Mycotoxin production

Previous studies found that isolates of Fos do not produce any mycotoxins of concern to animal and human health. In a study by Savard *et al*. (1997) it was determined that Fos isolate M12-4A produces fusaric acid (FA) and some of its variations. Amalfitano *et al*. (2002) also found isolates Foxy 2 and PSM 197 to produce FA and some of its esters. More recently Ndambi (2011) found that isolate Foxy 2 has the ability to produce beauvericin (BEA) in *Striga* (Ndambi,

2011). There was, however, no translocation of the mycotoxin to the sorghum plant (Ndambi, 2011). Application of BEA to *Striga* shoots showed degradation and ultimately necrosis of the cells at application point, and BEA is thus theorized to play a role in the later stages of infection by Foxy 2 (Ndambi, 2011).

Pathogenicity of Foc to *Striga*

Differences in virulence between Fos isolates to *Striga spp*. have been reported. Fos isolate PSM 197 was tested against *S. hermonthica*, *S. asiatica*, *S. gesnerioides* and the closely related plant parasitic species *Alectra vogelli* (Marley *et al*., 2005). The fungus infected the three *Striga* species, but the susceptibility of the *Striga* species differed. Isolate PSM 197 was, however, found to be not pathogenic to *A. vogelli* (Marley *et al*., 2005). Isolate Foxy 2 appears to be pathogenic to *S. hermonthica* and *S. asiatica* only, but not to *S. gesnerioides* (Elzein and Kroschel, 2004). It was also more virulent to *S. hermonthica* and *S. asiatica* than isolate PSM 197. When isolates Foxy 2, PSM 197 and M12-4A were compared, isolate M12-4A appeared to be less virulent than the other two Fos isolates (Venne *et al*., 2009).

The method used for inoculation influences the performance and virulence of Fos isolates to *Striga* plants. Venne *et al.* (2009) investigated two inoculation methods; spot inoculation and seed coating; and found that the seed coating was less effective than spot inoculation for isolate M12-4A under the same field conditions. In a separate study, Ciotola *et al*. (2000) noted that Arabic Gum, used as an adhesive to coat the seeds of sorghum and maize with isolate M12-4A, counteracted the negative effects that sorghum root-extracts had on the germination and germ tube development on isolate M12-4A (Venne *et al.*, 2009). Venne *et al.* (2009) further found that Foxy 2 and PSM 197 differed in efficacy at different geographical locations. Both isolates underperformed in Benin but showed good results in Burkina Faso when applied on sorghum. When Foxy 2 was applied to maize it did not affect *Striga* in Kenya (Avedi *et al*., 2014), which was attributed to different environments and Fos-inhibitive soils.

Effector proteins play an active role in the colonisation and infection of a host plant by *F. oxysporum*. Host specificity has been linked to these small proteins secreted in the xylem of host plants by different *formae speciales* of *F. oxysporum* (Houterman *et al*., 2007; Van der Does *et al*., 2008). Secreted In Xylem (SIX) proteins have been linked to pathogenicity and are unique to *F. oxysporum*, with the exception of the SIX1 protein that is also found in *Fusarium foetens* (Schroers, O'Donnell, Baayen & Hooftman) (Laurence *et al*., 2015), SIX2 that is found in *Fusarium verticillioides* (Ma *et al*., 2010), and SIX6 that is found in *Colletotrichum higginsianum* (Sacc.) and *C. orbiculare* (Berk.) Arx. (Kleemann *et al*., 2012; Gan *et al*., 2013). To date 14 SIX genes have been identified, of which the exact functions of each is not entirely known. SIX1

(Rep *et al*., 2005), SIX3 (Houterman *et al*., 2009), SIX4 (Thatcher *et al*., 2011), SIX5 (Ma *et al*., 2015) and SIX6 (Gawehns *et al*., 2014) proteins all play a role in fungal virulence. The SIX genes are regulated by *Sge*1 (Michielse *et al*., 2009), without which *F. oxysporum* shows diminished pathogenicity and a lack of SIX-gene function (Michielse *et al*., 2009).

DIVERSITY AND GLOBAL DISTRIBUTION OF *F. OXYSPORUM* F. SP. *STRIGAE*

Fos has been poorly studied. Phylogenetic analysis of the ITS gene region showed that the *Striga* pathogen is closely related to *F. oxysporum* f. sp. *radices-lycopersici* and *F. oxysporum* f. sp. *cubense* (Elzein *et al.,* 2008). The small number of Fos isolates studied, however, limited information available on the genetic variation of the fungus. Only a single vegetative compatibility group (VCG) has been reported, and it is unknown whether races exist for Fos (Watson *et al.,* 2007). A molecular marker has been developed for the rapid detection of the fungus in agricultural production systems (Zimmerman *et al*., 2015).

Vegetative compatibility groups

VCG analysis provides a useful technique to distinguish and subdivide *formae specialis* of *F. oxysporum* into genetically isolated clones (Kistler, 1997). The technique was originally used to divide *Aspergillus* into groups of individuals that are able to recognise each other, and has since also been used to separate *F. oxysporum* isolates into VCGs (Puhalla, 1985). More than 150 VCG's within 38 *formae speciales* of *F. oxysporum* are currently known (Katan and Di Primo, 1999). VCG-groupings are restricted within a *formae speciales*, meaning that the same VCG is not shared by different *formae speciales*.

VCG testing is not only useful when determining genetic similarity between fungal strains; it also provides insights to the population structure of a *forma specialis* (Kistler, 1997). To determination the VCG status of strains, nitrate non-utilizing auxotrophic mutants (*nit*-mutants) have to be generated and paired against each other (Leslie, 1993). Isolates belonging to the same VCG will form stable heterokaryons that become visible as wild-type growth on nutrient poor growth (Leslie, 1993). To date, only a single VCG had been identified from 14 Fos isolates collected from East and West Africa (Watson *et al*., 2007). It is imperative that further VCG testing is done on a larger population to Fos to understand the population dynamics of the *Striga* wilt fungus.

Mating type analysis

Sexually reproducing fungi are homothallic, containing both mating types, and are self-fertile. Asexual fungi, however, are mostly heterothallic, with only one of the mating types present in each strain. Mating type appears to be conserved within a VCG, with only one mating type present in each VCG (Fourie, 2008; Lievens *et al*., 2009; Lichius and Lord, 2014). A study by Southwood *et al*. (2012), however, found both mating type genes in the same VCG. It is believed that *F. oxysporum* lost its ability to reproduce sexually due to mutations in its mating type (*MAT*) genes, however, these genes were shown to be functional (Arie et al., 2000; Yun et al., 2000) (Arie *et al*., 2000; Yun *et al*., 2000). *MAT* genes regulate compatibility in sexually reproducing fungi.

The mating type genes in *F. oxysporum* occur as two idiomorphic functional alleles on a single locus (Lievens *et al*., 2009), known as *MAT1-1* or *MAT1-2*. The *MAT1-1* allele contains three genes, *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*, whereas the *MAT1-2* allele only consists of one gene, *MAT1-2-1* (Yun *et al*., 2000). The *MAT1* gene translates to functional proteins that have regulatory functions towards sex pheromone precursor and receptor genes, where communication between potential mating strains is mediated via these pheromones (Kim and Borkovich 2004; Lichius and Lord, 2014). The function of *MAT* genes in asexual fungi is, however, still unclear. Adam *et al*. (2011) found that *MAT* genes influenced carotenoid stimulation in *F. verticillioides*, and suggested that these genes have a regulatory function other than pheromone regulation in asexual fungi.

Global distribution of Fos

Fos is only found in *Striga*-affected areas in Africa. Very few efforts have been made to collect a population of Fos isolates, as the focus of past surveys was always to find suitable and highly virulent isolates of Fos for the development of a BCA. Isolates have been collected from Mali, Niger, Burkina Faso (Watson, personal communication, 2012), Nigeria (Marley et al., 2005), Ghana (Abbasher *et al.*, 1995) and Kenya (Avedi *et al*., 2014) before.

Detection of Fos

Molecular markers are valuable to rapidly and accurately detect fungal plant pathogens. In *F. oxysporum*, such markers are particularly important, as morphological features do not allow the identification of *formae speciales*, while pathogenicity testing is often laborious and time-consuming. To develop accurate molecular markers, a proper knowledge of the diversity of the fungus is required, and appropriate methods are required to identify variants within and between *formae speciales* of *F. oxysporum*.

Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) analysis uses restriction enzymes and PCR amplification to create a DNA fingerprint of restriction fragments. When compared with DNA fingerprints of other species or isolates, unique fragments can be identified to develop PCR markers that will then amplify only the selected gene area (Semagn *et al*., 2006; Chial, 2008). This method is extremely useful if no DNA sequence data is available (Chial, 2008). AFLP has a high reproducibility, and PCR markers derived from AFLP products are said to be specific (Nayaka *et al*., 2011). However, if the screening for a unique AFLP fragment does not include a wide range of isolates, some similarities might be missed in the DNA fingerprints (Nayaka *et al*., 2011), and may therefore account for non-specificity. AFLP analysis only shows variation in fragment lengths and it is thus possible that mutations within fragments of similar lengths might not be observed (Nayaka *et al*., 2011).

Zimmerman *et al*. (2015) developed a DNA marker from an AFLP fingerprint to accurately determine the quantities of Fos in tropical soils by quantitative PCR. This study included 40 Fos isolates as well as different *formae speciales* of *F. oxysporum* (Zimmermann *et al*., 2015). The marker accurately detected all Fos isolates tested, but also amplified a DNA fragment of *F. oxysporum f. sp. melonis* (Fom) of Israeli origin with the same fragment size and DNA sequence to that of Fos (Zimmermann *et al*., 2015). This put the specificity of the marker to question. No other *F. oxysporum* isolates were found to be amplified, and Zimmerman *et al*. (2015) concluded that the marker would be specific in African soils, since the Fom isolate was restricted to Israel.

SCAR amplification

Sequence-characterized amplified region (SCAR)-based markers are developed from random amplified polymorphic DNA (RAPD) or AFLP-derived unique DNA fragments (Agarwal *et al*., 2008; Nayaka *et al*., 2011). SCAR markers are locus-specific and show high reproducibility (Semagn *et al*., 2006). A SCAR primer set was developed for Fos (Watson *et al*., 2007), but this was later found to be not as specific as previously determined (Watson, 2013, pers. comm.). The non-specificity of this marker could be attributed to the small number of isolates used to develop the marker. Since the identification of a suitable and unique fragment for SCAR markers relies on fragment size, other mutations such as single nucleotide polymorphisms (SNP's) may be missed (Nybom *et al*., 2014).

CONCLUSION

There are 28 known species of *Striga* within the family Orobanchaceae that are hemi-parasites (Mohamed *et al*., 2001). Three of the most agro-economically important species include *S. hermonthica* and *S.asiatica*; which attack maize, sorghum and millet; and *S. gesnerioides*, which attacks legumes such as cowpea (Mohamed *et al*., 2001). It is estimated that 3.7 mil ha in Africa is currently infested with the pathogen (De Groote, 2007), causing annual losses of up to US$ 7 billion (Atera *et al*., 2012). Several methods and technologies are available to combat the pest, including hand-weeding (Atera *et al*., 2013), push-pull crops (Khan *et al*., 2011) and IR-maize (De Groote *et al*., 2008). The high costs, time constraints and ineffectiveness when used individually hamper the uptake of these technologies among farmers (Oswald, 2005; Hearne, 2009).

Striga primarily affects subsistence farmers (Parker, 2009). Since new agricultural technologies and methods to combat *Striga* are not readily adopted by farmers, a more cost-effective, sustainable and dependable method is needed for the successful management of the parasite (Elzein *et al*. 2008). Biological control, as part of an IPM system, is gaining more favour because of its host specificity, environmental safety and sustainability (Seier, 2005). Fos isolates proved to be excellent BCA candidates (Venne *et al*., 2009, Kangethe *et al*., 2016), but little has been done to understand Fos diversity and its safety to humans and animals (Elzein *et al*. 2008). Watson *et al*. (2007) used only 14 Fos isolates, collected in countries throughout West Africa with one isolate from East Africa, and found that they belong to a single VCG. Additionally, Elzein *et al*. (2008) used two isolates (Foxy 2 and PSM 197) and showed that their ITS gene areas are phylogenetically similar. It is, therefore, important to analyse more Fos isolates to determine the true genetic diversity of the pathogen. In Chapter 2 of this study, isolates were collected from *Striga* in Kenya and Nigeria and VCG typed. Their phylogenetic relationship was also determined using two conserved gene areas, namely the translocation elongation factor 1α and the mitochondrial small sub-unit. MAT genes in the collected isolates were also determined. Understanding the diversity in Fos can help with the selection of Fos strains for development as a possible BCA of *Striga*.

A molecular marker was designed to rapidly identify Fos (Watson *et al*., 2007; Zimmermann *et al*., 2015). There is, however, also a need to accurately detect the representative groups within Fos (Zimmermann *et al*., 2015; Kangethe *et al*., 2016). In Chapter 3, single nucleotide polymorphisms (SNP's) were used for marker design of the Kenyan and Nigerian populations of Fos. SNPs are single nucleotide variations within a DNA sequence (Nybom *et al*., 2014) more

predominant in introns than exons. When present in an exon, the mutation can either change the amino acid sequence or have no effect (Agarwal *et al*., 2008). Due to low mutation rates, uniqueness and exclusivity of these SNP's within a species, group within a species or individual, SNP-based markers have a high reproducibility, which makes it a superlative tool to study diversity (Idrees and Irshad, 2014; Nybom *et al*., 2014). A large amount of sequence data is needed for comparison and detection of a true SNP (Semagn *et al*., 2006). Access to sequence databases online means that this method can easily be shared and used inter-laboratory (Nybom *et al*., 2014). SNP markers can easily be designed and when used, are highly reproducible and specific.

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TABLES AND FIGURES

Figure 1: Three economically important *Striga* species. From left to right: *Striga hermonthica* (Source: M. de Klerk), *S. asiatica* (Source: D.L. Nickrent (http://parasiticplants.siu.edu/Orobanchaceae/Striga.asiatica.html) and *S. gesnerioides* (Source: N. Dreber http://www.biota-africa.org/inc_showphoto_download_ba.php?ID=62& viewmode=0).

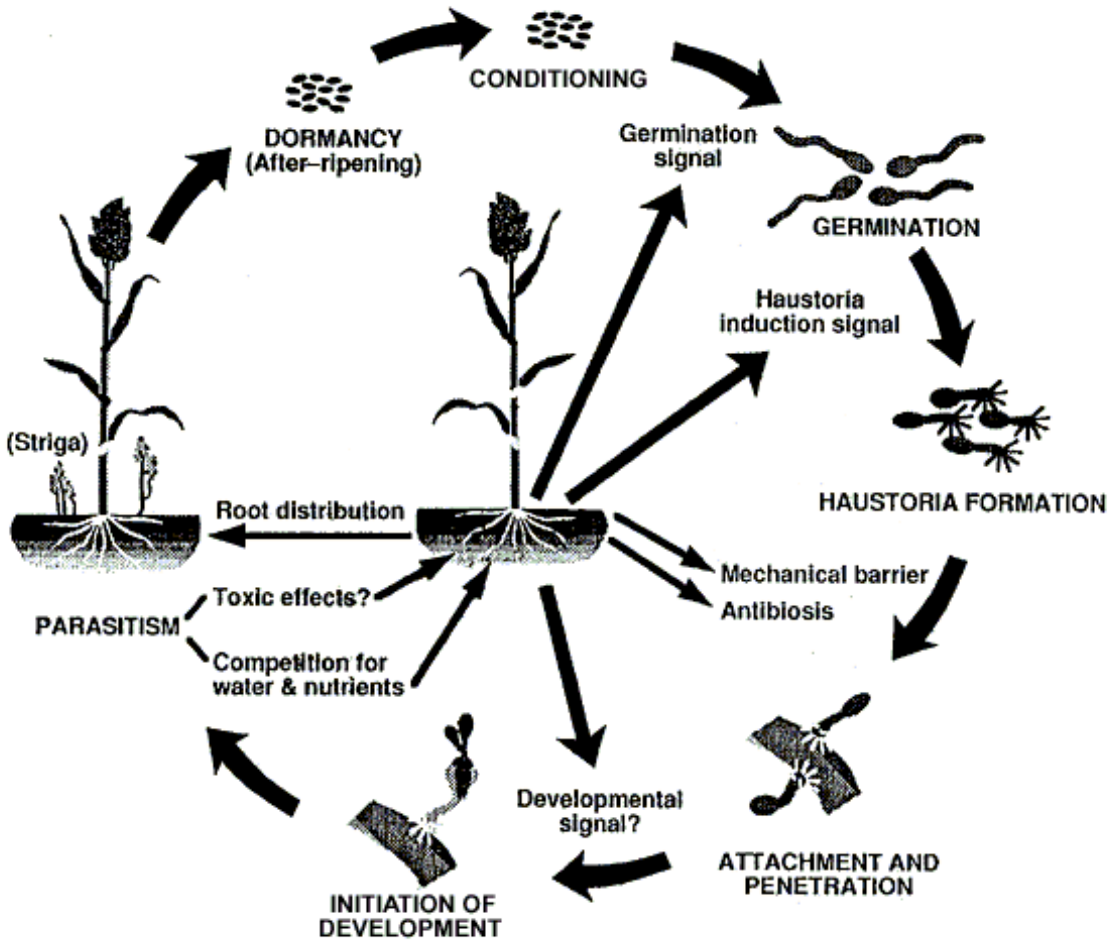

Figure 2: The life-cycle of *Striga* and its interactions with sorghum (Ejeta and Butler, 1993).

CHAPTER 2

The characterization of populations of *Fusarium oxysporum* f. sp. *strigae* from Kenya and Nigeria

ABSTRACT

Striga hermonthica, an obligate hemi-parasite of economically important staple food crops, is a major constraint to the production of maize and sorghum by subsistence farmers in Africa. To manage the parasitic weed, a soil-borne fungus *Fusarium oxysporum* f. sp. *strigae* (Fos) has been identified as potential biological control agent (BCA). Fos causes disease to *Striga* species, but does not affect other agricultural crops. In this study, 28 *F. oxysporum* isolates from diseased *Striga* plants in Kenya and 30 isolates from diseased plants in Nigeria were collected. The isolates were characterized by vegetative compatibility group (VCG) testing, phylogenetic analysis of the translation elongation factor 1α (TEF) and mitochondrial small sub-unit (MsSSU) gene areas, as well as mating type genotyping. Seven VCGs were identified in Nigeria and one in Kenya. A combined phylogenetic tree of the TEF and MtSSU gene regions revealed that Fos isolates from Nigeria and Kenya belong to separate clades. Mating type genotyping showed that the Kenyan population contained only one mating type gene, *MAT1-1*. Both mating type genes, however, were present in isolates from Nigeria. These results suggest that Fos has a polyphyletic nature, which could play a role in its efficacy as BCA.

INTRODUCTION

Striga hermonthica (Del) Beth is an obligate hemi-parasite of maize (*Zea mays* L.), sorghum (*Sorghum bicolour* (L.) Moench), millet (*Pennisetum glaucum* L.) and rice (*Oryza sativa* L.). The parasite is native to Sub-Saharan Africa, and is thought to have originated in Ethiopia or Sudan (Watson *et al*., 2007; Spallek *et al*., 2013). *Striga hermonthica* is considered one of the most destructive pests of agricultural crops in eastern and western Africa, where it causes losses of US$ 7 billion annually (Atera *et al*., 2012). Each *Striga* plant is able to produce up to 100 000 seeds that can easily spread to unaffected soils with contaminated crop seeds, humans and farming equipment, and to a lesser extent by animal movement, wind and water (Berner *et al*., 1994; Mourik, 2007; Hearne, 2009; Atera *et al*., 2012). The high number of seeds produced leads to a rapid build-up in the soil, where it can lie dormant until conditions are favourable for germination (Mourik, 2007).

Striga seeds germinate in response to a variety of volatiles released by the roots of the host plant, which include strigolactone and sorgolactone (Cardoso *et al*., 2011). The germinating seedling then parasitizes the host's roots for nutrients and water through the attachment of a haustorium (Cardoso *et al*., 2011; Ndambi *et al*., 2011). This causes severe stunting of the host plants, thereby reducing their ability to produce a harvest. Yield losses are estimated to be between 40 and 100% annually, which lead to devastating economic costs to commercial and subsistence farmers. Severe *Striga* infestations of farmers' fields can result in food insecurity and, in extreme cases, cause farmers to abandon their land (Mourik, 2007; Andersson and Halvarsson, 2011; Atera *et al*., 2012).

Non-pathogenic *F. oxysporum* strains have been successfully employed as biological control agents (BCAs) for the control of *Fusarium* wilt diseases of flax (Alabouvette and Couteaudier, 1992), watermelon (Larkin *et al*., 1996), cucumber (Mandeel and Baker, 1991), tomato (Fuchs *et al*., 1997) and carnations (Garibaldi *et al*., 1992). The non-pathogenic strains can be antagonistic or directly compete with plant pathogens, or they can induce the host's own resistance mechanisms (Fravel *et al*., 2003). Alabouvette and Couteaudier (1992) argued that a non-pathogenic *F. oxysporum* isolate can be applied to a non-compatible host as biocontrol agent, where it will protect the non-compatible host from pathogenic strains of *F. oxysporum*.

Two Fos isolates, Foxy 2 and FK3 (Watson *et al*., 2007), were proposed as BCA of *S. hermonthica* as part of an Integrated *Striga* Management system (ISM) (Abbasher *et al*., 1995; Marley *et al*., 1999; Watson *et al*., 2007). Isolate Foxy 2 was collected in Western Africa, and proposed as a BCA after it has been found to be highly virulent towards *Striga* in Western Africa

(Abbasher *et al*., 1995; Marley *et al*., 1999; Watson *et al*., 2007). Foxy 2, however, was not as effective in Eastern Africa (Avedi *et al*., 2014; Kangethe *et al*., 2016). Efficacy could differ due to the chosen application method, environmental differences and genetic differentiation in *Striga,* or due to geographical isolation (Bozkurt *et al.,* 2015; Zimmerman *et al.,* 2015). Isolate FK3, isolated from diseased *Striga* in Kenya, was found to be more effective in controlling *Striga* in eastern Africa and are now under development as BCA (Kangethe *et al*., 2016).

Vegetative compatibility is widely employed as a means to study diversity in *F. oxysporum.* Vegetative compatibility groups (VCG) are determined when the hyphae of complementary nitrate non-utilizing (*nit*)-mutants, generated on chlorate medium (CLM), anastomose to form stable heterokaryons on minimal medium (MM). Isolates in the same VCG are assumed to be essentially clones of each other (Leslie and Summerell, 2006). Studies on VCGs of Fos in Africa found only one VCG to be present (Watson *et al*., 2007). This study, however, was limited to 12 isolates from West Africa and one isolate from East Africa (Watson *et al*., 2007). VCGs, although very useful to characterize genetic diversity, does not measure the genetic distance between phenotypes (Fourie *et al*. 2009).

Phylogenetic studies are especially advantageous in the analysis of clonally reproducing species as mutations are conserved and can thus be used to accurately portray evolution within the species (Kistler, 1997). Elzein *et al*. (2008) compared the internal transcribed spacer (ITS) gene sequences of Fos isolates Foxy 2 and PSM 197, but did not find any sequence variability between the two isolates. The ITS gene region, however, has been reported to be unsuitable for accurate phylogenetic studies of *Fusarium* species, as two divergent ITS types exist that are non-orthologous (O'Donnell and Cigelnik, 1997). The translation elongation factor 1α (TEF) and the mitochondrial small sub-unit (MtSSU) genes have been shown to be concordant (O'Donnell *et al*., 1998; Skovgaard *et al*., 2001) and combined data sets provided better resolution regarding clade stability (O'Donnell *et al*. 1998). In addition, the presence of mating type (*MAT*) idiomorphs *MAT1-1* and *MAT1-2* in isolates and across phylogenetic clades can be informative on the potential of sexual reproduction or other mechanisms that may contribute to the generation of genetic diversity (O'Donnell *et al*., 2004). Even though both functional *MAT* genes were reported to be present in *F. oxysporum* (Yun *et al.*, 2000), no teleomorph has been observed before (Lievens and Thomma, 2008; Fourie *et al*., 2009; Michielse and Rep, 2009).

The objective of this study was to use VCG testing, a multi-gene phylogeny (TEF and MtSSU) and *MAT* identity to characterize diversity of Fos strains from eastern and western Africa. VCG analysis could provide insight into the genetic diversity of a larger number of Foc isolates, while phylogenetic studies could provide insight into genetic distance between VCGs.

MATERIALS AND METHODS

Fungal Isolates

Fungal isolates were collected from diseased *S. hermonthica* plants in Kenya in August 2012 (28 isolates) and in Nigeria in November 2012 (30 isolates). Primary isolations were made from the root collar of each plant by cutting off 15-mm pieces of the stems. These were then surface disinfected and divided into four pieces. The four pieces of each stem was placed onto ½ strength potato dextrose agar containing streptomycin (PDA+) and incubated until fungal growth was observed. The cultures were then purified on *Fusarium* selective medium (FSM), single-spored and maintained in 15% glycerol at -80^{o}C in the culture collection of the Department of Plant Pathology, Stellenbosch University (Table 1). Isolates, previously characterized as Fos by Prof Alan Watson at the McGill University, Montreal, Canada, were also obtained. These isolates included Foxy 2, PSM 197 and M12-4A. Five Fos isolates, named FK1–FK5, were further donated by Dr Henry Wainwright from Real IPM, Nairobi, Kenya. These isolates are also maintained in the culture collection at Stellenbosch University (Table 1).

Morphological identification

The fungal isolates collected from Kenya and Nigeria were plated out on carnation leaf agar (CLA) and ½ strength PDA for morphological and cultural identification, respectively, and incubated at 25^{o}C under white and near-UV light for 14 days. Isolates grown on PDA were inspected for colony colour and growth rate after 7 and 10 days; whereas micro- and macro-conidia, conidiophores and production of chlamydospores of cultures grown on CLA were studied using a light microscope after 14 days (Leslie and Summerell, 2006).

Vegetative compatibility group analysis

VCG testing of *F. oxysporum* isolates were performed as described by Leslie and Summerell (2006). All isolates were first plated onto 1.5-1.8% chlorate medium to generate *nit* mutants (Puhalla, 1985). *Nit*-mutants, identified as sparse, fast-growing colonies, were then typed as *nit*-1, *nit*-3 and *nit*-M mutants by using selective media containing different nitrogen sources. VCGs were determined by pairing *nit*-1 or *nit*-3 mutants of each isolate against the corresponding *nit*-M mutant of all the *F. oxysporum* isolates in collection respectively. A compatible reaction was recorded when a heterokaryon occurred between mutants (Leslie and Ploetz, 1990; Leslie and Summerell, 2006). All the mutants used for VCG analysis were stored on MM slants at the culture collection of the Department of Plant Pathology, Stellenbosch University.

Pathogenicity testing

Representative isolates of *F. oxysporum* were selected for pathogenicity testing based on their VCG status. These included 25 isolates from Nigeria and four isolates from Kenya. Pathogenicity was determined in two independent studies performed by Dr A. Elzein (International Institute of Tropical Agriculture) in Nigeria and Dr H. Wainwright (Real IPM, Nairobi) in Kenya. Only a preliminary pathogenicity test was done for a few strains in Kenya, which represented VCG 04708. This test was designed to consist of three treatments; an injection, a drench and a water-control (Wainwright, unpublished data). A liquid inoculum was prepared in 200 ml distilled water with dissolved rice (5 g) and peptone (2 g), grown for 72 hrs on a shaker at room temperature. The number of *S. hermonthica* plants that emerged, together with the number of wilted *S. hermonthica* plants, were recorded and compared. In Nigeria, the inoculum was prepared by growing the fungus on sterilized wheat for 3 weeks. The inoculum was then added to steam-sterilized soil at a concentration of 10 g/kg soil, where after *Striga* seed was added at a concentration of 15 g/kg soil. The soil was left for 1 week at 28°C to prime the *Striga* seed before the maize or sorghum seed was added (Elzein, unpublished data). The number of *Striga* plants that emerged for each pot, together with the number of *Striga* plants showing wilting symptoms, were recorded and compared. Dry weight of both the host and Striga plants across the whole experiment was recorded and compared (Elzein, unpublished data).

DNA isolation, PCR amplification and sequencing

Mycelium was harvested from *Fusarium* isolates grown on PDA at 25^{o}C for approximately 7 days. DNA extraction was performed with the Promega Wizard® SV DNA purification kit (Fitchburg, USA). The mycelia were scraped off the surface of PDA plates with a sterile scalpel blade, and deposited in Eppendorf tubes to which 400 µl of lysis buffer and glass beads were added, before vigorous shaking (30 Hertz for 5 min) using a MM301 shaker (Retsch®, Haan, Germany). The Eppendorf tubes were then incubated in a water bath at 65°C for 10 min before the lysate was centrifuged at 4 000 rpm for 4 min. The supernatant was then extracted and deposited into columns provided by the kit. The DNA extraction was performed according to the manufacturer's recommendations. DNA quality and concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

Amplification of the translation elongation factor 1α (TEF) and mitochondrial small sub-unit (MtSSU) genes was performed by PCR, using an Eppendorf Mastercycler® Gradient PCR machine (Eppendorf Scientific, Hamburg, Germany). The primer set EF1 and EF2 (O'Donnell *et al*. 1998) was used for amplification of the TEF 1α gene, with the following PCR conditions:

denaturation at 94°C for 2 min for one cycle; then amplification at 94°C for 45 s, 53°C for 45 s, and 72°C for 1 min for 40 cycles; with a final extension step at 72°C for 7 min. The MtSSU region was amplified by using the primers MS1 and MS2 (White *et al*. 1990) with the following PCR conditions: denaturation at 94°C for 2 min for one cycle; followed by 35 cycles consisting of 94°C for 45 s, 53°C for 45 s, and 72°C for 90 sec; with a final extension step at 72°C for 5 min. The PCR reaction for both gene-areas consisted of 1 unit of Taq polymerase (BIOTAQ, UK), 1x PCR buffer, 3.5 mM $MgCl_2$, 200 µM of each dNTP, bovine serum albumin (BSA) (only for TEF), 0.2 µM of each primer, and genomic DNA (50-100 ng), with a total reaction volume of 40 µl.

PCR products were visualized on 1% agarose gel, and purified for sequencing with the High Pure PCR Product purification Kit (Roche Applied Biochemicals, Indianapolis, IN, USA). Sequencing was performed in both directions with the original PCR primers, at the Central Analytical Facilities (CAF) at Stellenbosch University on an ABI 377 automated sequencer (Foster City, California, United States). Sequences were edited in Geneious Pro 5.3.3 (Biomatters, Auckland, New Zealand) and blasted against the nucleotide sequence database on GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm identity.

Phylogenetic analysis

A phylogenetic tree was prepared with the combined data set of the TEF and MtSSU gene regions of Fos isolates from Kenya and Nigeria. Several reference isolates representing different *formae speciales* and non-pathogens of *F. oxysporum*, obtained from GenBank, were also included in the study (O'Donnell *et al*., 2004; Fourie *et al*., 2009). The outgroup consisted of two isolates of *Fusarium commune* Skovgaard *et al*. (2001) (NRRL 22903 and 28387). The data sets was aligned on MAFFT, version 7 (http://mafft.cbrc.jp/alignment/server/) and edited with Seal (V 2.0 a11 Carbon). Maximum parsimony analysis was conducted on PAUP V4. The heuristic search option with ten random taxon additions was used, and tree bisection and reconstruction (TBR) was used as the branch swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. Bootstrap analysis of 1 000 heuristic search replicates was performed to estimate the reliability of inferred phylogenies. The conditions used for the bootstrap were the same as that used for the maximum parsimony (MP) analysis. A partition homogeneity test was completed in PAUP V4 to determine comparability of the two data sets phylogenies based on maximum likelihood (ML) analysis were inferred using PhyMLv3.0 (Guindon *et al.*, 2010) (http://www.atgc-montpellier.fr/phyml/). The best-fit model was inferred using the SMS Model test program (V1.8.1). The TEF and

MtSSU dataset used the GTR + I substitution model, and bootstrap confidence values were based on 1 000 repetitions.

Identification of *MAT* idiomorphs in Fos

The mating type of each Fos isolate was determined using primer pairs F*alpha1* and F*alpha2* (Arie *et al*., 2000) targeting the *MAT*1-1-1 gene, and GfMAT2c (Steenkamp *et al*. 2000) and FF1 Foc (Visser *et al*., 2010) primers to target the *MAT*1-2-1 gene (Table 2). Conditions for the PCRs were as follows: 95°C for 7 min; 35 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 1 min; with a final extension step of 7 min at 72°C. PCR reactions were conducted with KAPA Ready Mix (Kapa Biosystems, Cape Town, South Africa), containing 0.2 µM of each primer and 2 µl genomic DNA (50-100 ng) in a total volume of 25 µl. The presence of both mating type amplicons were confirmed by sequencing of the PCR products and blasting of the sequence on Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Morphological and molecular identification

In total, 28 isolates from Kenya and 30 isolates from Nigeria were identified as *F. oxysporum*. These isolates produced macroconidia in false heads on short monophialides, and chlamydospores singly or in pairs, which is typical of the species. Chlamydospores were produced at the earliest after 8 days, with 70% of the isolates producing chlamydospores after 14 days. The colony colour of isolates ranged from cream with pink to purple shades.

Vegetative Compatibility Grouping

Seven VCG's were identified in the Nigerian Fos population, and were designated VCG 04701-04707. VCG 04701 included seven, and VCG 04702 five isolates, respectively. VCG 04703-04707 consists of only two isolates each. Eight single member VCGs (SMVs) were also identified and provisionally named SMV 1–8. Four isolates were self-incompatible, and two isolates were unable to produce stable *nit*-mutants and excluded from further analysis (Table 1). All Fos isolates from Kenya were compatible to each other, and designated VCG 04708. This VCG included FK 1–5, which were previously isolated from *S. hermonthica* in Kenya. Additionally, two Fos isolates from the Kenyan collection were found to be self-incompatible (CAV 6215 and CAV 6209).

Pathogenicity testing

Only two out of four isolates selected from Kenya for pathogenicity testing, were pathogenic towards *S. hermonthica*, based on wilting symptoms in *S. hermontica* plants after treatment. Two VCG 04708 isolates (CAV 6073 and CAV 6081) did not cause any disease symptoms on *S. hermonthica* plants (Table 1). Twenty one isolates from Nigeria, which included at least one isolate per VCG and SMV 1, 2, 3, 4, 5 and 7, were all pathogenic to *Striga* (Table 1).

Phylogenetics

The partition homogeneity test supported the combination of the TEF and MtSSU datasets (*P*> 0.1). The MP and ML analysis yielded similar topologies for Fos isolates. The Fos isolates showed to be polyphyletic and were separated into two different clades according to country origin (Fig. 1). The isolates collected in Nigeria (VCG 04701-04707 and SMV1-8) clustered in a separate lineage with 100% bootstrap support, but were related to representatives of *F.oxysporum f. sp. aechmeae*, *F. oxysporum f. sp. batatas,* a non-pathogenic *F. oxysporum* isolated from onion, and two non-pathogenic *F. oxysporum* isolates in Clade 4. The isolates collected from Kenya (VCG 04708) grouped in Clade 2 with *F. oxysporum f. sp. lini, F. oxysporum f. sp. dianthi, F. oxysporum f. sp. melonis*, and two *F. oxysporum* isolates described as non-pathogens (O'Donnell *et al.* 2004). All Fos isolates were separated in one phylogenetic species (PS2) as described by Laurence *et al*. (2014) (Fig. 1).

Identification of *MAT* idiomorphs

The mating type idiomorphs in Fos isolates were confirmed by the amplification of the 370-bp *MAT1-1* fragment and the 750-bp *MAT1*-2 fragment. *MAT1-1* was present in VCGs 04701, 04702, 04704, 04705, 04708 and SMV's 4, 6 and 7, and *MAT1-2* in VCGs 04701, 04702, 04703, 04706, 04707 and SMV's 1, 2, 5 and 8 (Fig. 1). Both mating types were found in VCGs 04701 and 04702. In the Kenyan Fos population, all VCG 04708 isolates in Clade 2 (O'Donnell *et al.,* 2004) contained the *MAT1-1* idiomorph only. Both mating type idiomorphs were present in Fos isolates from Nigeria in Clade 4 (O'Donnell *et al.,* 2004). *MAT1-1* genes of Fos shared a 100% similarity to *MAT1-1-1* on GenBank, and the *MAT1*-2 gene was 100% similar to the *MAT1-2-1* gene.

DISCUSSION

The diversity of Fos in Africa was greater than previously anticipated, with isolates from Nigeria and Kenya forming genetically separated lineages in the combined TEF and MtSSU phylogenetic analysis. This suggests that Fos has a polyphyletic origin, similar to *F. oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *melonis* (Kistler, 1997), *F. oxysporum* f. sp. *betae* (Harveson and Rush, 1997), *F. oxysporum* f. sp. *cepae* (Swift *et al*., 2002) and *F. oxysporum* f. sp. *vasinfectum* (Wang *et al*., 2010). Earlier studies by Watson *et al*. (2007) and Elzein *et al*. (2008) on a limited number (14 isolates) of Fos isolates did not detect this polyphyletic nature of Fos, probably because of the small samples size that they used. It is believed that Fos evolved in at least two separate occasions, once in West Africa, and once in Kenya. The polyphyletic origin of a *formae speciales* could be ascribed to geographical separation (Skovgaard *et al*., 2001; Fourie, 2009; Silva *et al*., 2014), as genetic exchange could be hindered by the lack of gene flow when certain populations become geographically isolated (Wang *et al*., 2004; Wang *et al*., 2010). It could also point to a possible host specialization, as the *S. hermonthica* population in Kenya has been found to be genetically different to those found in Eastern Africa (Bozkurt *et al.,* 2015).

VCGs within a *formae specialis* of *F. oxysporum* are assumed to be clonally related (Gordon and Martyn, 1997; Kistler, 1997). The Fos isolates collected from *Striga* were divided into eight VCGs, of which seven VCGs as well as eight SMVs were present in the Nigerian population. More VCGs within a population often indicate an older population (Swift *et al*., 2002), which suggests that the Fos in Nigeria has evolved earlier than in Kenya. All isolates from Kenya belonged to only one VCG (VCG 04708), which contained a single mating type idiomorph within a monophyletic phylogenetic clade. This would suggest that VCG 04708 shares a single, successful pathogenic ancestor. The isolates from Nigeria also formed a single lineage, but consisted of multiple VCGs that contain both mating types. These VCGs could be assumed to result from a single or multiple mutations in any of the loci governing vegetative compatibility (Correll, 1991; Taylor *et al*., 1999; Jimenez-Gasco *et al*., 2004). The greater number of VCGs could also suggest the presence of an ancient or cryptic sexual stage (Leslie, 1993; Desjardins, 1995) which, together with the presence of both mating types with a 1:1 distribution within the population have contributed to genetic diversity (Arie *et al*., 2000; Yun *et al*., 2000; Ramirez-Prado *et al*., 2008). This, however, is unlikely as all VCGs and SMVs group within the same lineage.

Asexual reproduction has the advantage of preserving advantageous adaptations and mutations, even though this type of reproduction is seen as an evolutionary stalemate (Alby and Bennett, 2010; Seidl and Thomma, 2014). When Fos isolates were first collected with the goal to explore the possibility of a BCA in the 1990's, only one isolate was collected in Kenya (K7-5A/ CAV 6000) (Watson *et al*., 2007). In the current study, no isolates from Kenya could match isolate K7-5A genetically, or VCG 04701 which it belongs to. Rather, isolate K7-5A belonged to the same VCG (VCG 04701) and phylogenetic clade as Foxy 2, which is from West Africa. It is, therefore, possible that the original population that isolate K7-5A belonged to disappeared in Kenya due to a lack of fitness, or due to changes in the host (Bozkurt *et al*. 2015). Interestingly, when Foxy 2 was tested as a potential biocontrol agent in Kenya, the fungus could also not be recovered from diseases *Striga* plants afterwards. Rather, VCG 04708 were recovered, which is the only Fos VCG in the country, and contains both pathogenic and non-pathogenic isolates.

VCGs can be used as an indicator of an unknown *F. oxysporum* isolates' pathogenic potential (Leslie, 1993). In this study, however, two isolates in VCG 04708 were non-pathogenic. This was unusual, as VCG 04708 also contained several Fos isolates in Kenya. The discrepancy might be due to experimental error, and it is recommended that pathogenicity tests with these cultures be repeated. Still, it worthy to note that Appel and Gordon (1994) also found non-pathogenic isolates in the same VCGs of pathogenic isolates of *F. oxysporum* f. sp. *melonis.* They argued that the non-pathogenic isolates belonging to these VCGs could have lost their pathogenic ability or virulence, as they found one isolate that was remarkably similar to the isolates within one of the VCGs, sharing mtDNA with those isolates. According to Gordon and Martyn (1997), non-pathogenic isolates could share alleles responsible for vegetative compatibility with the pathogenic isolates. Wang *et al*. (2010), however, argued that non-pathogenic isolates that group with a pathogenic VCG could be the antecedents of the pathogenic isolates within that VCG, and that this demonstrates that these VCGs developed independently.

As Fos will be used by subsistence farmers as a BCA to combat *Striga*, it is importance to consider Fos diversity and their geographical locations, as there is evidence that the different Fos strains performs differently in diverse locations (Avedi *et al*., 2014; Zimmerman *et al*., 2015). Ideally, several strains should be included together in a 'ready to use' product, which could account for Fos diversity. It is also important to develop molecular markers to rapidly and accurately detect all strains of Fos, for which diversity within Fos needs to be considered. The current study, thus, provide baseline information on the diversity of Fos for screening and detection of BCA for use against *S. hermonthica* in West and East Africa.

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TABLES AND FIGURES

Table 1. *Fusarium oxysporum* isolates collected from diseased *Striga hermonthica* plants in East and West Africa.

Original Number	CAV[a] number	ID	VCG [b]	Pathogenicity status [c]	Mating type	Field trail/ farmer	Collector	Country	Crop
K7-5A	6000	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	Marie Ciotola	Kenya	Unknown
M12-4A	6001	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	Marie Ciotola	Mali	Unknown
M5-1B	6002	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	Marie Ciotola	Mali	Unknown
M6-1A	6003	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	Marie Ciotola	Mali	Unknown
M13-1B	6004	*F. oxysporum* f. sp. *strigae*	04701	P	1	Unknown	Marie Ciotola	Mali	Unknown
N1-12A	6005	*F. oxysporum* f. sp. *strigae*	04701	P	1	Unknown	Marie Ciotola	Niger	Unknown
N2-5A	6006	*F. oxysporum* f. sp. *strigae*	04701	P	1	Unknown	M. Ciotola	Niger	Unknown
N5-1A	6007	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	M. Ciotola	Niger	Unknown
B7-3A	6008	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	M. Ciotola	Burkina Faso	Unknown
F oxy 2	6009	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	A. Abbasher	N Ghana	Unknown
PSM 197	6010	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	PS. Marley	Nigeria	Unknown
M8-5A	6011	*F. oxysporum* f. sp. *strigae*	04701	P	2	Unknown	M. Ciotola	Mali	Unknown
N95/1	6135	*F. oxysporum*	04701	P	2	Farmer	A. Elzein/US	Nigeria	Sorghum
N192/2	6156	*F. oxysporum*	04701	P	2	Farmer	A. Elzein/US	Nigeria	Sorghum
N222	6167	*F. oxysporum*	04701	P	2	Farmer	A. Elzein/US	Nigeria	Millet
N222/B	6233	*F. oxysporum*	04701	-	1	Farmer	A. Elzein/US	Nigeria	Millet
N230/1	6171	*F. oxysporum*	04701	-	2	Farmer	A. Elzein/US	Nigeria	Millet
N248/A	6180	*F. oxysporum*	04701	-	2	Bio-control trial	A. Elzein/US	Nigeria	Maize
N271/3	6199	*F. oxysporum*	04701	-	2	On farm trial	A. Elzein/US	Nigeria	Maize
N4/1	6092	*F. oxysporum*	04702	P	1	Farmer	A. Elzein/US	Nigeria	Sorghum
N39/3	6116	*F. oxysporum*	04702	P	1	Farmer	A. Elzein/US	Nigeria	Maize
N55/1	6126	*F. oxysporum*	04702	P	1	Farmer	A. Elzein/US	Nigeria	Maize
N183/1	6153	*F. oxysporum*	04702	P	2	Farmer	A. Elzein/US	Nigeria	Sorghum
N220	6166	*F. oxysporum*	04702	P	2	Farmer	A. Elzein/US	Nigeria	Millet
N3/1	6091	*F. oxysporum*	04703	P	2	Farmer	A. Elzein/US	Nigeria	Sorghum

N260/4	6236	*F. oxysporum*	04703	-	2	Farmer	A. Elzein/US	Nigeria	Maize
N155/3	6142	*F. oxysporum*	04704	P	1	Farmer	A. Elzein/US	Nigeria	Millet
N155/2	6143	*F. oxysporum*	04704	P	1	Farmer	A. Elzein/US	Nigeria	Millet
N2/1	6090	*F. oxysporum*	04705	P	1	Farmer	A. Elzein/US	Nigeria	Sorghum
N158/1	6144	*F. oxysporum*	04705	P	1	Farmer	A. Elzein/US	Nigeria	Maize
N106/1	6137	*F. oxysporum*	04706	P	2	Farmer	A. Elzein/US	Nigeria	Millet
N96/2	6230	*F. oxysporum*	04706	-	2	Farmer	A. Elzein/US	Nigeria	Sorghum
N196/1	6160	*F. oxysporum*	04707	P	2	Farmer	A. Elzein/US	Nigeria	Sorghum
N126/3	6140	*F. oxysporum*	04707	P	2	Farmer	A. Elzein/US	Nigeria	Millet
N112/2	6139	*F. oxysporum*	SMV 1	P	2	Farmer	A. Elzein/US	Nigeria	Millet
N175/1	6148	*F. oxysporum*	SMV 2	P	2	Farmer	A. Elzein/US	Nigeria	Sorghum
N208/1	6162	*F. oxysporum*	SMV 3	P	-	Farmer	A. Elzein/US	Nigeria	Sorghum
N203/1	6161	*F. oxysporum*	SMV 4	P	1	Farmer	A. Elzein/US	Nigeria	Sorghum
N177/1	6149	*F. oxysporum*	SMV 5	P	2	Farmer	A. Elzein/US	Nigeria	Sorghum
N256/4A	6186	*F. oxysporum*	SMV 6	-	1	Mother trial	A. Elzein/US	Nigeria	Maize
N39/4	6115	*F. oxysporum*	SMV 7	P	1	Farmer	A. Elzein/US	Nigeria	Maize
N288/3	6240	*F. oxysporum*	SMV 8	-	2	Farmer	A. Elzein/US	Nigeria	Sorghum
A6/1	6016	*F. oxysporum*	04708	-	1	Farmer	H. Wainwright/US	Kenya	Unknown
A7/3	6019	*F. oxysporum*	04708	-	1	Farmer	H. Wainwright/US	Kenya	Unknown
A8/1	6020	*F. oxysporum*	04708	-	1	Farmer	H. Wainwright/US	Kenya	Unknown
A8/2	6021	*F. oxysporum*	04708	-	1	Farmer	H. Wainwright/US	Kenya	Unknown
A12/1	6023	*F. oxysporum*	04708	P	1	Farmer	H. Wainwright/US	Kenya	Unknown
C2/1	6039	*F. oxysporum*	04708	-	1	Farmer	H. Wainwright/US	Kenya	Unknown
C6/2	6043	*F. oxysporum*	04708	-	1	Farmer	H. Wainwright/US	Kenya	Unknown
C6/3	6044	*F. oxysporum*	04708	P	1	Farmer	H. Wainwright/US	Kenya	Unknown
C7/1	6045	*F. oxysporum*	04708	-	1	Farmer	H. Wainwright/US	Kenya	Unknown
F1/1	6050	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F4/1	6057	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F7/1	6063	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F8/3	6067	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F10/3	6073	*F. oxysporum*	04708	NP	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize

F12/1	6075	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F12/3	6077	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F13/2	6079	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F14/1	6081	*F. oxysporum*	04708	NP	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F18/1	6087	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F20.3	6210	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F22.1	6211	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F22.2	6212	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
F26.1	6218	*F. oxysporum*	04708	-	1	Foxy 2 Field Trail	H. Wainwright/US	Kenya	Maize
FK1	6222	*F. oxysporum* f. sp. *strigae*	04708	-	1	Unknown	H. Wainwright/US	Kenya	Unknown
FK2	6223	*F. oxysporum* f. sp. *strigae*	04708	-	1	Unknown	H. Wainwright/US	Kenya	Unknown
FK3	6224	*F. oxysporum* f. sp. *strigae*	04708	-	1	Unknown	H. Wainwright/US	Kenya	Unknown
FK4	6225	*F. oxysporum* f. sp. *strigae*	04708	-	1	Unknown	H. Wainwright/US	Kenya	Unknown
FK5	6226	*F. oxysporum* f. sp. *strigae*	04708	-	1	Unknown	H. Wainwright/US	Kenya	Unknown

[a]Culture collection at the Deptartment of Plant Pathology, University of Stellenbosch
[b]Vegetative compatibility groups of *F. oxysporum* associated with *Striga*
[c]Pathogenic status: P = Pathogenic, NP = Not Pathogenic, - = Not tested. Unpublished data kindly provided by Dr Watson, Dr Elzein and Dr Wainwright.

Table 2. Primers, primer sequences and annealing temperatures used as putative molecular markers for the identification of *Fusarium oxysporum* f. sp. *strigae*.

Target area	Primers	Sequence	Annealing Temp (°C)
TEF-1α	EF1 / EF2	ATGGGTAAGGA(A/G)GACAAGAC / GGA(G/A)GTACCAGT(G/C)ATCATGTT	53
MtSSU	MS1 / MS2	CAGCAGTCAAGAATATTAGTCAATG / GCGGATTATCGAATTAAATAAC	53
MAT1-1	Falpha1-F / Falpha2-R	CGGTCAYGAGTATCTTCCTG / GATGTAGATGGAGGGTTCAA	58
MAT1-2	Gfmat2c-F / FF1 Foc-R	AGCGTCATTATTCGATCAAG / GTATCTTCTGTCCACCACAG	58

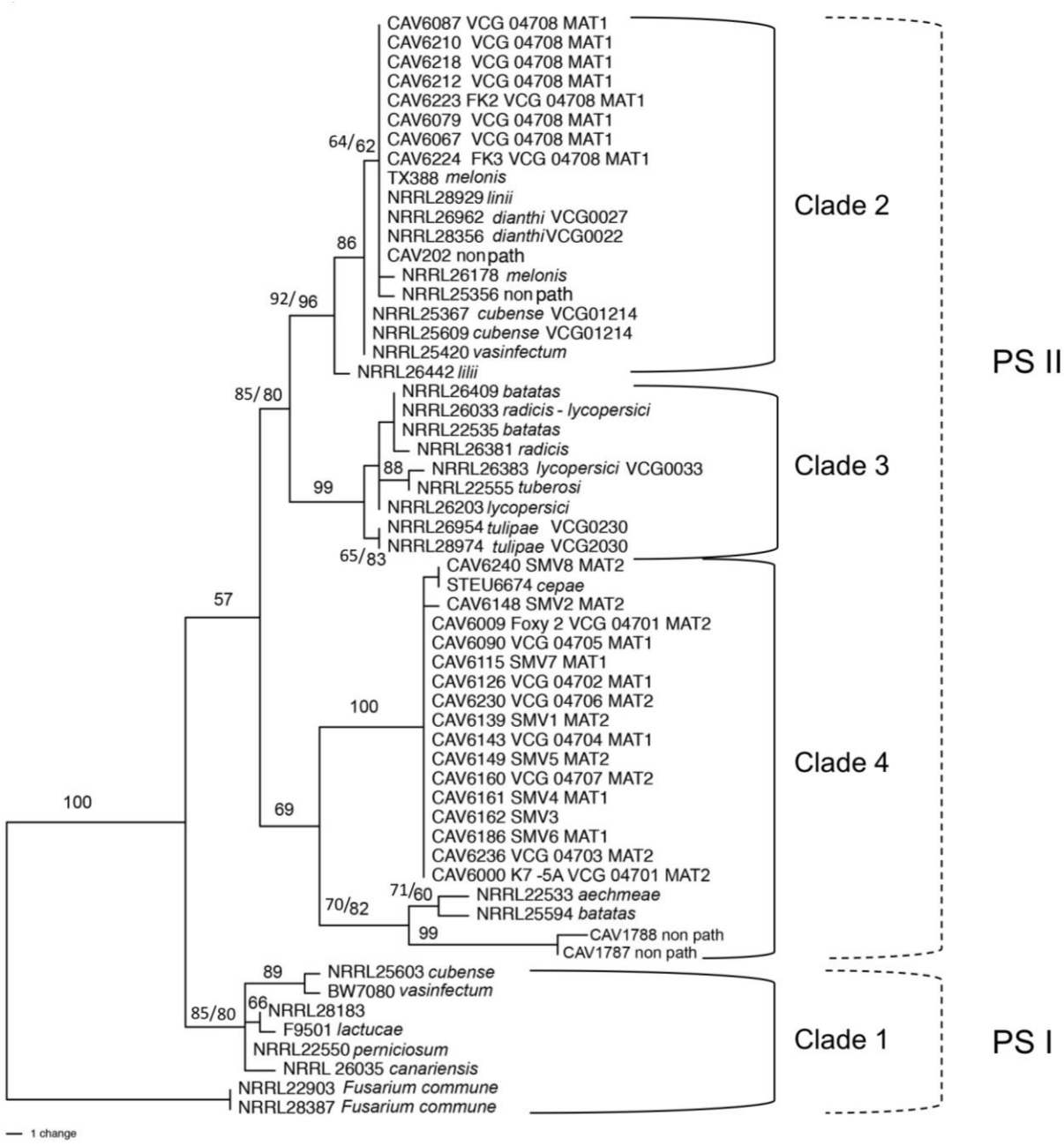

Figure 1. A phylogenetic tree (RI = 0.959 and CI = 0.802) inferred from the combined data sets of the Translation Elongation Factor 1α and Mitochondrial Small Sub-Unit gene regions for *Fusarium oxysporum* f. sp*. strigae* isolates. Bootstrap values generated from the maximum parsimony analysis, followed by the values generated from the maximum likelihood analysis, are shown at the internodes. Clade designation (1-4) is according to O'Donnell *et al*. (2004), while the Phylogenetic Species boundary *sensu* Laurence *et al*. (2014) is indicated (PS I and PS II). The tree is rooted with two *Fusarium commune* isolates, NRRL 22903 and NRRL 28387.

CHAPTER 3

Development of molecular tools to identify the two *Fusarium oxysporum* f. sp. *strigae* biocontrol agents Foxy2 and FK3

ABSTRACT

Fusarium species are frequently associated with *Striga hermonthica*, a destructive parasitic weed of maize and sorghum in Africa. Of the *Fusarium* species, *F. oxysporum* f. sp. *strigae* (Fos) can kill the parasite, but does not affect maize and sorghum. Two highly virulent Fos isolates, Foxy2 and FK3, have been identified as potential biological control agents (BCA) for application against *S. hermonthica*. The first objective of this study was to identify all *Fusarium* species associated with *Striga*, and the second was to design molecular markers to distinguish the Foxy2 and FK3 from other strains of Fos and all other *Fusarium* species associated with *S. hermonthica*. *Striga hermonthica* plants were collected in Nigeria and Kenya, *Fusarium* species isolated from stem tissue and identified using morphological and molecular techniques. Six *Fusarium* species were associated with *S. hermonthica*, of which *F. longipes* and the *F. incarnatum-equiseti* species complex were associated with the weed for the first time. Markers specific to Foxy 2 and closely related vegetative compatibility groups present in Nigeria were successfully developed from the translocation elongation factor α-1 gene region, and for FK3 and VCG 04708 in Kenya from the Secreted-In-Xylem 14 gene region. Both markers could accurately detect target Fos DNA in the presence of *S. asiatica* DNA. The molecular markers can be used to test for the efficacy and dissemination of the BCAs following field application.

INTRODUCTION

The parasitic plant *Striga hermonthica* (Del.) is known as 'witchweed' in the tropical savannas and Sahelian region of Africa. This is due to their early attachment and parasitism of economically important crops like maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench), and their late emergence above ground (Mohamed *et al*., 2001). The genus *Striga* consists of 28 species, of which the more important species are *S. hermonthica*, *S. asiatica* (L.) Kuntze and *S. gesnerioides* (Willd.) (Mohamed *et al.,* 2001; Spallek *et al.,* 2013). All three species can cause extensive yield losses to their host crops (Parker, 2009; Atera *et al.,* 2013).

Various strategies have been suggested to manage *Striga* species, of which imidazolinone-resistant (IR) maize (De Groote *et al*., 2008), the push-pull technology (*Desmodium* species*,* interplanting) (Khan *et al*., 2011) and hand weeding (Atera *et al*., 2013) proved promising. An integrated management system is, however, needed to maximise efficiency and minimise the development of resistant *Striga* plants. The task to sustainably manage *Striga* is further complicated by the existence of massive seed-banks that have built up due to poor management efforts, and the reluctance of farmers to adopt new technologies (Marley *et al*., 2004; Oswald, 2005; Franke *et al*., 2006). One possible means to improve *Striga* management is to use biological control agents (BCA's), that infect and kill the parasite, in an integrated pest management strategy. The BCAs, however, need to be host-specific and poses a low risk to humans, animals and the environment (Hearne, 2009; Zarafi *at al*., 2014)

Studies on the fungal flora of *Striga* plants showed that the genus *Fusarium* is most commonly associated with the parasite (Venne *et al*., 2008). Of the *Fusarium* species, *F. oxysporum* was most common and caused disease to *S. hermonthica* in Nigeria and Kenya (Marley *et al*., 1999; Kagot *et al*., 2014). Strains pathogenic to *Striga* plants were not pathogenic to any agricultural crops, and were consequently designated *F. oxysporum* f. sp. *striga*e (Fos) (Elzein and Kroschel, 2006; Elzein *et al*., 2008). It was also found that Fos produces no mycotoxins of concern to mammalian health, and the fungus was thus deemed safe for development as a BCA (Savard *et al*., 1997; Amalfitano *et al*., 2002). Several strains of Fos was explored for their potential as BCA's (Ciotola *et al*., 2000; Marley *et al*., 2004; Yonli *et al*., 2004; Venne *et al*., 2009; Sunda *et al*., 2012), of which two highly virulent isolates showed most potential. These were Foxy2, which was isolated in Ghana (Abbasher *et al.*, 1995), and FK3 that was isolated in Kenya (Avedi *et al*., 2014).

The survival, competitive ability and multiplication of a BCA isolate after field applications is important when determining its efficacy and distribution (Recorbet *et al*., 2003; Chandra *et al*., 2011). In the past, monitoring of BCAs include sampling from soil and

plant material, for the counting of colony-forming units and immunoassays. These traditional techniques were time consuming, laborious and frequently identified false positives (Schmit and Lodge, 2005). DNA technologies, such as DNA sequencing and fingerprinting, now provide an opportunity to develop molecular markers to rapidly and accurately detect microbes in environmental samples such as plants and soil (Schmit and Lodge, 2005; Watson *et al.*, 2006). Watson *et al.* (2006) developed a SCAR marker for Fos, which was later found to be non-specific (Watson, personal communication, 2013). This could be due to using only 14 isolates to test the marker (Watson *et al*., 2006). Zimmerman *et al.* (2015) has since used an AFLP amplicon to design a PCR marker for Fos. The marker was successful, but also amplified *F. oxysporum f. sp. melonis* (Zimmerman *et al*., 2015).

The objectives of this study were: (1) To determine the most prevalent species of *Fusarium*, associated with *Striga*. (2) To develop molecular markers to accurately identify two potential *Striga* BCA; Foxy2 and FK3, respectively. These markers would be used to monitor the distribution and efficacy of the BCA during and after field application, and to differentiate them from other closely related *Fusaria*.

METHODS AND MATERIALS

Fungal Isolates

Isolates used in this study were collected from diseased *Striga* plant material that showed wilting symptoms in Kenya (53 samples) and Nigeria (67 samples) in 2012. Isolations were made by cutting off 15-mm pieces of the stems at the root-collar, surface-sterilising them, and placing them on ½ strength potato dextrose agar containing streptomycin (PDA+). Plates were incubated at room temperature until fungal growth appeared, where after hyphal material was transferred to *Fusarium* selective media (FSM). *Fusarium* cultures were then selected, single-spored and stored in 15% glycerol at -80ºC at the culture collection of the Department of Plant Pathology, Stellenbosch University. Twelve isolates previously characterized as Fos were also obtained from Prof A. Watson from the McGill University, Montreal in Canada, including Foxy2 and PSM 197. A further five isolates, FK1-FK5, were obtained from Dr. Henry Wainwright from Real IPM, Nairobi, Kenya.

Morphological characterization

The fungal isolates were plated out on carnation leaf agar (CLA) and ½ PDA for morphological identification, and incubated at 25°C under white and near-UV light for 14 days. Isolates grown on CLA were studied under a light microscope after 14 days and inspected for micro- and macroconidia, conidiophores and the production of chlamydospores

(Leslie and Summerell 2006). Isolates grown on PDA were inspected to determine colony colour and growth rate after 7 and 10 days (Nelson *et al*., 1983).

DNA extraction

Fungal DNA was extracted from 7-day-old mycelia and deposited into Eppendorf tubes. DNA extraction was then performed by using the Promega Wizard® SV DNA purification System kit (Fitchburg, USA), according to the manufacturer's recommendations. Quality and concentrations were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

DNA from *S. asiatica* seed, donated by the Horticulture Department of Stellenbosch University, was extracted using cetyl trimethyl ammonium bromide (CTAB) (1.4 M NaCl, 2% (w/v) CTAB, 0.1 M Tris at pH 8) extraction buffer. The seed were first freeze dried and milled to a powder, after which it was suspended in 750 µl 2% CTAB buffer, and incubated for 60 min at 65°C. The supernatant (500 µl) was diluted with an equal amount of chloroform, briefly vortexed and centrifuged at 12 000 g for 5 min. The upper phase of the supernatant (400 µl) was then added to an equal volume of cold (≈ -20°C) isopropanol, and incubated for 20 min at room temperature. The isopropanol mix was centrifuged for 5 min at 12 000 g, after which the supernatant was discarded and the pellet was suspended in 500 µl 70% ethanol (≈ -20°C) and incubated again at room temperature for 20 min. The mixture was centrifuged for 5 min at 12 000 g, the ethanol discarded and the pellet dried. Once dry, the pellet was re-suspended in 200 µl TE buffer (pH 8.0) and incubated at 4°C overnight. After the incubation step, 20 µl 7.5 M ammonium acetate (NH_4OAc) and 200 µl chloroform was added to the tube, mixed, and centrifuged for 5 min at 12 000 g. The supernatant was transferred to an Eppendorf tube and 500 µl of cold (≈ -20°C) 100% ethanol added, incubated for 2 hrs at -20°C, and centrifuged for 15 min at 12 000 g. The pellet was then washed twice with cold 70% ethanol and dried before being suspended in 75 µl nuclease-free water.

PCR and sequencing

Species identities of the *Fusarium* isolates were confirmed by sequencing of the translocation elongation factor 1α (TEF) gene area, and by blasting the consensus sequences against the Genbank database. For marker development, unique single nucleotide polymorphisms (SNPs) specific to Foxy2 and FK3 were identified from the TEF, Secreted-In-Xylem 14 (*SIX14)*, Calmodulin, β-tubulin, Intergenic spacer region (IGS), Mitochondrial Small Subunit (MtSSU), RNA polymerase II subunits 1 and 2 (RPB1 and RPB2) genes.

Amplification of the TEF, MtSSU and IGS gene areas was performed with published primers (Table 1). The reaction volume consisted of 40 µl, and contained 1x PCR buffer, 3.5 mM $MgCl_2$, 200 µM for each dNTP, 1 unit of Taq polymerase (BIOTAQ, UK), 10x bovine serum albumin (BSA), 0.4 µM, 0.4 µM and 0.2 µM of each primer for each gene area, respectively, and genomic DNA (50-100 ng). The following PCR conditions were followed for the TEF gene region: Denaturation at 94°C for 2 min for one cycle; then for 40 cycles at 94°C for 45 s, 53°C for 45 s, and 72°C for 1 min; with the final extension step at 72°C for 7 min. PCR conditions for the MtSSU gene region were: Denaturation at 94°C for 2 min for one cycle; then 35 cycles consisting of 94°C for 45 s, 53°C for 45 s, and 72°C for 90 sec; with the final extension step at 72°C for 5 min. PCR conditions for the IGS gene region were as follows: Denaturation at 94°C for 2 min for one cycle; then for 35 cycles at 94°C for 30 s, 55°C for 1 min 30 s, and 72°C for 1 min; with the final extension step at 72°C for 5 min.

PCR amplification of the Calmodulin and β-tubulin and gene areas was done using published primers (Table 1), with a total reaction volume of 20 µl, containing 1x KAPA ready mix (Kapa Biosystems, Cape Town, South Africa) 0.4 µM of each primer and genomic DNA (50-100 ng). Reaction cycling conditions for the Calmodulin gene area were as follows: Denaturation at 94°C for 2 min for one cycle, 94°C for 50 s, 58°C for 50 s, 72°C for 1 min in a cycle of 35 times, with final extinction at 72°C for 7 min. Reaction cycling conditions for the β-tubulin and gene were as follows: Denaturation at 94°C for 5 min for one cycle, 94°C for 45 s, 55°C for 30 s, 72°C for 1 min 30 s, in a cycle of 35 times, with final extinction at 72°C for 6 min.

Amplification of the *SIX14,* RPB1 and RPB2 gene areas was done using published primers (Table 1) with a reaction volume of 25 µl, containing 1x KAPA ready mix (Kapa Biosystems, Cape Town, South Africa), 0.2 µM of each primer and genomic DNA (50-100 ng). Reaction cycling conditions for *SIX14* were as follows: Denaturation at 94°C for 2 min for one cycle, 94°C for 45 s, 63°C for 30 s, 72°C for 1 min in a cycle of 35 times, with final extinction at 72°C for 5 min. PCR conditions for RPB1 were: Denaturation at 95°C for 7 min for one cycle, 95°C for 45 s, 60°C for 45 s, 72°C for 1 min, in a cycle of 35 times, with final extinction at 72°C for 7 min. Reaction cycling conditions for RPB2 were: Denaturation at 95°C for 7 min for one cycle, 95°C for 45 s, 55°C for 45 s, 72°C for 1 min, in a cycle of 35 times, with final extinction at 72°C for 7 min.

All PCR products were visualized on 1% agarose gels, cleaned with the Promega Wizard® SV DNA purification System kit (Fitchburg, USA), and sequenced at the Central Analytical Facilities (CAF) at Stellenbosch University on an ABI 377 automated sequencer (Foster City, California, United States). The sequences were edited in Geneious Pro 5.3.3 (Biomatters, Auckland, New Zealand).

Sequence analysis, SNP identification and primer design

Consensus sequences of the TEF, *SIX14*, Calmodulin, β-tubulin, IGS, MtSSU, RPB1 and RPB2 genes were obtained from Genbank for several *formae speciales* of *F. oxysporum*. These include *F. oxysporum* f. sp. *cubense, lycopersici, phaseoli, pisi, lini, dianthi, narcissi, cepae, melonis, batatas, cucumerinum, canariensis, fragariae, radices, ciceris, radices-lycopersici, lilii, passiflorae, perniciosum, raphani, narcissi, tuberosi, vasinfectum, canariensis, erythroxyli, glycines, cyclaminis, conglutinans, chrysanthemi* and *rapae*. Non-pathogenic *F. oxysporum* strains were also included. Consensus sequences of representative isolates (Foxy2 and FK3) were added to the respective databases. Databases alignment were done using MAFFT, version 7 (http://mafft.cbrc.jp/alignment/server/) and reviewed in MEGA (Version 7, http://www.megasoftware.net/). Databases were manually inspected to find SNPs unique to each target.

Primers were designed with SNPs at the 3' side of both forward and reverse primers, where possible. The suitability of candidate primer sequences was determined by calculating the T_m delta, and by checking for self and cross primer-dimers. PCR amplification were done in a reaction volume of 25 µl using 1 x KAPA ready mix (Kapa Biosystems, Cape Town, South Africa), containing 0.2 µM of each primer and 2 µl of genomic DNA (50-100 ng). Reaction cycling conditions were as follows: Denaturation at 95°C for 5 min for one cycle, 95°C for 45 s, annealing temperature (Ta) for 30 s, 72°C for 30 s in a cycle of 35 times, with final extension at 72°C for 5 min. All PCR products were visualized using 1% agarose gels.

Primer specificity and sensitivity

The primer pairs were first tested against a subset of 22 target isolates to confirm specificity (Table 2), before the test was extended for the validation to a larger population (Table 3). The subset of 22 target isolates included representatives of all known Fos VCGs, including FK3 and Foxy 2, *Fusarium* species isolated from *Striga*, and other *formae speciales* of *F. oxysporum*. The markers were then validated against 67 *F. oxysporum* isolates associated with *Striga* plants, 60 *Fusarium* strains, isolated from *Striga* plants, as well as 27 other *formae speciales* of *F. oxysporum* (Table 3).

To test the sensitivity of markers, FK3 and Foxy2 were diluted to concentrations of 5 µg/µl, 0.5 µg/µl and 0.05 µg/µl. PCR amplification was done in a reaction volume of 25 µl containing 1 x KAPA ready mix (Kapa Biosystems, Cape Town, South Africa), 0.2 µM of each primer and 2 µl of genomic DNA. PCR reactions were performed using the optimized protocols and genomic DNA at the three different concentrations, both with and without *S. asiatica* genomic DNA at a concentration of 50 ng/µl. PCR's were repeated twice to confirm results.

RESULTS

Identification of *Fusarium* species

Fusarium species isolated from *S. hermonthica* plants in this study included *Fusarium equiseti* (Corda) Saccardo, *Fusarium incarnatum* (Roberge) Saccardo, *Fusarium incarnatum-equiseti* species complex (O'Donnell *et al*., 2009), *Fusarium longipes* (Wollenweber & Reinking), *Fusarium semitectum* (Berkeley & Ravenel) and *F. oxysporum* (Table 3). *Fusarium oxysporum* was most common, with 28 strains collected in Kenya and 30 in Nigeria. *Fusarium longipes* and *F. semitectum* were only isolated from Nigerian plant material, and *F. incarnatum-equiseti species complex* from Kenyan plant material (Table 3).

Culturally, *F. equiseti* produced white mycelia which, over time, became discoloured to display a brown tint. Macroconidia were present and was sickle shaped with 4-6 septa. *Fusarium incarnatum* had white mycelia, which grew in a scale-like fashion, with mycelia gaining an orange tint over time. Their macroconidia were straight with 3-4 septa. *Fusarium longipes* produced mycelia gaining an orange tint over time, and their macroconidia had 5-7 septa. *Fusarium semitectum* produced macroconidia with 3-5 septa, slightly curved, and formed white mycelia that gained a brown tint over time. The *Fusarium incarnatum-equiseti species complex* produced white mycelia that gained a brown tint over time, and macroconidia that was slightly curved with 3-5 septa. The morphological identities of all *Fusarium* species were confirmed by TEF sequencing.

Primer selection and development

Markers for Foxy2 were designed from the TEF (two primer sets) and RPB2 (three primer sets) genes, and markers for FK3 from SIX14 (two forward and three reverse primers) and RPB2 (two primer sets) genes. The calmodulin, β-tubulin, IGS, RPB1 and MtSSU gene areas did not contain any unique SNPs for Foxy2 and FK3. RPB2 contained unique SNPs, but the primers developed from these SNP's were not specific to Foxy2 or FK3, and non-target DNA was amplified.

For Foxy2, Foxy2-F1 (SNP transversion C/A at site 245 bp) and Foxy2-R1 (SNP transversion T/G at site 370 bp and transition A/G at site 382 bp) amplified a fragment of 130 bp for the target DNA, and did not produce any non-specific amplicons (Fig. 1). The Foxy2 primer set consisted of Foxy2-F1 (ATCGCGCGTCCTTTGCCA) and Foxy2-R1 (GCTCATTGAGGCTGTGAGAATGGC) (Fig 1), with an optimal annealing temperature of 61°C. Three primer sets were designed for FK3 from the *SIX*14 gene. Of these, primer sets FK3-F (SNP transitions C/T at site 21 bp, C/T at site 23 bp, GG/AA at site 24 bp and transversion C/G at site 28 bp) and FK3-R (SNP transitions C/T at site 301 bp and C/T at

site 317 bp) (Fig. 2) were chosen as they only amplified target DNA and not any non-specific amplicons. The FK3 marker was designed to amplify a fragment of 272 bp, and PCR amplification was done using primer set FK3F (GACTTCTGCCCACTGTAAATG) and FK3R (AATCAGGATTCAGTGACAACA) at an optimal annealing temperature of 56°C (Fig 2).

Primer specificity and sensitivity

The primer pairs successfully amplified the correct fragment length for targeted strains, but not for any isolates representing other *formae speciales* of *F. oxysporum* or other *Fusarium* species associated with *Striga* plants (Table 3; Figs. 3, 4). The primer pair specific to FK3 detected only Fos VCG 04708 isolates, and produced a fragment with an amplicon size of 272 bp (Fig. 3). The Foxy2 marker detected all Fos isolates associated with *Striga* plants in Nigeria, including VCGs 04701-04707 and SMVs 1-8. These VCGs and SNPs are all closely related (Chapter 2), and the PCR produced an amplification product of 130 bp (Fig. 4).

The FK3 primer set detected DNA from pure fungal mycelia at 0.1 ng/µl, and from fungal DNA mixed with *S. asiatica* DNA at 1 ng/µl (Fig. 5). The primer set for Foxy2 were less sensitive, and could only detect Fos DNA at a concentration of 10 ng/µl, both in the presence and absence of *S. asiatica* DNA (Fig. 6). The bioassay was repeated with Fos DNA in the presence of *S. asiatica* DNA at 10 ng/µl with the TEF primer set to confirm that Fos DNA was unaffected by *S. asiatica* DNA and the extraction protocol.

DISCUSSION

In this study, molecular markers were developed that can accurately identify FK3 and Foxy2. These markers would allow scientists to reliably detect the BCAs by PCR following field-application, while reducing the time and labour required for traditional methods such as immune-assays and CFU counting (Ward *et al.*, 2004). Both Fos markers were validated against a large population of isolates that included several Fos and other *Striga*-associated *Fusarium* species, as well as other *formae speciales* of *F. oxysporum*. The FK3 marker detected FK3 and all Fos VCG 04708 isolates present in Kenya, whereas the Foxy2 marker detected the BCA from Nigeria and all other Fos VCGs and SMVs in the country. It is, thus, believed that they would not result in specificity problems when additional Fos strains are collected in the two countries, as did the SCAR markers developed by Watson *et al*. (2006). The Fos markers developed in this study can also be used together with the Fos-specific marker developed by Zimmerman *et al*. (2015). They can assist in studies on the epidemiology, spread and population structure of Fos in Kenya and Nigeria, and to monitor Fos BCAs of *Striga* plants when introduced into farmer fields. They should, however, be

tested for the detection of Fos isolates from other African countries, even though the Foxy2 marker detected Fos strains from Mali, Niger, Burkina Faso and Ghana in the current study.

It is important to consider the risk of molecular markers losing functionality when they are developed, even if they are accurate during development and validation. Loss of function for molecular markers can be attributed to mutations in the gene region where the marker was designed to bind. The risk of marker failure for Foxy2 due to a mutation is minimal, as it was developed from the TEF gene region (Geiser *et al*., 2004; Chandra *et al*., 2011, Bertoldo *et al*., 2015), however, primer specificity can be influenced if non-*striga* related *F. oxysporum* strains already exists with the same mutations. The TEF gene region is involved in protein translation processes, and is highly conserved (Chandra *et al*., 2011, Bertoldo *et al*., 2015). The TEF gene region has been one of the most phylogenetically informative gene regions for *F. oxysporum* (O'Donnell *et al*., 1998; Bertoldo *et al*., 2015). Risk for marker failure is more probable for FK3, as it was designed from the *SIX*14 gene region. This gene region forms part of a mobile chromosome, and mutations are more likely to occur due to the presence of transposable elements associated with horizontal gene transfer (Ma *et al*., 2010; Ma *et al*., 2013; Schmidt *et al*., 2013). *SIX* genes, are effectors involved with pathogenicity in *F. oxysporum* (Rep *et al*., 2005; Houterman *et al*., 2009; Thatcher *et al*., 2011; Gawehns *et al*., 2014; Ma *et al*., 2015), and have previously been used to distinguish between races of *F. oxysporum* f. sp. *lycopersici* (Lievens *et al*., 2009) and *F. oxysporum* f. sp. *cubense* (Czislowski *et al*., 2017), and between geographically different isolates of *F. oxysporum* f. sp. *vasinfectum* (Chakrabarti *et al*., 2011).

The application of BCAs in agricultural fields would be most valuable if they could be quantitatively detected in environmental samples such as plants and soil. It was possible to detect Fos DNA with the Fos markers when it was combined with *Striga* DNA. This suggests that it is possible to identify the *Striga* fungus in diseased plants, even though a higher concentration of fungal DNA was required to detect it in the precence of plant DNA. To improve detection, Fos collected in environmental samples might require the PCR to be performed in a nested or semi-nested fashion to ensure amplification of very low Fos DNA. Alternatively, quantitative real-time PCR could also be used. Such assays, however, would require higher skilled personnel, as well as improvement on the design of current markers.

The decline in *Striga* damage in some agricultural fields is often attributed to '*Striga* suppressive soils', a phenomenon caused by natural soil-borne antagonists (Abbasher *et al*., 1998). Several *Fusarium* species are pathogens of *Striga*, and were thus proposed to play a role in the decline of *Striga* damage in certain areas (Abbasher *et al*., 1996; Abbasher *et al*., 1998; Yonli *et al*., 2010; Kagot *et al*., 2014). Of these, *F. oxysporum* has always been most prevalent (Marley *et al*., 1999; Kagot *et al*., 2014), as was demonstrated in the current study. This dominance of *F. oxysporum* could be explained by the greater fitness as a pathogen

compared to that of other *Fusarium* species, as was demonstrated in earlier studies (Abbasher *et al*., 1998; Marley *et al*., 1999; Kagot *et al*., 2014). This makes *F. oxysporum* attractive as a BCA. The *F. incarnatum-equiseti* species complex and *F. longipes* have been associated with *Striga* for the first time, but their virulence to the parasite has not been determined. The *F. incarnatum-equiseti* species complex occurs on a wide range of plants and in subtropical areas (O'Donnell *et al*., 2009), but is considered a weak pathogen of plants as their disease associations are difficult to demonstrate (Leslie and Summerell, 2011). *Fusarium longipes* has been described as a sub-tropical species, occurring in the soils of grasslands (Summerell *et al.*, 2011).

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TABLES AND FIGURES

Table 1. Primer sets used for the sequencing of gene areas of *Fusarium oxysporum* f. sp. *strigae*.

Gene area	Primers	Sequence F/R	Annealing Temp (°C)	PCR/Sequencing	Reference
TEF-1α	EF1	ATGGGTAAGGA(A/G)GACAAGAC	53	PCR and Sequencing	O'Donnell *et al*. 1998
	EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT		PCR and Sequencing	O'Donnell *et al*. 1998
MtSSU	MS1	CAGCAGTCAAGAATATTAGTCAATG	53	PCR and Sequencing	White *et al*. 1990
	MS2	GCGGATTATCGAATTAAATAAC		PCR and Sequencing	White *et al*. 1990
SIX14	SIX14Fol-F	ATAAAGTGCGACTGGACTTCTGCC	63	PCR and Sequencing	Taylor *et al*., 2016
	SIX14Fol-R	ACCCCCATCCACATTCCTAAGCGA		PCR and Sequencing	Taylor *et al*., 2016
Calmodulin	Cal-228F	GAGTTCAAGGAGGCCTTCTCCC	58	PCR and Sequencing	Carbone and Kohn, 1999
	CAL-737R	CATCTTTCTGGCCATCATGG		PCR and Sequencing	Carbone and Kohn, 1999
β-tubulin	T1	AACATGCGTGAGATTGTAAGT	55	PCR and Sequencing	O'Donnell and Cigelnik, 1997
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		PCR and Sequencing	Glass and Donaldson, 1995
IGS	PNFO	CCCGCCTGGCTGCGTCCGACTC	55	PCR and Sequencing	Edel *et al*., 1995)
	PNF22	CAAGCATATGACTACTGGC		PCR and Sequencing	Edel *et al*., 1995)
RPB1	Fa	CAYAARGARTCYATGATGGGWC	60	PCR and Sequencing	Hofstetter *et al*., 2007
	R8	CAATGAGACCTTCTCGACCAGC		Sequencing	O'Donnell *et al*., 2010
	F5	ATGGGTATYGTCCAGGAYTC		Sequencing	O'Donnell *et al*., 2010
	F6	CTGCTGGTGGTATCATTCACG		Sequencing	O'Donnell *et al*., 2010
	F7	CRACACAGAAGAGTTTGAAGG		Sequencing	O'Donnell *et al*., 2010
	F8	TTCTTCCACGCCATGGCTGGTCG		Sequencing	O'Donnell *et al*., 2010
	R9	TCARGCCCATGCGAGAGTTGTC		Sequencing	O'Donnell *et al*., 2010
	G2R	GTCATYTGDGTDGCDGGYTCDCC		PCR and Sequencing	O'Donnell *et al*., 2010

RPB2	5f2	GGGGWGAYCAGAAGAAGGC	55	PCR and Sequencing	Reep *et al*., 2004
	7cr	CCCATRGCTTGYTTRCCCAT		Sequencing	Liu *et al*., 1999
	7cf	ATGGGYAARCAAGCYATGGG		Sequencing	Liu *et al*., 1999
	11ar	GCRTGGATCTTRTCRTCSACC		PCR and Sequencing	Liu *et al*., 1999

Table 2. A set of 22 *Fusarium* species used to pre-screen primers designed for FK3 and Foxy2.

CAV [a]	Species	Clade	VCG
6001	*F. oxysporum* f. sp. *strigae*	Nigeria	VCG 04701
6153	*F. oxysporum* f. sp. *strigae*	Nigeria	VCG 04702
6236	*F. oxysporum* f. sp. *strigae*	Nigeria	VCG 04703
6142	*F. oxysporum* f. sp. *strigae*	Nigeria	VCG 04704
6144	*F. oxysporum* f. sp. *strigae*	Nigeria	VCG 04705
6230	*F. oxysporum* f. sp. *strigae*	Nigeria	VCG 04706
6160	*F. oxysporum* f. sp. *strigae*	Nigeria	VCG 04707
6139	*F. oxysporum* f. sp. *strigae*	Nigeria	SMV1
6148	*F. oxysporum* f. sp. *strigae*	Nigeria	SMV2
6161	*F. oxysporum* f. sp. *strigae*	Nigeria	SMV4
6149	*F. oxysporum* f. sp. *strigae*	Nigeria	SMV5
6186	*F. oxysporum* f. sp. *strigae*	Nigeria	SMV6
6115	*F. oxysporum* f. sp. *strigae*	Nigeria	SMV7
6240	*F. oxysporum* f. sp. *strigae*	Nigeria	SMV8
6023	*F. oxysporum* f. sp. *strigae*	Kenya	VCG 04708
6040	*F. oxysporum* f. sp. *strigae*	Kenya	VCG 04708
6012	*F. oxysporum* f. sp. *strigae*	Kenya	VCG 04708
6111	*Fusarium incarnatum*	NA	NA
6112	*Fusarium longipes*	NA	NA
6123	*Fusarium semitectum*	NA	NA
330	*F. oxysporum* f. sp. *lycopersici*	NA	NA
337	*F. oxysporum* f. sp. *vasinfectum*	NA	NA

[a]Culture collection hosted at the Department of Plant Pathology, Stellenbosch University

Table 3. *Fusarium* species used in validation of molecular markers FK3 and Foxy2.

Number	CAV[a]	Species	Country	VCG [b]	FK3 marker	Foxy2 marker
K7-5A	6000	F.o. f. sp. *strigae*	Kenya	4701	-	+
M12-4A	6001	F.o. f. sp. *strigae*	Mali	4701	-	+
M5-1B	6002	F.o. f. sp. *strigae*	Mali	4701	-	+
M6-1A	6003	F.o. f. sp. *strigae*	Mali	4701	-	+
M13-1B	6004	F.o. f. sp. *strigae*	Mali	4701	-	+
N1-12A	6005	F.o. f. sp. *strigae*	Niger	4701	-	+
N2-5A	6006	F.o. f. sp. *strigae*	Niger	4701	-	+
N5-1A	6007	F.o. f. sp. *strigae*	Niger	4701	-	+
B7-3A	6008	F.o. f. sp. *strigae*	Burkina Faso	4701	-	+
F oxy 2	6009	F.o. f. sp. *strigae*	Ghana	4701	-	+
PSM 197	6010	F.o. f. sp. *strigae*	Nigeria	4701	-	+
M8-5A	6011	F.o. f. sp. *strigae*	Mali	4701	-	+
N95/1	6135	F. *oxysporum*	Nigeria	4701	-	+
N192/2	6156	*F. oxysporum*	Nigeria	4701	-	+
N222	6167	*F. oxysporum*	Nigeria	4701	-	+
N222/B	6233	*F. oxysporum*	Nigeria	4701	-	+
N230/1	6171	*F. oxysporum*	Nigeria	4701	-	+
N248/A	6180	*F. oxysporum*	Nigeria	4701	-	+
N271/3	6199	*F. oxysporum*	Nigeria	4701	-	+
N4/1	6092	*F. oxysporum*	Nigeria	4702	-	+
N39/3	6116	*F. oxysporum*	Nigeria	4702	-	+
N55/1	6126	*F. oxysporum*	Nigeria	4702	-	+
N183/1	6153	*F. oxysporum*	Nigeria	4702	-	+
N220	6166	*F. oxysporum*	Nigeria	4702	-	+
N3/1	6091	*F. oxysporum*	Nigeria	4703	-	+
N260/4	6236	*F. oxysporum*	Nigeria	4703	-	+
N155/3	6142	*F. oxysporum*	Nigeria	4704	-	+
N155/2	6143	*F. oxysporum*	Nigeria	4704	-	+
N2/1	6090	*F. oxysporum*	Nigeria	4705	-	+
N158/1	6144	*F. oxysporum*	Nigeria	4705	-	+
N106/1	6137	*F. oxysporum*	Nigeria	4706	-	+
N96/2	6230	*F. oxysporum*	Nigeria	4706	-	+
N196/1	6160	*F. oxysporum*	Nigeria	4707	-	+
N126/3	6140	*F. oxysporum*	Nigeria	4707	-	+
N112/2	6139	*F. oxysporum*	Nigeria	SMV 1	-	+
N175/1	6148	*F. oxysporum*	Nigeria	SMV 2	-	+
N208/1	6162	*F. oxysporum*	Nigeria	SMV 3	-	+

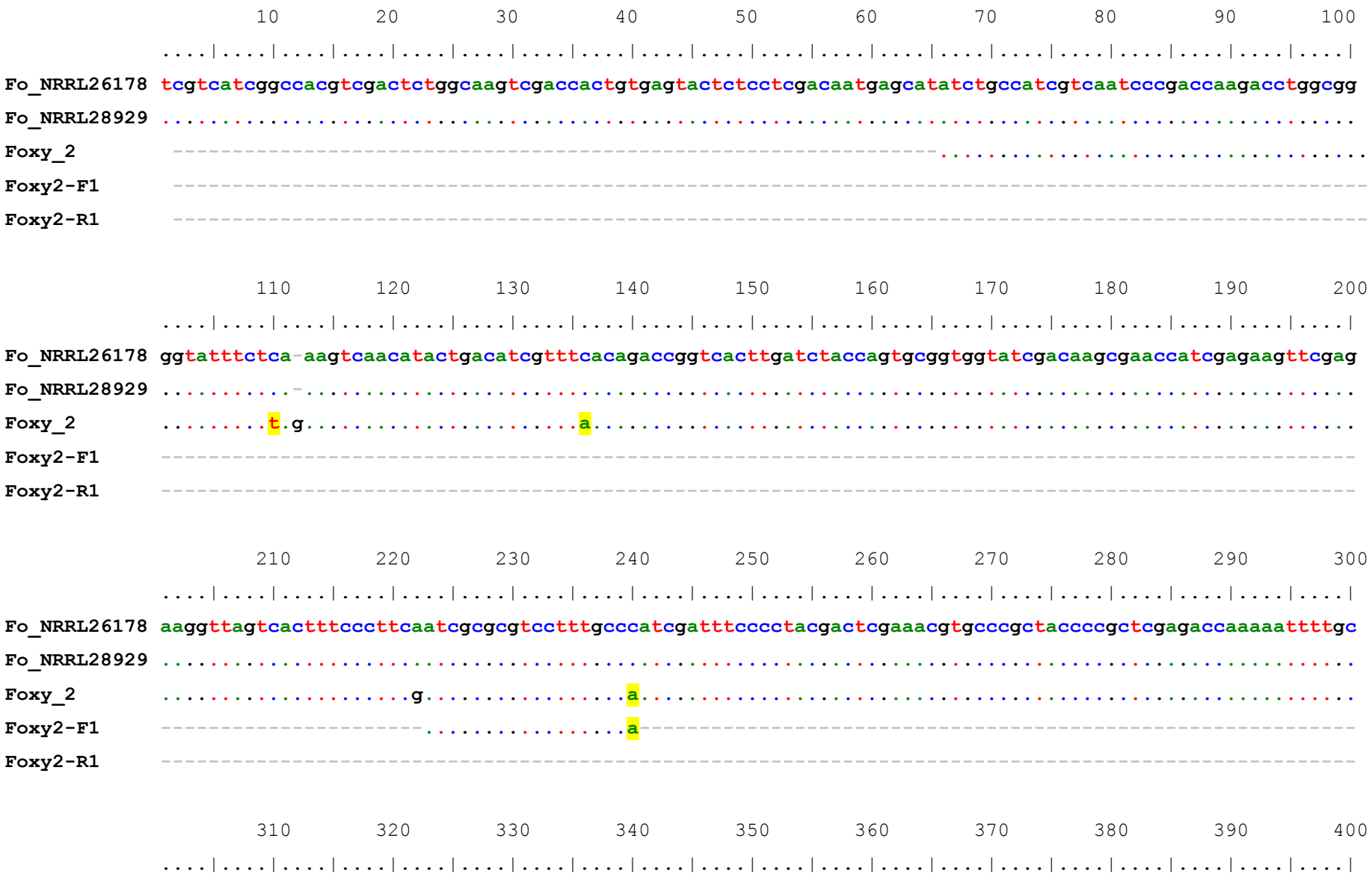
N203/1	6161	*F. oxysporum*	Nigeria	SMV 4	-	+
N177/1	6149	*F. oxysporum*	Nigeria	SMV 5	-	+
N256/4A	6186	*F. oxysporum*	Nigeria	SMV 6	-	+
N39/4	6115	*F. oxysporum*	Nigeria	SMV 7	-	+
N288/3	6240	*F. oxysporum*	Nigeria	SMV 8	-	+
A6/1	6016	*F. oxysporum*	Kenya	4708	+	-
A7/3	6019	*F. oxysporum*	Kenya	4708	+	-
A8/1	6020	*F. oxysporum*	Kenya	4708	+	-
A8/2	6021	*F. oxysporum*	Kenya	4708	+	-
A12/1	6023	F.o. f. sp. *strigae*	Kenya	4708	+	-
C2/1	6039	*F. oxysporum*	Kenya	4708	+	-
C6/2	6043	*F. oxysporum*	Kenya	4708	+	-
C6/3	6044	F.o. f. sp. *strigae*	Kenya	4708	+	-
C7/1	6045	*F. oxysporum*	Kenya	4708	+	-
F1/1	6050	*F. oxysporum*	Kenya	4708	+	-
F4/1	6057	*F. oxysporum*	Kenya	4708	+	-
F7/1	6063	*F. oxysporum*	Kenya	4708	+	-
F8/3	6067	*F. oxysporum*	Kenya	4708	+	-
F10/3	6073	*F. oxysporum*	Kenya	4708	+	-
F12/1	6075	*F. oxysporum*	Kenya	4708	+	-
F12/3	6077	*F. oxysporum*	Kenya	4708	+	-
F13/2	6079	*F. oxysporum*	Kenya	4708	+	-
F14/1	6081	*F. oxysporum*	Kenya	4708	+	-
F18/1	6087	*F. oxysporum*	Kenya	4708	+	-
F20.3	6210	*F. oxysporum*	Nigeria	4708	+	-
F22.1	6211	*F. oxysporum*	Kenya	4708	+	-
F22.2	6212	*F. oxysporum*	Kenya	4708	+	-
F26.1	6218	*F. oxysporum*	Kenya	4708	+	-
FK1	6222	F.o. f. sp. *strigae*	Kenya	4708	+	-
FK2	6223	F.o. f. sp. *strigae*	Kenya	4708	+	-
FK3	6224	F.o. f. sp. *strigae*	Kenya	4708	+	-
FK4	6225	F.o. f. sp. *strigae*	Kenya	4708	+	-
FK5	6226	F.o. f. sp. *strigae*	Kenya	4708	+	-
B5/1	6030	*F. oxysporum*	Kenya	Non-pathogen	-	-
C8/1	6047	*F. oxysporum*	Kenya	Non-pathogen	-	-
C8/2	6048	*F. oxysporum*	Kenya	Non-pathogen	-	-
F25.2	6216	*F. oxysporum*	Kenya	Non-pathogen	-	-
A7/1	6017	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
A7/2	6018	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
B3/3	6028	*F. equiseti*	Kenya	N/A	-	-

B7/2	6033	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
B11/2	6035	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
B12/2	6037	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
C1/1	6038	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
C5/1	6041	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
C7/2	6046	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
C8/3	6049	*F. incarnatum-equiseti sc*	Kenya	N/A	-	-
F1/2	6051	*F. incarnatum*	Kenya	N/A	-	-
F2/2	6053	*F. incarnatum*	Kenya	N/A	-	-
F2/3	6054	*F. equiseti*	Kenya	N/A	-	-
F3/2	6056	*F. incarnatum*	Kenya	N/A	-	-
F4/2	6058	*F. equiseti*	Kenya	N/A	-	-
F5/1	6059	*F. equiseti*	Kenya	N/A	-	-
F6/1	6061	*F. equiseti*	Kenya	N/A	-	-
F7/2	6064	*F. incarnatum*	Kenya	N/A	-	-
F8/2	6066	*F. incarnatum*	Kenya	N/A	-	-
F9/2	6069	*F. incarnatum*	Kenya	N/A	-	-
F9/3	6070	*F. incarnatum*	Kenya	N/A	-	-
F12/2	6076	*F. incarnatum*	Kenya	N/A	-	-
F13/3	6080	*F. incarnatum*	Kenya	N/A	-	-
F14/2	6082	*F. incarnatum*	Kenya	N/A	-	-
F16/1	6084	*F. incarnatum*	Kenya	N/A	-	-
F16/2	6085	*F. equiseti*	Kenya	N/A	-	-
N5/2	6093	*F. equiseti*	Nigeria	N/A	-	-
N8/1B	6095	*F. equiseti*	Nigeria	N/A	-	-
N8/1A	6096	*F. equiseti*	Nigeria	N/A	-	-
N13/2	6097	*F. incarnatum*	Nigeria	N/A	-	-
N17/2	6099	*F. longipes*	Nigeria	N/A	-	-
N19/4	6100	*F. incarnatum*	Nigeria	N/A	-	-
N20/3	6102	*F. incarnatum*	Nigeria	N/A	-	-
N21/3	6103	*F. longipes*	Nigeria	N/A	-	-
N23/2	6106	*F. longipes*	Nigeria	N/A	-	-
N24/1	6107	*F. incarnatum*	Nigeria	N/A	-	-
N32/1	6108	*F. incarnatum*	Nigeria	N/A	-	-
N35/1	6109	*F. longipes*	Nigeria	N/A	-	-
N36/1	6111	*F. incarnatum*	Nigeria	N/A	-	-
N36/4	6112	*F. longipes*	Nigeria	N/A	-	-
N37/2	6113	*F. longipes*	Nigeria	N/A	-	-
N37/4	6114	*F. incarnatum*	Nigeria	N/A	-	-
N45/1	6119	*F. incarnatum*	Nigeria	N/A	-	-

N49/1	6122	*F. incarnatum*	Nigeria	N/A	-	-
N50/7	6123	*F. semitectum*	Nigeria	N/A	-	-
N50/3	6124	*F. incarnatum*	Nigeria	N/A	-	-
N53/1	6125	*F. semitectum*	Nigeria	N/A	-	-
N106/2	6138	*F. semitectum*	Nigeria	N/A	-	-
N236/1	6176	*F. semitectum*	Nigeria	N/A	-	-
N238/6	6177	*F. semitectum*	Nigeria	N/A	-	-
N245	6179	*F. semitectum*	Nigeria	N/A	-	-
N254/2	6183	*F. semitectum*	Nigeria	N/A	-	-
N257/3	6187	*F. semitectum*	Nigeria	N/A	-	-
N258/5	6188	*F. semitectum*	Nigeria	N/A	-	-
N258/3	6189	*F. semitectum*	Nigeria	N/A	-	-
N259/2	6190	*F. semitectum*	Nigeria	N/A	-	-
N262/1	6193	*F. semitectum*	Nigeria	N/A	-	-
N262/2	6194	*F. semitectum*	Nigeria	N/A	-	-
N267/C	6195	*F. semitectum*	Nigeria	N/A	-	-
N269/C	6196	*F. semitectum*	Nigeria	N/A	-	-
N273	6200	*F. semitectum*	Nigeria	N/A	-	-
N279/1	6201	*F. semitectum*	Nigeria	N/A	-	-
N281/2	6202	*F. semitectum*	Nigeria	N/A	-	-
PPRI 5456	315	*F.o.* f. sp. *lycopersici*	N/A	N/A	-	-
PPRI 5457	316	*F.o.* f. sp. *lycopersici*	N/A	N/A	-	-
PPRI 4923	317	*F.o.* f. sp. *melonis*	N/A	N/A	-	-
PPRI 4915	318	*F.o.* f. sp. *melonis*	N/A	N/A	-	-
PPRI 4954	319	*F.o.* f. sp. *melonis*	N/A	N/A	-	-
PPRI 4950	320	*F.o.* f. sp. *melonis*	N/A	N/A	-	-
SP. 709	321	*F.o.* f. sp. *melonis*	N/A	N/A	-	-
SP. 717	322	*F.o.* f. sp. *melonis*	N/A	N/A	-	-
SP. 257	324	*F.o.* f. sp. *niveum*	N/A	N/A	-	-
F2-56	325	*F.o.* f. sp. *cubense*	N/A	N/A	-	-
CBS 186.53	326	*F.o.* f. sp *conglutin*	N/A	N/A	-	-
CBS 491.97	327	*F.o.* f. sp. *dianthi*	N/A	N/A	-	-
CBS 137.97	328	*F.o.* f. sp. *gladioli*	N/A	N/A	-	-
CBS 259.51	329	*F.o.* f. sp. *lini*	N/A	N/A	-	-
CBS 413.90	330	*F.o.* f. sp. *lycopersici*	N/A	N/A	-	-
CBS 195.65	331	*F.o.* f. sp. *tulipae*	N/A	N/A	-	-
CBS 935.73	333	*F.o.* f. sp. *phaseoli*	N/A	N/A	-	-
CBS 127.73	334	*F.o.* f. sp. *pisi*	N/A	N/A	-	-
CBS 101587	335	*F.o.* f. sp. *radicis-lycopersici*	N/A	N/A	-	-
CBS 488.76	336	*F.o.* f. sp. *raphanni*	N/A	N/A	-	-

CBS 411.90	337	*F.o.* f. sp. *vasinfectum*	N/A	N/A	-	-
CBS 783.83	338	*F.o.* f. sp. *elaeidis*	N/A	N/A	-	-
CBS 179.32	339	*F.o.* f. sp. *nicotianae*	N/A	N/A	-	-
CBS 744.79	340	*F.o.* f. sp. *passiflorae*	N/A	N/A	-	-
CBS 794.70	341	*F.o.* f. sp. *perniciosum*	N/A	N/A	-	-
	980	*F.o.* f. sp. *cubense*	N/A	N/A	-	-
	789	*F.o.* f. sp. *cubense*	N/A	N/A	-	-
	967	*F.o.* f. sp. *cubense*	N/A	N/A	-	-
	189	*F.o.* f. sp. *cubense*	N/A	N/A	-	-

[a]Culture collection hosted at the Department of Plant Pathology, University of Stellenbosch
[b]Country of origin
[c]Vegetative compatibility groups of *F. oxysporum* associated with *Striga*



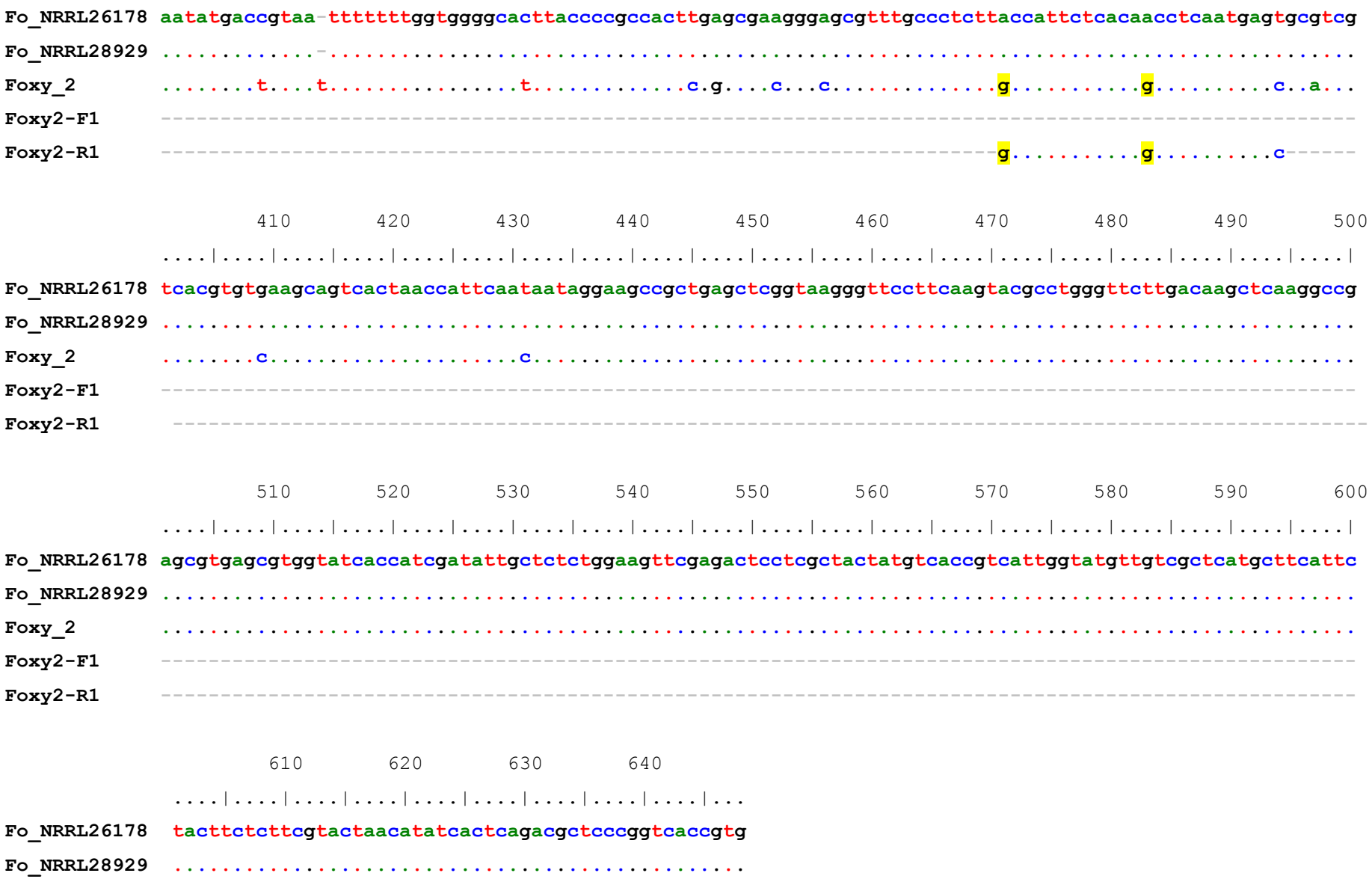
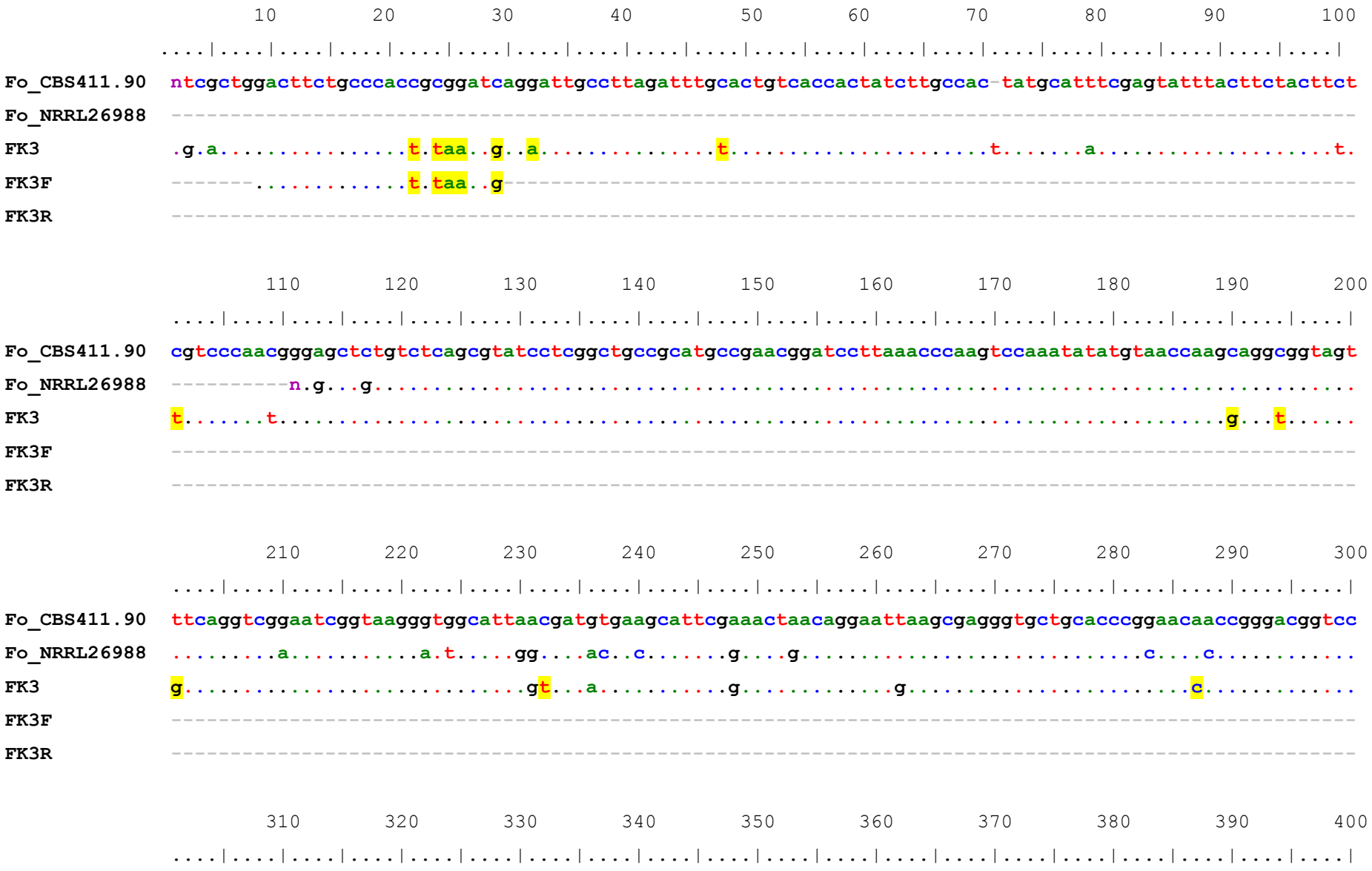


Figure 1. Aligned sequences of the translocation elongation factor α-1 gene area, visualized in Bio-edit. The sequences include the forward (Foxy2-F1) and reverse (Foxy2-R1) primers designed for isolate Foxy2 based on single nucleotide polymorphisms (SNPs). Unknown nucleotides are indicated with an “n”, and deletions with a “-“. The two reference isolates are NRRL28929, a *Fusarium oxysporum* f. sp. *lini* isolate, and NRRL26178, a *F. oxysporum* f. sp. *melonis* isolate obtained from Genbank. True SNPs for Foxy2 are highlighted in yellow.



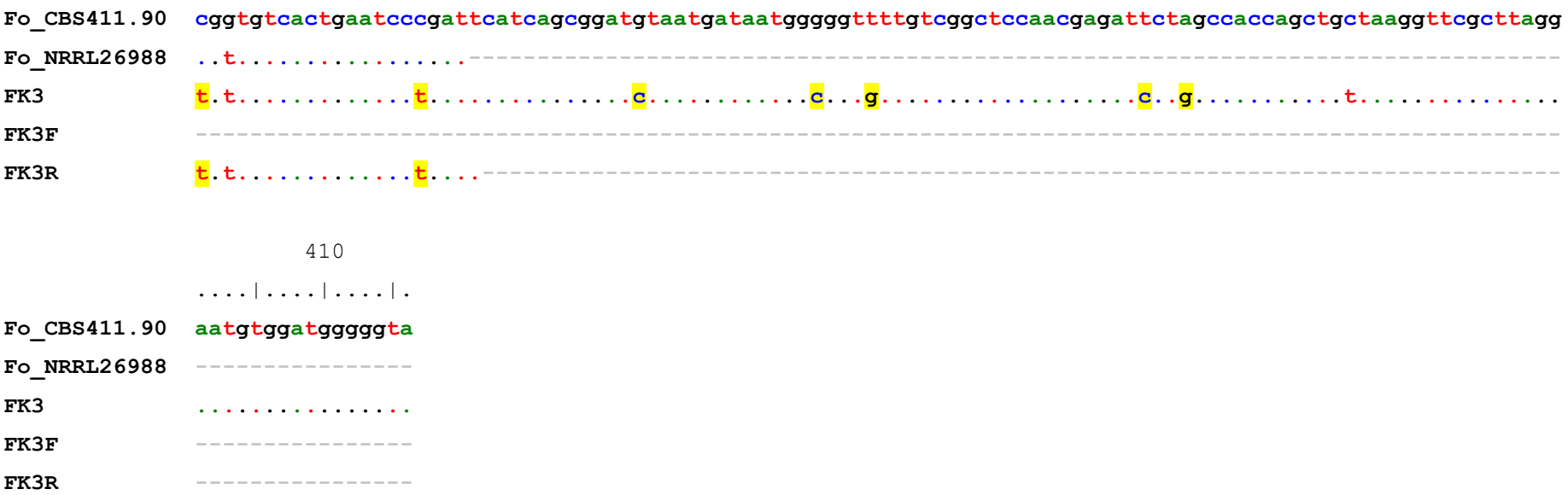


Figure 2. Aligned sequences of the Secreted in Xylem 14 gene region, visualized in Bio-edit, including the forward (FK3-F) and reverse (FK3-R) primers designed on single nucleotide polymorphisms (SNPs) present in isolates of VCG 04708, which contain isolate FK3. Unknown nucleotides are indicated with an “n”, and deletions with a “-“. The two reference isolates are CBS411.90, a *Fusarium oxysporum* f. sp. *vasinfectum* isolate sequenced in this study, and NRRL26988, a *F. oxysporum* f. sp. *gladioli* isolate obtained from Genbank. True SNPs for FK3 are marked in yellow.

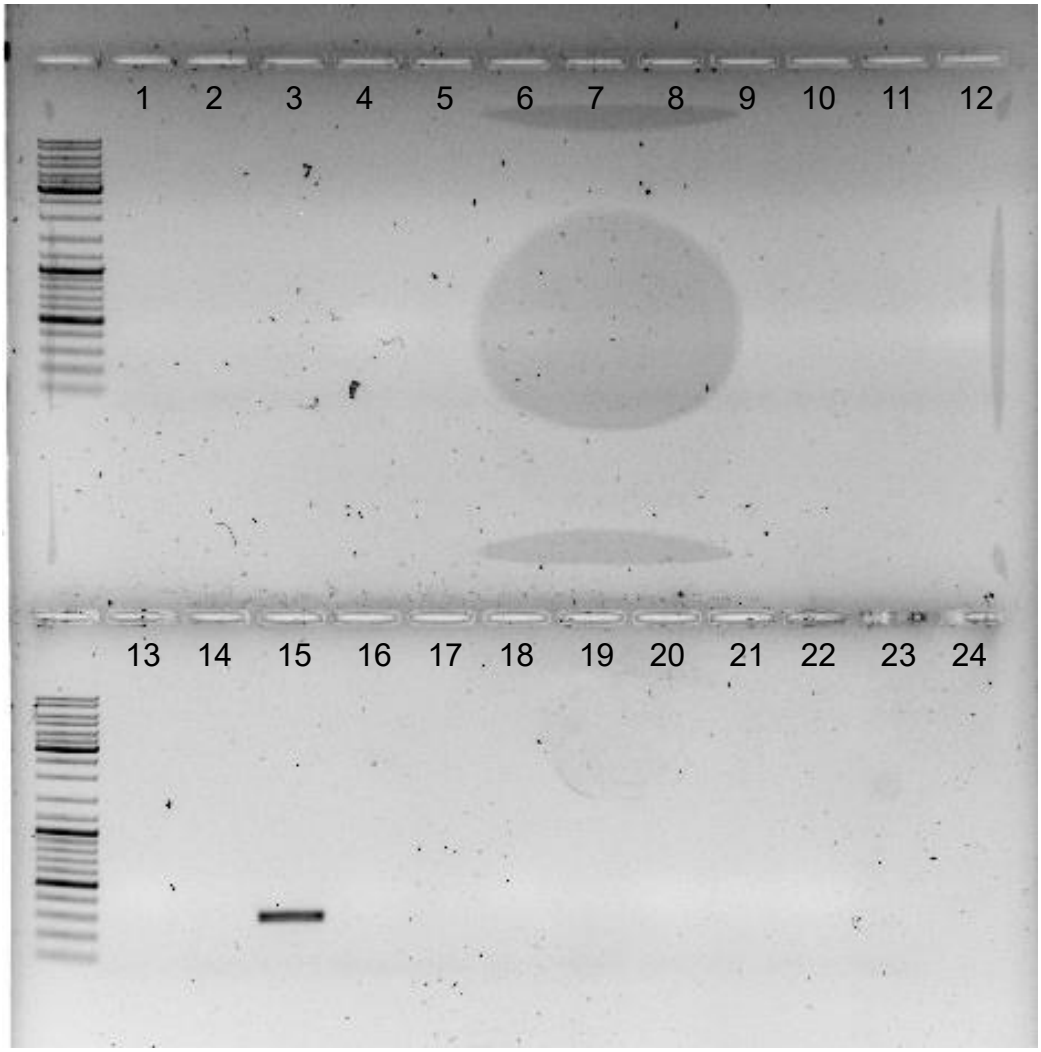


Figure 3. The testing of primer set FK3-F/R against Fos isolates representing Nigerian and Kenyan VCGs, SMVs, other *Fusarium* species and other *formae speciales* of *F. oxysporum*. Lanes 1-7: Fos VCGs from Nigeria (1=VCG 04701, 2=VCG 04702, 3=VCG 04703, 4=VCG 04704, 5=VCG 04705, 6=VCG 04706, 7=VCG 04707). Lanes 8-14: Fos SMVs from Nigeria (8=SMV1, 9=SMV2, 10=SMV4, 11=SMV5, 12=SMV6, 13=SMV7, 14=SMV8). Lanes 15-17: Isolates representing VCG 04708 from Kenya. Lanes 18-22: Other *Fusarium* spp. and *formae speciales* of *F. oxysporum* associated with *Striga* (18=*F. incarnatum*, 19=*F. longipes*, 20=*F. semitectum* 21=*F. oxysporum* f. sp. *lycopersici*, 22=*F. oxysporum* f. sp. *vasinfectum*). Lane 23: Water control.

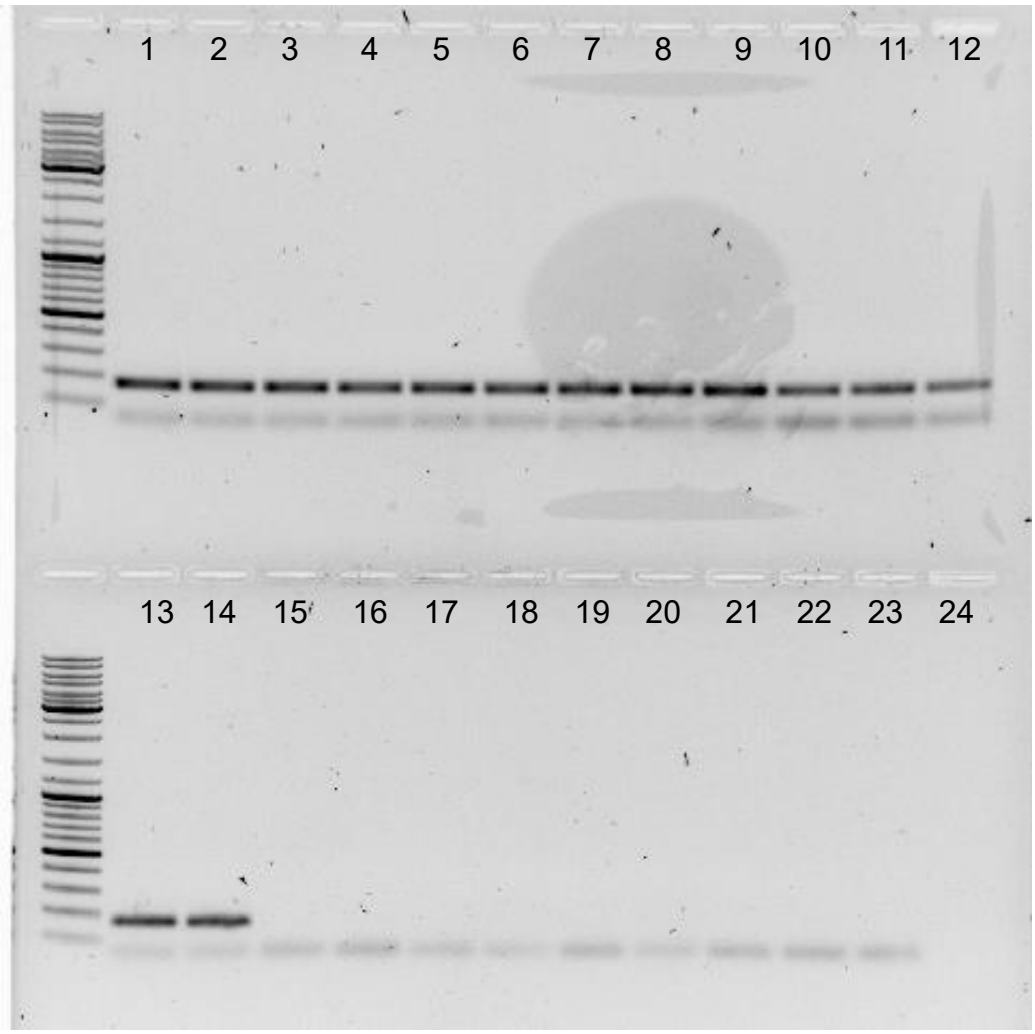


Figure 4. Specificity testing of primer set Foxy2_F/R for detection of the Nigerian lineage, tested against 22 isolates, representing Nigerian and Kenyan VCG's, Nigerian SMV's, other *Fusarium* species and other *F. oxysporum formae speciales*. Lanes 1-7: Fos VCGs from Nigeria (1=VCG 04701, 2=VCG 04702, 3=VCG 04703, 4=VCG 04704, 5=VCG 04705, 6=VCG 04706, 7=VCG 04707). Lanes 8-14: Fos SMVs from Nigeria (8=SMV1, 9=SMV2, 10=SMV4, 11=SMV5, 12=SMV6, 13=SMV7, 14=SMV8). Lanes 15-17: Isolates representing VCG 04708 from Kenya. Lanes 18-22: Other *Fusarium* spp. and *formae speciales* of *F. oxysporum* associated with *Striga* (18=*F. incarnatum*, 19=*F. longipes*, 20=*F. semitectum* 21=*F. oxysporum* f. sp. *lycopersici*, 22=*F. oxysporum* f. sp. *vasinfectum*). Lane 23: Water control.

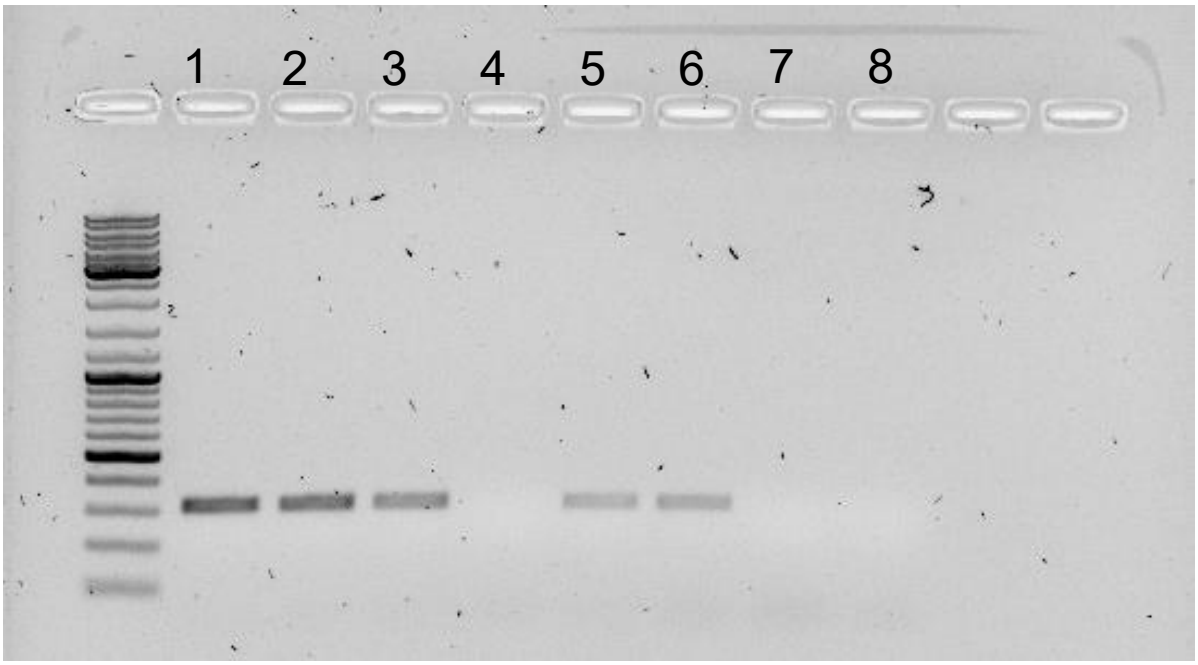


Figure 5. Sensitivity testing of primer set FK3-F/R, for detection of FK3. From left to right: VCG 04708 DNA, 1=10 ng/µl, 2=1 ng/µl, 3=0.1 ng/µl and 4=water control. VCG 04708 DNA, diluted in 50 ng/µl *Striga asiatica* DNA, 5=10 ng/µl, 6=1 ng/µl, 7=0.1 ng/µl and 8=water control.

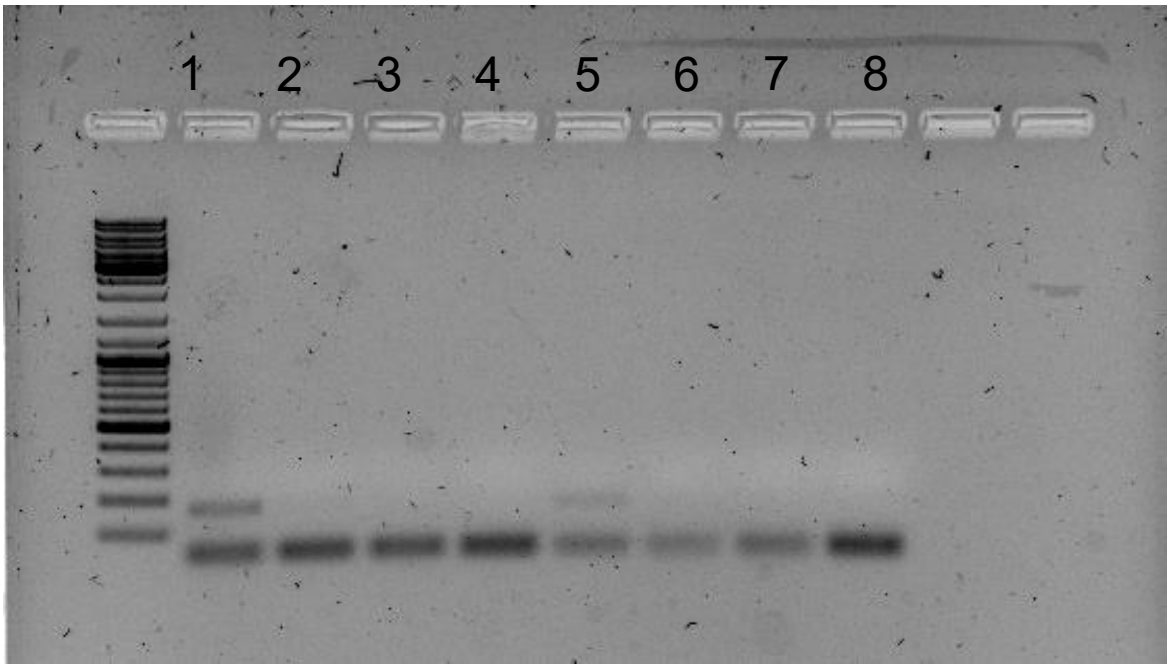


Figure 6. Sensitivity testing of primer set Foxy2-F1/R1, for detection of Foxy2. From left to right: VCG 04702 DNA, 1=10 ng/µl, 2=1 ng/µl, 3=0.1 ng/µl and 4=water control. VCG 04702 DNA, diluted in 50 ng/µl *Striga asiatica* DNA, 5=10 ng/µl, 6=1 ng/µl, 7=0.1 ng/µl and 8=water control.

CONCLUSION

Striga is one of the most devastating threats that subsistent farmers face in sub-Saharan Africa. It causes substantial yield losses to grain crops such as maize and sorghum, and makes farmland unsuited for subsistence farming. Efforts to develop an integrated *Striga* management program involved the inclusion of a biological control agent (BCA) that does not harm fauna and flora other than the intended target. *Fusarium oxysporum* f. sp. *strigae* (Fos), a soil-borne fungus, was identified as potential BCA, and several studies have since investigated its host range and secondary metabolite production. In the current study, the population diversity of Fos in two African countries was investigated by means of vegetative compatibility group (VCG) analysis and the phylogenetic analysis of two housekeeping genes, the translocation elongation factor (TEF) 1α and mitochondrial small sub-unit (MtSSU) genes. The distribution of mating type genes in Fos was determined, and molecular markers developed to identify BCA applied to farmer fields.

VCG analysis and the phylogenetic study indicated that Fos in Africa may have evolved during at least two separate events. This could be due to geographic isolation of the regions where the fungus was collected, as well as host specialization. The *S. hermonthica* population in Kenya is different from populations elsewhere in Africa (Bozkurt *et al*., 2014), which could promote the development of a new pathotype (Skovgaard *et al*., 2001; Fourie, 2008; Silva *et al*., 2014), as has been observed in other *formae speciales* of *F*. *oxysporum* before; including *F*. *oxysporum* f. sp. *betae* (Harveson and Rush, 1997), *F. oxysporum* f. sp. *cepae* (Swift *et al*., 2002) and *F. oxysporum* f. sp. *vasinfectum* (Wang *et al*., 2010). It could also explain why Foxy 2, which was isolated in West Africa, did not attack *S. hermonthica* in Kenya (Avedi *et al*., 2014). It is further believed that the Kenyan population developed more recently than the Nigerian population, as the Kenyan population consists of a single VCG whereas the Nigerian population include several VCGs and single member VCGs (SMVs) that are phylogenetically closely related (Swift *et al*., 2002). The Fos population from Kenya consisted of one mating type idiomorph, whereas both mating types were present in VCGs found in Nigeria.

The housekeeping gene, TEF, in Nigerian Fos isolates were identical for all the isolates, and could have shared an ancient parent. It is difficult to establish why this group contain both mating types within a VCG, and it is speculated that the ancient parent could have been homothallic and contained both mating type idiomorphs, or that a second mating type idiomorph was obtained during parasexuality or a horizontal gene transfer (HGT) event (Lee *et al*., 2003; O'Donnell *et al*., 2004). The presence of both mating type idiomorphs in the same fungal isolate,

however, is more consistent with what is found in a sexually reproduction system (Desjardins, 1995; Ramirez-Prado *et al*., 2008), and add more merit to the hypothesis that an ancient or cryptic sexual stage had been present in *F. oxysporum* (Leslie, 1993). No sexual stage of *F. oxysporum* has, however, been observed to date, and the fungus is thus regarded as a heterothallic asexual organism.

Molecular markers that distinguish between the Fos populations present in Kenya and Nigeria have been developed in this study. The primer set FK3-F/R targets the Kenyan population consisting of VCG 04708, while Foxy2-F/R1 targets the Nigerian population that includes all phylogenetically-related VCGs and SMVs present in the country. Isolate FK3, which had been collected in Kenya, is currently under development as a BCA for Kenyan conditions (Kangethe *et al*., 2016), and Foxy 2, as representative of the Nigerian population, is being developed as a BCA for West Africa (Elzein *et al*., 2009). Sensitivity assays revealed that the Foxy2 marker is less sensitive than the FK3 marker in detecting target Fos DNA in the presence of *Striga asiatica* DNA. This might affect is value in detecting the fungus *in planta* when it is applied in the field, even though both markers can still be used to amplify the target DNA in cultures re-isolated from infected plants and infested fields.

The development of a BCA for *Striga* plants in Africa should take into account the geographic region, Fos diversity and the host plant. PCR markers developed in the current study are valuable for Fos identification purposes, but monitoring the activity of Fos in soil is also important, as soil type has been shown to affect the efficacy and proliferation of Fos as a BCA (Zimmerman *et al*., 2015). The quantification of Fos in agricultural soils and *in planta* can be done by quantitative (q) real-time PCR assays, which would require a modification of the markers developed in this study. The qPCR markers can be used to track the distribution of Fos in treated areas, as well as its unintended spread and effects thereof. It could also be used to clarify and provide evidence in cases where the BCA may be accused of causing disease (Louda *et al*., 2003). Since this study, together with those of Avedi *et al*. (2014) and Kangethe *et al*. (2016) have shown the possible effects of selective pressure on Fos, it is important to monitor the efficacy and distribution of Fos after field application.

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