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Occurrence of the entomopathogenic Fungi of Spodoptera frugiperda (Lepidoptera: Noctuidae) in selected areas of Tanzania

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Abstract

Spodoptera frugiperda (J.E. Smith) is a highly destructive pest that affects major food and cash crops in Sub-Saharan Africa. Maize, in particular, is a preferred host for this pest, posing a significant threat to food security. Initially, conventional synthetic pesticides were widely used to combat the pest. However, the potential of entomopathogenic fungi (EPF) as cost-effective and safe alternative has been recognized. The objective of this study was to collect and identify the local EPF species in selected areas of Tanzania's mainland, namely Mwanza, Morogoro, Coast, and Songwe regions. Morphological and molecular methods were employed to identify the fungal species recovered from 100 *S. frugiperda* cadavers. The findings revealed that 90% of the recovered fungi belonged to the genera of Fusarium while the remaining 10% were Clonostachys. These results suggest that Fusarium species hold promise as effective bio-control agents against *S. frugiperda* due to their wide distribution and tolerance to field disturbances. However, additional studies are necessary to validate the effectiveness of these recovered fungi against *S. frugiperda*.

Keywords: *biological control; Clonostachys rosea; entomopathogenic fungi; Spodoptera frugiperda; Zea mays*

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Introduction

Maize (*Zea mays* L.) belongs to the Poaceae family and is one of the three most important cereal crops worldwide (Rouf *et al.*, 2016). In Tanzania, *Z.mays* is considered a major crop for both food and commercial purposes, with an average annual production of 10 million tons (FAO, 2022). Maize consumption is highly valued due to its rich content of essential nutrients such as carbohydrates and proteins in addition to minerals (iron) and some important vitamins (Day *et al.*, 2017). Furthermore, maize provides ethanol and starch, which are utilized as fuels; starch can be enzymatically converted into sorbitol, dextrin, sorbic acid, and lactic acid (Naqvi *et al.*, 2011). These products are used in various applications, including the production of beer, ice cream, syrup, shoe polish, glue, fireworks, ink, batteries, mustard, cosmetics, aspirin, and paint.

Poor technologies, inadequate infrastructure, limited capital, flooding, poor soil fertility, alongside pest infestations, have significantly impacted maize production despite its inherent value (Mkonda & He, 2016). The fluctuation in maize productivity in recent years has not only impacted food security but has also disrupted the livelihoods of many people who directly or indirectly rely on this crop (Suleiman & Kurt, 2015). Maize Lethal Necrosis (MLN), Maize Streak Virus (MSV) and Grey Leaf Spots diseases have also been reported to negatively affect production (Onwunali & Mabagala, 2022; Wangai *et al.*, 2012). Among the field insect pests prevalent in Tanzania are maize stalk borers (*Busseola fusca* and *Chilo partelus*), white grubs (*Phyllophaga implicita*), and armyworms (*Spodoptera exempta* and *Spodoptera frugiperda*) (G. Rwegasira, unpublished data). The invasive American fall armyworm, *S. frugiperda*, holds particular significance as it has recently instigated chaos and uncertainty in the food sector, primarily due to the challenges surrounding its control (Sisay *et al.*, 2019).

In Tanzania, the introduction of *S. frugiperda* in 2017 resulted in extensive devastation to cereal and horticultural crops, along with ornamental plants, causing significant economic losses. The infestation rates of it in maize-growing areas have been reported to range between 80% and 100% (FAO, 2017). Over time, the pest has rapidly spread across all regions of Tanzania and has even crossed borders into neighbouring countries (Nagoshi *et al.*, 2018). Being a significant pest, it has caused yield losses of up to 21 million tons, equivalent to US$ 6.1 billion (Sisay *et al.*, 2018). Consequently, the emergence of this pest has led to an increased reliance on conventional insecticides as a quick solution to mitigate the potential consequences in the food sector (Cruz-Avalos *et al.*, 2019). However, despite their effectiveness, pesticides are costly and only provide temporary relief, as pests have been reported to develop resistance over time (Zhang *et al.*, 2020). As a result, the problem persists, posing an on-going threat to food security (FAO, 2020). Additionally, the overreliance on conventional pesticides is associated with human and livestock toxicity, the decline of naturally occurring biological control agents (BCAs), and overall environmental degradation (Akutse *et al.*, 2019). Growing global interest is focused on safer and eco-friendly pest control methods, (Rajula *et al.*, 2020). However, many countries have yet to fully harness the potential of this older technology (Zekeya *et al.*, 2019). Among the naturally occurring BCAs, entomopathogenic fungi (EPF) offer numerous advantages over conventional strategies, such as cost-effectiveness, persistence, ease of multiplication, reliability, effectiveness, and non-hazardous nature (Chandler, 2017) and hence regarded as promising alternatives (Lacey *et al.*, 2015). Previous studies have shown that *B. bassiana* and *M. brunneum* inflict mortalities in *S. frugiperda* (Hernandez-Trejo *et al.*, 2019).

Despite their widely known potentials and diversity, Tanzania has a single commercialized *Aspergillus oryzae*-based biocide for controlling *Tuta ablosuta* and *S. frugiperda* (Zekeya *et al.*, 2019). The drawback is due to little scientific attention invested in indigenous EPF and consequently, it is essential to keep importing EPF-based products for pest control programs. However, relying excessively on a limited number of solutions may lead to unexpected ecological issues. Hence, it is imperative to comprehend, advocate for, and optimize the efficacy of local populations of natural enemies. This work aimed to investigate local occurrence and genetic diversity of fungal species which could partly contain integrated pest management (IPM) programs against *S. frugiperda* in Tanzania.

Materials and methods

Collection of S. frugiperda cadavers

S. frugiperda cadavers were collected during surveys which were conducted from February 2022 to April 2022 in selected areas of Morogoro, Mwanza, Coast and Songwe regions (Figure 1 near here) to contain a diversity of conditions (Bueno-Pallero *et al.*, 2020). Morogoro is located between 6.8278°S and 37.6591°E at 511 m asl, eastern central of Tanzania, tropical sub-humid with bimodal rainfall system and annual rainfall and temperature of 740 mm and 25.1°C respectively (Kacholi, 2020). Mwanza is found between 2.5164°S and 32.9175°E at 1210 m asl in the north-western part of Tanzania, almost warm throughout the year with temperatures ranging between 17°C to 28°C and precipitation of 1050 mm. Moreover, the Coast region is found between 7.3238°S and 38.8205°E, 191 m asl with annual temperatures ranging between 22.1°C and 31.1°C, almost hot throughout the year with two rainy seasons and Songwe is found in between 8.5238°S, 32.5373°E at 1929 m asl (URT, 2012).

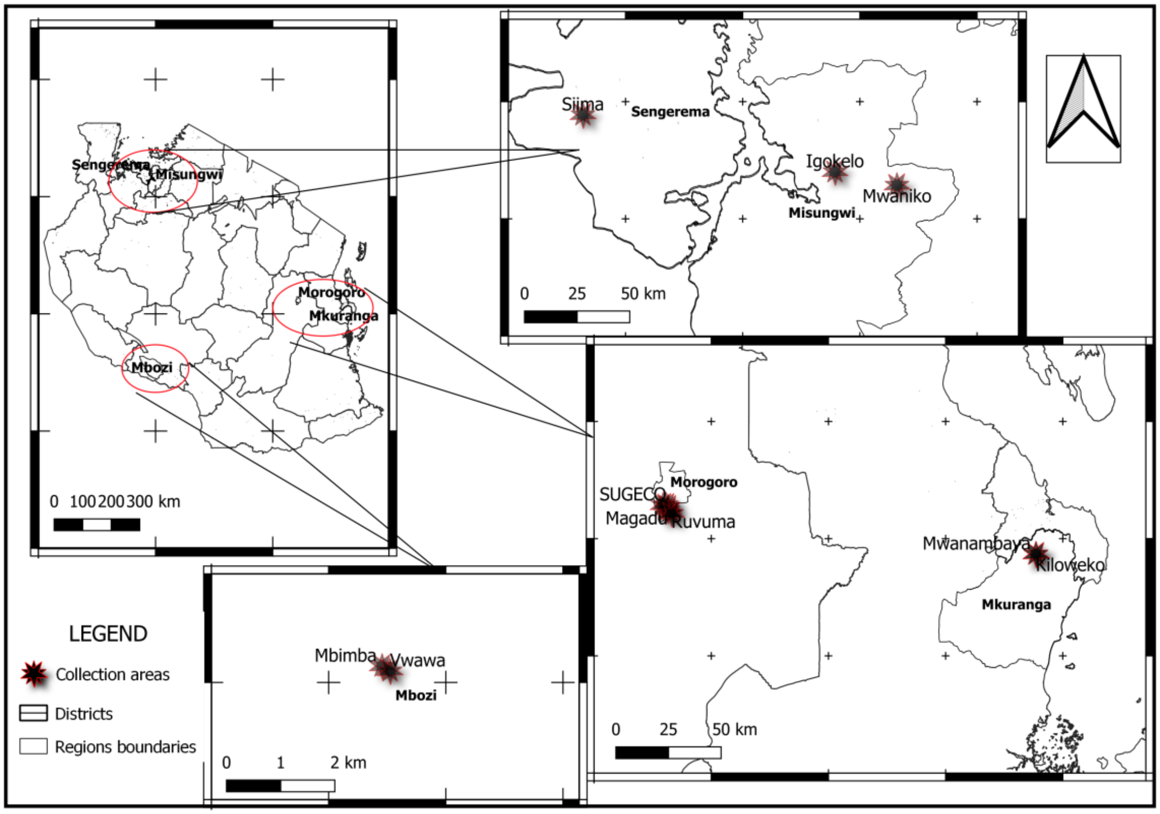

Figure 1. A map showing S. frugiperda collection points in selected areas of Mwanza, Morogoro, Songwe and Coast regions.

Careful observations were invested to whorl of V2-V6 maize plants for guaranteeing the collection of cadavers which appeared whitish or greenish as a prior indication of EPF incidence, a procedure by Hungria *et al*. (2010). The same procedure was further implemented in other 19 fields to collect a total population of 100. Cadavers were kept in well-labelled plastic vials capped with sterile cotton wool as per Thaochan and Sausa-Ard (2017) and immediately transported to the mycology laboratory of the Department of Crop Science and Horticulture at the Sokoine University of Agriculture (SUA) in Morogoro-Tanzania.

Isolation of the entomopathogenic fungi

Each cadaver was prior surface sterilized by dipping into 0.1% NaOCl for a minute followed by twice washing with sterile water before being placed in sterile plastic Petri dishes (90x10 mm) which were lined with moistened, sterile blotter paper for conidial germination as per Mnyone *et al.* (2011). Settings were incubated in the dark for three days at 28 ± 2°C, 75% RH until mycelial outgrowth become visible, a procedure by Verma *et al.* (2020). Emerging mycelia were picked using a sterile inoculating needle under a dissecting microscope (Leica Zoom 2000 No. Z45V) and transplanted into plates containing sterile media (autoclaved at 121°C for 15 min) of oatmeal agar, OTA (basal medium) and cetyltrimethylammonium bromide, CTAB (selective media), 50 g/L oat, 0.6 g/L CTAB, 15 g/L agar, 0.5 g/L chloramphenicol. The cultures were then subjected to incubation as per Ávila-Hernández *et al.* (2020) until they grew fully (14-15 days of incubation). Sub-culturing was accomplished repeatedly until pure colonies were obtained.

Morpho-cultural characterization of fungal isolates

The characterization of the isolates was accomplished through spores' morphological features besides colony pigmentation with the aid of expertise and taxonomic references by Bischoff *et al.* (2009). However, confirmation of their identities was later achieved by molecular methods. Moreover, 22 fungal isolates were recovered and their purified states were transferred to slants (1.5×10 cm) of OTA+CTAB and incubated in the dark at 28 ± 2°C, 75% RH until sporulation, afore being stored at 4°C, procedures as per Quesada-Moraga *et al.* (2006).

Molecular characterization; DNA extraction, amplification, sequencing and phylogenetic analysis

Extraction of DNA material from the mycelia of recoveries was achieved as per Mahuku (2004) with some minor modifications. The internal transcribed spacer (ITS) regions of the fungi were amplified by using the ITS1 (F5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as per Zhang *et al.* (2013). The Polymerase chain reaction (PCR) mixture was amplified using applied biosystems 4375305 machines with an initial denaturation of 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54.2°C for 45 s, extension at 68°C for 1 min and final elongation at 68°C for 5 min. The amplicon was separated in 1% agarose gel using 100 volts for 90 min. The gel was further stained in 0.5 µg/ml of ethidium bromide solution, visualized and documented in an alliance uvitec machine (Cambridge, United Kingdom). The positive PCR samples were further amplified in 50 µl reaction volume and shipped to Macrogen Europe BV (Amsterdam, Netherlands) for Sanger sequencing. The sequenced products were analysed using bioinformatics software, whereby reads were cleaned and assembled using MacVector with assembler software version 14. Generated consensus sequences were compared by the BLAST algorithm in the NCBI GenBank database as per Kamau *et al.* (2022). Phylograms were constructed out of 1000 replications of bootstrap tests using a neighbour joining (NJ) method with MEGA 7 software as per Kumar *et al.* (2018).

Results

Macroscopic and microscopic identification

Morpho-cultural characteristics of the recovered isolates from various points revealed domination of the species under genera Fusarium (>50%) (Figure 2 near here) (Table 1 near here). Some isolates failed to sporulate on OTA+CTAB media and therefore the molecular approaches contained the stated difficulty. Furthermore, it was observed that most of the colonies appeared white in the initial stages but continually changed to light and strongly yellow (Figure 3 near here) either on top, at a base or at the margins of the culture (Mathur & Kongsdal, 2003; Summerell *et al.*, 2003). Numerous isolates' macroconidia were found to have hyaline and sickle-shaped morphology with some variations on their apical cells with 3-5 septation. Moreover, the texture of the colonies was ranging from woolly to cottony with a buff appearance in their centres while others appeared powdery from points where inoculation took place towards the sides of the Petri dishes.

Table 1

Morpho-cultural characteristics of various fungal isolates collected during surveys in selected areas of Tanzania's mainland

Isolate	Propagule	Shape	Colour	Septation
EM 1	colony	Flat and irregularly shaped mycelium	At first white, then light to deep yellowish towards the agar base.	none

	macroconidia	none	none	none
EM 4	colony	Flat and irregularly shaped mycelium	Deep yellowish on margins and at agar base	None
	macroconidia	none	none	none
EM 5	colony	Flat and irregularly shaped mycelium	light yellowish	none
	macroconidia	none	none	none
EM 6	colony	Raised and cylindrical fluffy mycelium	Whitish with pale yellow at the agar base	none
	colony	none	none	none
EM 11	Colony	Powdery, flat and irregularly shaped	Pale yellowish on margins and base	septate
	macroconidia	sickle-shaped	hyaline	3-5 septation
EM 15	colony	Powdery, flat and cylindrical shaped	deep yellow at agar base	septate
	macroconidia	Sickle-shaped with the tapered shape of apical cells	hyaline	3-5 septation
EM 16	colony	powdery, flat and irregularly shaped	whitish with deep yellow at the centre and base	septate
	macroconidia	sickle-shaped	hyaline	3-5 septation
EM 17	colony	cylindrical mycelium	pale yellow at margins and agar base	None
	macroconidia	none	none	none
EM 20	colony	Lobate-shaped margins	whitish	none
	macroconidia	none	none	none
EM 21	colony	cylindrical and fluffy raised colony	light yellow at the base	none
	macroconidia	none	none	none

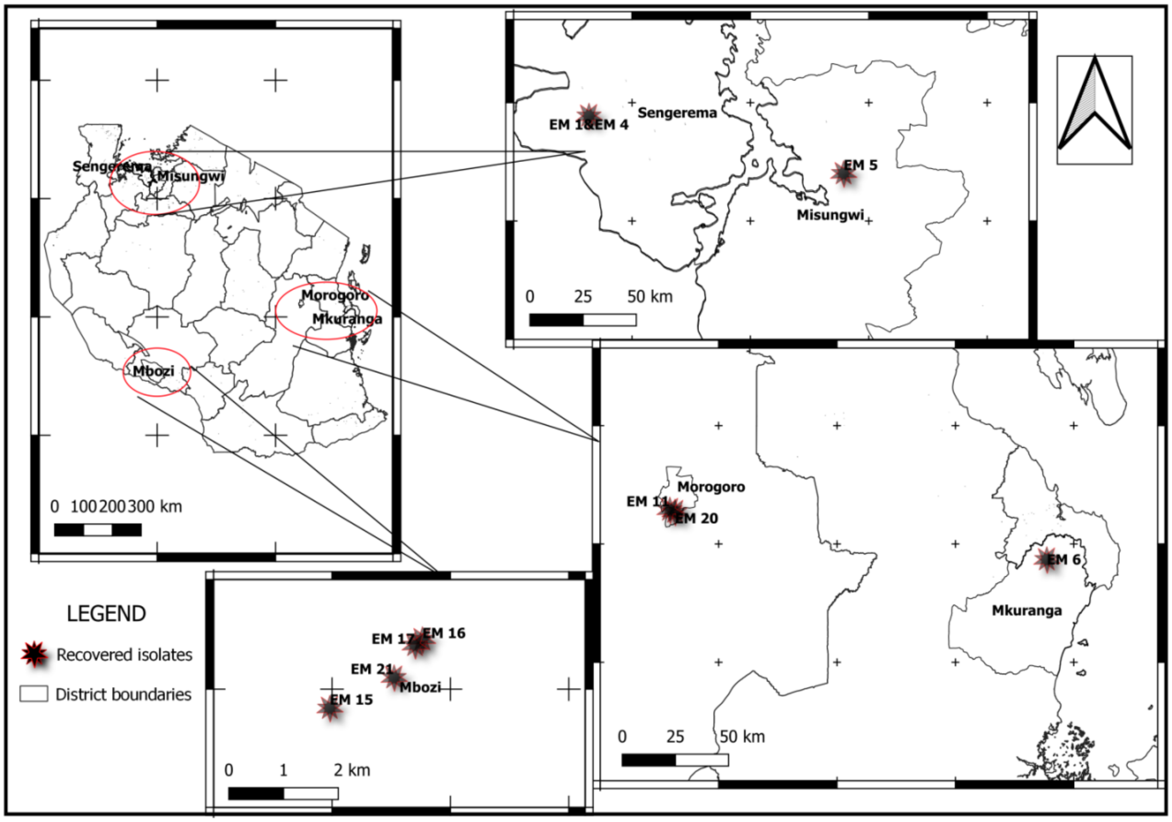

Figure 2. A map showing the recovered fungal isolates from the S. frugiperda cadavers sourced from different selected points

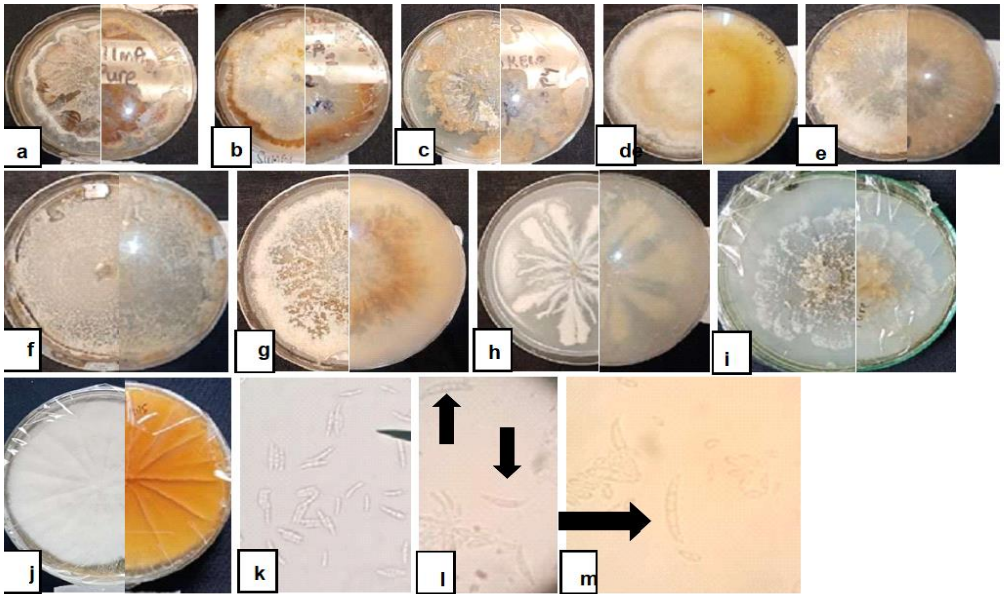

Figure 3. Morpho-cultural features of recovered fungal isolates from S. frugiperda cadavers sourced from various areas of Tanzania mainland. Front and back appearances of the cultures are presented (a)EM 1 (b)EM 4 (c)EM 5 (d)EM 6 (e)EM 17 (f)EM 11 (g)EM 15 (h)EM 20 (i)EM 6 (j)EM 21 (k)macroconidia of EM 11 (l)macroconidia of EM 16 (m)macroconidia of EM 5.

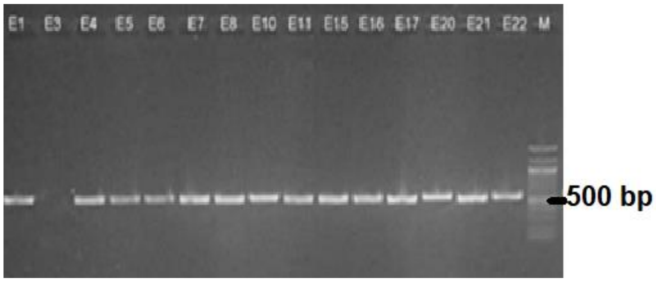

Figure 4.PCR results demonstrating a hit of 500 bp for fungal isolates which were recovered from S. frugiperda cadavers. M=molecular markers; isolates numbers indicated.

Table 2

The recovered fungal species from S. frugiperda cadavers with their matching sequence ID from NCBI Gene bank collections

Isolate	**Collection site**	**Species with a top hit**	**Reference fungi nucleotide % from NCBI and accession No.**	**Source and origin**
EM 1	Siima (Mwanza)	*Fusarium brachygibbosum*	95.51 (OL699888.1)	*Zea mays*, Iran
EM 4	Siima (Mwanza)	*Fusarium longifundum*	98.66 (OP482367.1)	genomic DNA, China
EM 5	Igokelo(Mwanza	*Fusarium equiseti*	94.65 (MN258583.1)	*Solanum tuberosum,* Jordan
EM 6	Mwanambaya(Coast)	*Fusarium clamydosporum*	95.98 (MT032393.1)	Soil, Egypt
EM 11	Kasanga(Morogoro)	*Fusarium solani*	98.03 (KF918580.1)	Mangrove soil, Malaysia
EM 15	Vwawa(Songwe)	*Fusarium equiseti*	99.59 (OM899948.1)	*Oryza sativa,* Kenya
EM 16	Vwawa(Songwe)	*Fusarium humuli*	97.58 (OL954504.1)	Trigo, Mexico

EM 17	Vwawa(Songwe)	*Fusarium incarnatum*	99.39 (MN522963.1)	cucurbita stem, USA
EM 20	SUA crop museum(Morogoro)	*Clonostachys rosea*	92.01 (ON705469.1)	Air, China
EM 21	Vwawa(Songwe)	*Fusarium equiseti*	94.75 (MN452639.1)	Glycine max, USA

Molecular characterization

The genomic DNA extracted from fungi yielded sharp bands on 0.8% agarose gel with quality ranging from 1.84 to 2 while concentration ranged from 18.5 to 25.5 ng/µl at A260/A280 of a nano-drop spectrophotometer. Those qualities made the bands useful for downstream applications. The fungal genomic DNA amplified by PCR from all of the recovered isolates were positive yielding an amplicon of 500 bp (Figure 4) hence were taken appropriately for Sanger sequencing procedures (Wang *et al.,* 2021). Bioinformatics analysis of the sequenced fungal isolates revealed 9 isolates out of 10 of the consensus sequences had more than 94% nucleotide identity with different species of Fusarium (Table 2). However, a consensus sequence with a high hit to *Clonostachys rosea* was found from isolate EM 20.

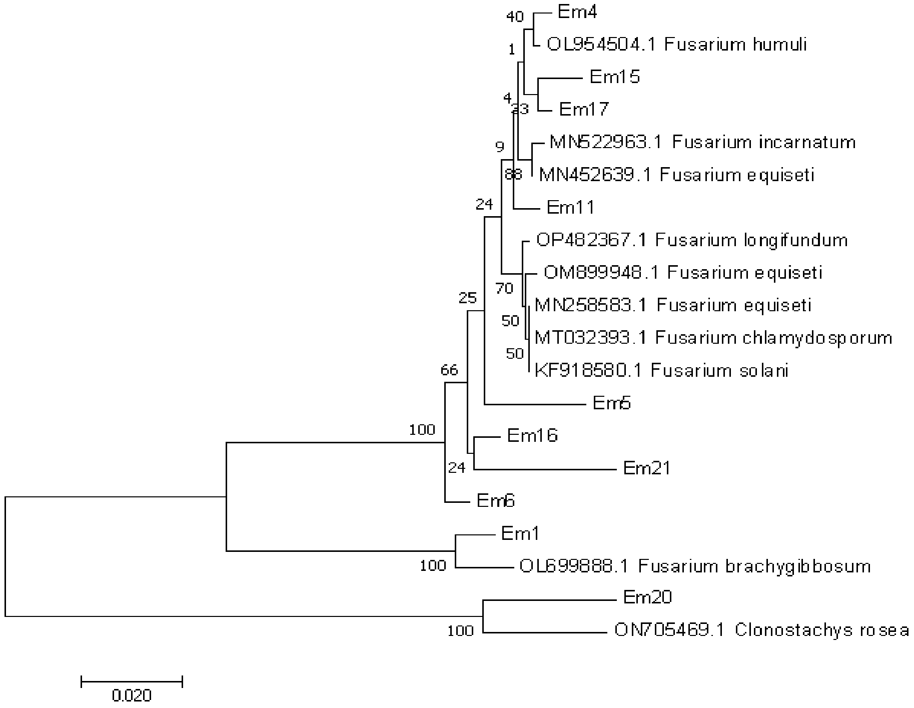

Figure 5. Phylogenetic relationships of the recovered fungal species from S. frugiperda cadavers and the corresponding species from the GenBank collections. The isolates labelled 'EM' are those isolated during the study while those with accession numbers are the ones from GenBank.

Phylogenetic relationships of the recovered fungal species with those found in GenBank collections

Evolutionary lineages among the recovered species with those found in GenBank (http://www.ncbi.nlm.nih.gov) were observed

(Figure 5 near here). Recovered Fusarium species from *S. frugiperda* cadavers were found in a clade of other Fusarium in GenBank collections with the support of a 100% bootstrap score. Apart from Fusarium species that were dominating, the peculiar specie *C. rosea* was found grouped uniquely with its single lineage and with the support of a 100% bootstrap score.

Discussion

The present results have shown the dominance of Fusarium species (90%) from the S. *frugiperda* cadavers which were sourced from various locations of Mwanza, Morogoro, Coast and Songwe. This indicates their wide coverage towards diverse ecologies and agroecosystems in the country. Despite the fact that, data on soil attributes like PH, moisture, temperature and general climatic conditions of surveyed areas were not done it is obvious that the sampled locations were highly varied. The diverse occupation of the species in dissimilar ecological attributes and weather conditions indicates the robustness and suitability of the species for the control of *S. frugiperda* which also occupies different ecologies (da Silva Santos *et al.,* 2020). Additionally, Fusarium species are reported to reside in plants and animals which make them labelled opportunists (Wang *et al.,* 2019). Though the sampled fields were receiving regular agronomic undertakings, including thinning, weeding and at some instances a single round of insecticide application at seedling stage (for those with early infestation), the recovery of fungal isolates from *S. frugiperda* cadavers shows the species' abilities in withstanding continual disturbances. Therefore the larvae mortality suggests the species' potential in combating the pest wherever it will be used (Thaochan & Sausa-Ard, 2017). Some scholars for instance Sandoval-Denis and Crous, (2018) reported the production of long-term surviving structures as one of the strategies the fungi use to survive adverse conditions. Therefore the results of this study seemed consistent with that observation as the recovered isolates are believed to hold the attribute irrespective of the disruptions which were happening in the fields. Likewise, it is reported that the structures have aptitudes of remaining in soils for extended periods until they encounter potential insect hosts (Pelliza *et al.,* 2011).

On the other hand, recovering fungi directly from *S. frugiperda* cadavers is consolidating the fact that they are EPF and somehow attributed host specific since occupying bodies of insects is their habit preference, yet other studies need to confirm. Uninterestingly, OTA+CTAB media failed to ease conidia production to some common Fusarium strains contrary to the hypothesis (Abdullah *et al.,* 2015). Therefore as a result identification using only the morphological characteristics became difficult, the shortcoming which was then overcame by molecular approaches. Therefore culturing of these collected fungal strains to various media is proposed.

Among the identified fungi, the notable member of the *Fusarium solani* species complex, *F. solani* is linked to mortalities of various insect pests of economic importance. For instance, Hernandez-Trejo *et al.* (2019) report *F. solani* inflicts 30-100% mortalities in *S. frugiperda* and *Periplaneta americana* (Blattodea: Blattidae). Moreover, *Fusarium equiseti*, a member of the *Fusarium incarnatum-equiseti* species complex (FIESC) has also been reported to kill *Cephus cinctus* (Hymenoptera: Cephidae) and *Bemisia tabaci* (Hemiptera: Aleyrodidae) by 34-100% (Anwar *et al.,* 2017). The strains' pathogenicity activity has been also demonstrated against gall wasp, *Dryocosmus kuriphilus*. Some Fusarium species have been further developed into commercialized formulations against some problematic pests (Al-Ani *et al.,* 2018). Therefore, the findings of this study accelerate thinking of using Fusarium-based EPF as a sound strategy for managing insect pests in fields (Anwar *et al.,* 2017).

However, some of the isolated fungal species are linked to plant and animal diseases. *F. brachygibbosum* for instance is linked to potato tubers and wheat seeds diseases. Likewise, *F. clamydosporum*, *F. solani* and *F. equiseti* are also reported to cause wilt diseases in chilli (Parihar *et al.,* 2022). Additionally, *F. equiseti*, in particular, is linked to ear and kernel rot in maize. Likewise, the mycotoxins they produce i.e. fumonisin B (FB), trichothecene and moniliformin are directly linked to human oesophageal cancer, infant neural tube abnormalities and equine leukoencephalomalacia (Zhou *et al.,* 2018). However, some recent enormous usage of molecular techniques and genetic engineering, in particular, have steered thinking to possibilities of disentangling those lethal attributes so that the fungi values may be exploited. A study by Navarro *et al.* (2011) reports the deletion of several loci in *F. oxysporum* strains for example, eliminated parasitism behaviour towards tomato plants but again increased their entomopathogenic behaviour against larvae of *Galleria mellonella* L. (Pyralidae: Lepidoptera). This scenario exceeds the urge towards an exploration of many other Fusaria species with entomopathogenic ability regardless of inadvertent phytopathogens and other toxins they produce.

The report of *C. rosea* (Hypocreales: Bionectriaceae) among the fungi which were obtained from *S. frugiperda* cadavers is a fascinating outcome of this research work and therefore it is here reported for the first time in Tanzania. The specie has been profiled as entomopathogenic, antagonistic and mycoparasite against various field pests (Lopez & Sword, 2015). It is used widely against wheat yellow mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) besides reinforcing antagonism against other fungal strains such as *Fusarium circinatum* and *Alternaria spp.* (Sun, *et al.,* 2020). Separately from parasitizing quite several fungal strains, *C. rosea* has been reported to improve the ability of plants to withstand arrays of salt and pest stresses when they reside plants as endophytes (de Carvalho *et al.,* 2020). With similar concern, Lopez and Sword (2015) reported increased dry mass, number of nodes and reproductive tissues in cotton plants following inoculation of *C. rosea*. These few observations outline the potentials of the specie and therefore can be utilized as BCA in agricultural settings. However, the isolate failed to develop conidia on OTA+CTAB and therefore this creates a research question for the forthcoming studies.

Conclusion

This study displayed in situ existence of *Fusaria* and *Clonostachys* fungal strains across wide ranges of Tanzania ecologies; the strains that can be exploited in formulating biopesticides as they are equipped with insecticidal properties. Though a thorough analysis of metabolites was not done, other reports still show clearly the natural potentials of the isolates against various insect pests including *S. frugiperda*. Hence provide a guarantee of sustainable agricultural production. However, it has remained unclear on their efficacy against *S. frugiperda* and therefore this study warrants the opportunity for other upcoming studies to contain the mentioned. Moreover, more exploration surveys across the country are recommended to recover diverse arrays of EPF species of worth that will be engaged in IPM programs.

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