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ORIGINAL ARTICLE

Fungal root endophytes of tomato from Kenya and their nematode biocontrol potential

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Abstract The significance of fungal endophytes in African agriculture, particularly Kenya, has not been well investigated. Therefore, the objective of the present work was isolation, multi-gene phylogenetic characterization and biocontrol assessment of endophytic fungi harbored in tomato roots for nematode infection management. A survey was conducted in five different counties along the central and coastal regions of Kenya to determine the culturable endophytic mycobiota. A total of 76 fungal isolates were obtained and characterized into 40 operational taxonomic units based on the analysis of ITS, *β-tubulin* and *tef1α* gene sequence data. Among the fungal isolates recovered, the most prevalent species associated with tomato roots were members of the *Fusarium oxysporum* and *F. solani* species complexes. Of the three genes utilized for endophyte characterization, *tef1α* provided the best resolution. A combination of ITS, *β-tubulin* and *tef1α* resulted in a better resolution as compared to single gene analysis. Biotests demonstrated the ability of selected non-pathogenic fungal isolates to successfully reduce nematode penetration and subsequent galling as well as reproduction of the root-knot nematode *Meloidogyne incognita*. Most *Trichoderma asperellum* and *F. oxysporum* species complex isolates reduced root-knot nematode egg densities by 35–46 % as compared to the non-fungal control and other isolates. This study provides first insights into the culturable endophytic mycobiota of tomato roots in Kenya and the potential of some isolates for use against the root-knot nematode *M. incognita*. The data can serve as a framework for fingerprinting potential beneficial endophytic fungal isolates which are optimized for abiotic and biotic environments and are useful in biocontrol strategies

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against nematode pests in Kenyan tomato cultivars. This information would therefore provide an alternative or complementary crop protection component.

Keywords Endophytes · Tomato · Kenya · Multi-gene phylogeny · Biocontrol · Nematodes

Introduction

Modern agriculture is facing new challenges in which ecological and molecular approaches are being integrated to achieve higher crop yields while minimising negative impacts on the environment. In this context, enhancing plant growth and plant resistance by using beneficial microorganisms is currently considered as an important key strategy (Pineda et al. 2010). Tomato (*Solanum lycopersicum*) production has been increasing worldwide due to the properties of its edible fruit which is an important source of carotenoids, flavonoids, vitamins and minerals (Debjit et al. 2012). In Kenya, tomato is currently the second leading vegetable in terms of production and value after potato. It accounts for 14 % of the total vegetable production and 6.72 % of the total horticultural crops grown (Geoffrey et al. 2014). Kenya is among Africa's leading producer of tomato and is ranked 6th in Africa with a total production of 397,000 t per year (FAO 2012).

Globally, tomato production is restricted by different biotic constraints such as pests, insects, plant parasitic nematodes, begomoviruses and fungal pathogens. In particular, the plant-parasitic root-knot nematode *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949, is considered a major problem in the tropics and subtropics, such as the East African rift valley areas of Kenya and Ethiopia (Onkendi et al. 2014). Therefore, increase in tomato yield is partly dependent on proper nematode control. Plant-parasitic nematodes are mainly controlled by application of nematicides or use of resistant tomato cultivars. Pesticide application has several drawbacks such as high costs, which may discourage resource-challenged farmers in sub-saharan Africa, and negative impacts on the environment and human health (Nicol et al. 2011). The consistent use of pesticides is also considered to cause a low level of natural antagonists, e.g. nematophagous fungi and beneficial insects (Atcha-Ahowé et al. 2009). The use of resistance cultivars is not a reliable alternative due to the fact that, in the currently available tomato cultivars, the *M. incognita* resistance gene is broken down at temperatures above 28 °C (Roberts and Thomason 1989). From an integrated pest management (IPM) perspective, additional methods are therefore required. In this context, biological control of soil-borne pathogens and nematodes by using antagonistic rhizosphere or endophytic microorganisms is a promising approach (Martinuz et al. 2012a, b).

It is becoming evident that virtually every plant in a natural or agricultural setting is colonized by a diversity of soil-borne microbes, e.g., root endophytes, mycorrhizal fungi, and plant growth-promoting rhizobacteria (Rodriguez and Redman 2008). Endophytes live within plant tissues for all or part of their life cycles and cause no apparent disease symptoms (Hyde and Soytong 2008). Endophytic fungi have been reported to be key elements in plant symbiosis, affecting plant host tolerance to stressful conditions (Rodriguez et al. 2009), plant defense (Omacini et al. 2001) and plant growth and development (Vogelsang et al. 2006).

The magnitude of fungal diversity is estimated to be 1.5 million species, but only 5 % of those have been described (Hawksworth 2001). The available data also indicate that fungal diversity in the tropics is richer than that in temperate regions (Berrin et al. 2012). A more in-depth knowledge on the status of microbial endophytes and their beneficial activities in the sub-Saharan African cropping systems is, however, still lacking. Fungal root endophytes are a phylogenetically diverse group primarily occurring within the Ascomycota, although some belong to the Basidiomycota. *Piriformospora indica* (phyllum Basidiomycota) and particular isolates within *Trichoderma* and *Fusarium* species (phylum Ascomycota) have been reported to enhance growth of various plant species and to induce resistance to pests like insects and nematodes (Daneshkhah et al. 2013; Vu et al. 2006). Some research has also revealed positive effects of dark septate endophytes (DSE) which comprise a group of asexual ascomycetes and are characterized by dark melanized septa on plant growth, yield and nutrient uptake (Andrade-Linares et al. 2011; Jumpponen 2001).

Several fungi have received serious attention as important antagonists in natural suppressive soils and as candidates for biocontrol (Stirling 2011). Possible mechanisms that have been suggested to be responsible for biocontrol activity include: the production of antifungal metabolites, competition for space and nutrients, mycoparasitism, plant growth promotion and induction of the defense responses in plants (Howell 2003; Aly et al. 2011).

The objective of this study was to isolate, identify and characterize the culturable endophytic mycobiota in the roots of tomato in Kenya, and to screen different fungal endophytes for their biocontrol potential towards the root-knot nematode *M. incognita*. Our study adds to the very incomplete information available on the endophytic mycobiota in Kenyan agriculture by providing an overview of the diversity of species that are present in tomato. It also sheds some light on the phylogenetic relationships among the different isolates studied. We believe that the knowledge obtained in this work will lead to fingerprinting of competent fungal endophytes for nematode management.

Materials and methods

Study sites and sampling strategy

To assess the endophytic mycobiota of fungal endophytes associated with tomato plants, one agro-ecological zone, zone IV, which represents the semi-humid to semi-arid area was selected. A survey was conducted to cover major tomato-growing areas along the central and coastal regions of Kenya. These areas included five different counties, namely Mwea in Kirinyaga county (central region), Mombasa, Lamu, Kilifi and Taveta counties (coastal region). More areas were sampled from the coastal region due to higher temperatures, because root-knot nematodes are known to be more dominant at higher temperatures than at lower temperatures (Karssen and Moens 2006). Sampling was done between March and December 2012. This period covered both the short and the long rainy seasons. The geographic locations and altitude descriptions of the central and coastal areas are summarized in Table 1.

During the field surveys, a purposeful sampling technique was applied (Suri 2011). Information was obtained directly from farmers. After interviewing the farmers, plants were chosen on the basis of three criteria: plants had to be in a physiological maturity stage, healthy in appearance, and free from any type of chemical application. From each selected farm, 3 plants were chosen at random from different positions within the field and uprooted. A total of 188 plants were collected from different farms in the five counties. The sample from each plant was placed individually in polythene bags, tied and labeled. Samples were labeled indicating the locality, date and farmer's name. They were kept cold before being transported to the laboratory. Root samples were stored at 4 °C in a refrigerator until processing.

Isolation of endophytic fungi

Within 48 h of collection, roots samples were further processed in the laboratory at the Department of Plant Sciences, Kenyatta University, Kenya. The roots were gently washed under running tap water and cut into 1-cm-long fragments using a sterile scalpel. The root pieces were surface-sterilized under a laminar flow hood through sequential immersion in 70 % (v/v) ethanol (Carl-Roth, Germany) for 2 min, 2 % (w/v) sodium hypochlorite (Carl-Roth) solution for 3 min, and rinsed three times with sterile water. The root pieces were then air-dried on sterile blotting paper.

Prior to plating, tissue imprints were made on potato dextrose agar (PDA) plates to assess the effectiveness of the surface sterilization procedure (Schulz and Boyle 2005). If no fungal growth was observed in the imprints, this indicated that the sterilization procedure was successful. Five segments per sample were platted on 1/10 strength of potato dextrose agar (Carl-Roth, Germany), to restrain mycelial growing speed, supplemented with 150 mg/l streptomycin sulfate (Sigma-Aldrich, Germany) to control bacterial growth.

The plates were incubated at 25 °C in darkness in an incubator (Memmert, Schwabach, Germany) and assessed after every 2 days for emerging fungi. Newly appearing mycelium was subcultured onto fresh PDA media until pure cultures were obtained.

A rough differentiation of the fungal strains was done on the basis of the macroscopic parameters such as colony appearance, mycelium color and growth rate on PDA medium. Texture and pigmentation from above and below were observed for each isolate. After that, morphological characteristics of each isolate were assessed visually. For this, the spores or mycelia of each isolate was harvested and then stained with lactophenol blue stain for 15 min. The slide was then mounted on a light microscope (Leica, Wetzlar, Germany) and assesed

Table 1 Geographic location and altitude of areas where plants were isolated in Kenya

Location	Altitude (m)	Coordinates	Mean annual rainfall (mm/year)	Average temperature (°C)	Moisture index (%)	Soil type	Habitat description
Central	1160	0°32′–0°46′ S, 37°13′–37°30′E	880–2200	19 °C	52–67	Deep, well-drained red to reddish-brown soils	Grasslands and open savannah
Coastal	50	3°80′–4°10′ S, 39°60′–39°80′E	889–1397	26.3 °C	40–50	Firm clay-loam soils, dark grayish brown	Grasslands and open savannah

(Nyamai et al. 2012; Chira 2003; Munga et al. 2005)

at 100x magnification. Isolates were grouped into morphospecies. Multi-locus DNA sequencing techniques were then used to support morphological identification of the isolated fungal endophytes to genus or species level. Molecular characterization was carried out at the University of Bonn, Germany. Isolates are available from the authors upon request and have been deposited in the culture collection of Molecular Phytomedicine, University of Bonn.

DNA extraction, amplification, and sequencing

Mycelia were obtained by inoculating 3 plugs from a 7-day-old culture on PDA into 250-ml flasks containing 100 ml potato dextrose broth (Carl-Roth) and then incubating for 5 days at room temperature in a rotary shaker (Edmund Bühler, Hechingen, Germany) at 150 rpm. Mycelia were harvested by filtration through Miracloth (Calbiochem, USA) and subsequently frozen, then lyophilized for 24 h before grinding them to fine powder. Total DNA was extracted from 20 mg of lyophilized mycelia using the Wizard Magnetic DNA Purification System for Food (Promega, Mannheim, Germany) according to the manufacturer's instructions. The ITS region was amplified using ITS1-F (5′-TCCGTAG GTGAACCTGCGG-3′) and ITS4-R (5′-TCCTCCGC TTATTGATATGC-3′) primers (White et al. 1990), while the *tef1α* was amplified using EF-1 (5′-ATGGGTAA GGARGACAAGAC-3′), EF-2 (5′-GGARGTACCA GTSATCATG-3′) primers (O'Donnell et al. 2004) and EF1-728F(5′-CATCGAGAAGTTCGAGAAGG-3′), EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) primers for *Trichoderma* specific isolates (Carbone and Kohn 1999). The *ß-tubulin* region was amplified using Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) primers (Glass and Donaldson 1995). The total reaction volume of all PCR mixtures was 50 µl. The reaction mixture consisted of distilled water, 25 mM dNTP, 1.5 units of GoTaq polymerase, (Promega), 10 µl 5× GoTaq Green reaction buffer (Promega), 0.2 µM of each primer and 5 ng genomic DNA template PCR amplification was conducted in a C1000 Thermocycler (Bio-Rad, CA, USA) by applying an initial denaturation step at 95 °C for 4 min, followed by 34 cycles of 95 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min. PCR products were purified using the illustraTM GFX TM PCR, DNA and Gel Band Purification kit (GE Healthcare UK, Amersham, UK).

Prior to purification, the PCR amplicons were separated on a 1 % (w/v) agarose Tris-acetate-EDTA (TAE) gel supplemented with 10 µg/ml ethidium bromide and analyzed with the Gel Doc XR+ Molecular Imager, (Bio-Rad). DNA fragments of proper size were excised and the PCR product recovered using the procedures outlined in the purification kit manual. Purified DNA fragments were quantified using a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Passau, Germany) and sent to GATC Biotech for sequencing (GATC Biotech, Konstanz, Germany).

Edited sequences were blasted against nucleotide databases (e.g. NCBI). The most homologous sequences (species) were then used to search for their corresponding type strains or well-characterized strains (Ko Ko et al. 2011). Representative fungal isolates from this study and their reference strains are summarized in Table 4. In addition, all *Fusarium* spp. isolates were determined by comparing their TEF1-α sequences with those in *Fusarium*-ID (http://isolate.fusariumdb.org) and *Fusarium* MLST (http://www.cbs.knaw.nl/Fusarium) databases (Aoki et al. 2014; O'Donnell et al. 2008) and their results are indicated in Supplementary Table S1. It is known that sequence-based identification strategies can be meaningful only with the availability of well-curated, robust, and reliable databases that are populated with sequence data from type or reference strains (where possible). It is therefore important to compare sequences with ex-type strains to avoid misinterpretation and relying on the GenBank. Errors in fungal sequences in GenBank have been found to be as high as 20 % (Nilsson et al. 2006). All sequences used in this study were submitted to GenBank and are available under the accession numbers listed in Table 2.

Phylogenetic analysis

Sequences were assembled and edited using the software Geneious v.5.6.3 (2012 Biomatters). Further alignment of the sequences was performed by MAFFT webserver v.6 using the Q-INS-i strategy and standard settings (Katoh and Toh. 2008). A first phylogenetic analysis was carried out with MEGA 5.0 (Tamura et al. 2011). A minimum evolution (ME) tree was computed using the Tamura-Nei substitution model and 1000 bootstrap replicates (Felsenstein 1985). Further parameters were set to default values. Phylogenetic inference was done at the Biodiversity and Climate Research Centre (BiK-F), Frankfurt, Germany, with the help of the TrEase server (Mishra et al. unpublished; http://www.thines-lab.senckenberg.de/trease), which runs several phylogeny software packages as outlined below. Before phylogenetic inference, multiple sequence alignments were subjected to refinement using Gblocks (v.0.91b) (Castresana 2000) with default settings. Phylogenetic inference was done with three different methods for all datasets. RAxML (7.2.6) (Stamatakis 2014; Ott et al. 2007) was used with the GTRGAMMA substitution model and 1000 bootstrap replicates for maximum likelihood (ML) inference. Fasttree (v.2.1.7) (Price et al. 2010) was used with the default parameters for ME phylogenetic reconstruction. For Bayesian phylogenetic inference MrBayes (v.3.2.2 x64) (Ronquist et al. 2012) was used with ncat = 4, 5 million generations and 30 % burnin.

Table 2 Fungal isolates recovered from tomato roots in this study and their GenBank accession numbers

Isolates code	Fungal isolates	Geographical origin		GenBank accession number		
		County	Region	ITS	*ß-tubulin*	*tef1α*
KC 1	*Trichoderma asperellum*	Kirinyaga	Central	KT357597	KU296847	KT357558
KC 2	*Trichoderma asperellum*	Kirinyaga	Central	KT357598	KU296848	KT357555
KC 3	FSSC	Kirinyaga	Central	KT357582	KT316674	KT357542
KC 4	FSSC	Kirinyaga	Central	KT357583	KT316676	KT357543
KC 5	FOSC	Kirinyaga	Central	KT357565	KT316685	KT357532
KC 6	FOSC	Kirinyaga	Central	KT357567	KT316688	KT357527
KC 7	FOSC	Kirinyaga	Central	KT357568	KT316686	KT357531
KC 8	FOSC	Kirinyaga	Central	KT357569	KT316683	KT357525
MKC 9	FSSC	Mombasa	Coastal	KT357584	KT316670	KT357547
MKC 10	FSSC	Mombasa	Coastal	KT357585	KT316667	KT357541
MKC 11	FSSC	Mombasa	Coastal	KT357586	KT316668	KT357544
MKC 12	FSSC	Mombasa	Coastal	KT357587	KT316661	KT357545
MKC 13	FSSC	Mombasa	Coastal	KT357588	KT316673	KT357540
MKC 14	FSSC	Mombasa	Coastal	KT357589	KT316671	KT357546
MKC 15	FSSC	Mombasa	Coastal	KT357590	KT316669	KT357548
MKC 16	FSSC	Mombasa	Coastal	KT357591	KT316662	KT357549
MKC 17	FSSC	Mombasa	Coastal	KT357592	KT316672	KT357539
MKC 18	*Fusarium nygamai*	Mombasa	Coastal	KT357566	KU296849	KT357537
MKC 19	FOSC	Lamu	Coastal	KT357577	KT368162	KT357528
MKC 20	FOSC	Lamu	Coastal	KT357578	KT368159	KT357524
MKC 21	FOSC	Lamu	Coastal	KT357579	KT316679	KT357535
MKC 22	*Fusarium* spp.	Lamu	Coastal	KT357572	KU296850	KT357530
MKC 23	FSSC	Lamu	Coastal	KT357593	KT316663	KT357551
MKC 24	*Aspergillus sclerotiorum*	Lamu	Coastal	KT357603	KU296851	KT357559
MKC 25	*Fusarium* spp.	Lamu	Coastal	KT357575	KU296852	KT357552
MKC 26	FSSC	Lamu	Coastal	KT357594	KT316664	KT357550
MKC 27	*Hypocrea lixii*	Lamu	Coastal	KT357604	KU296853	KT357557
MKC 28	*Curvularia aeria*	Kilifi	Coastal	KT357596	KU296854	KT357553
MKC 29	FOSC	Kilifi	Coastal	KT357574	KT368160	KT357533
MKC 30	FOSC	Kilifi	Coastal	KT357576	KT368158	KT357534
MKC 31	FOSC	Kilifi	Coastal	KT357580	KT316689	KT357529
MKC 32	*Stemphylium lycopersici*	Kilifi	Coastal	KT357599	KT316666	KT357561
MKC 33	*Stemphylium lycopersici*	Kilifi	Coastal	KT357600	KT316677	KT357563
MKC 34	*Stemphylium lycopersici*	Kilifi	Coastal	KT357601	KT316675	KT357562
MKC 35	*Stemphylium lycopersici*	Kilifi	Coastal	KT357602	KT316665	KT357560
MKC 36	*Fusarium verticillioides*	Kilifi	Coastal	KT357570	KU296855	KT357538
MKC 37	*Fusarium* spp.	Taveta	Coastal	KT357571	KU296856	KT357536
MKC 38	FOSC	Taveta	Coastal	KT357573	KT368161	KT357526
MKC 39	*Altenaria solani*	Taveta	Coastal	KT357605	KU296857	KT357564
MKC 40	*Cochliobolus* spp.	Taveta	Coastal	KT357595	KU296858	KT357554
Ref 1	FOSC 162	Bonn	Germany	KT357581	KT316682	KT357523
Ref 2	*Trichoderma asperellum* 1	Real IPM	Kenya	KT357606	KU296859	KT357556

FSSC Fusarium solani species complexes, *FOSC Fusarium oxysporum* species complexes

Operational taxonomic units (OTUs) were assigned based on phylogenetic distinctiveness, i.e. grouping together with most homologous type strains (well-characterized strains) on terminal branches or being excluded from them with high support in at least two of the three phylogenetic methods. Dothiomycetes and

Eurotiomycetes also collected in this survey were used as outgroup.

Plants and substrate

Tomato cultivar Moneymaker, which is susceptible to *M. incognita*, was used in all experiments. Seeds were surface-sterilized by first submersing them in a 70 % ethanol solution for 1 min followed by rinsing them twice with sterile de-mineralized water for 5 min. The water was discarded, and the seeds soaked in a 1.5 % (w/v) NaOCl solution for 10 min under constant shaking in a rotary shaker. The seeds were again rinsed twice with water and subsequently hydro-primed by shaking over night to facilitate water uptake and synchronize germination.

Two kinds of substrates were used: standard soil substrate Type ED 73 (Gebrüder Patzer, Germany) for seed germination and a mixture of field soil (15 % clay, 78 % silt and 8 % sand) and sand in a ratio of 2:1 (v/v). Before mixing, the field soil was passed through a sieve. The substrates were autoclaved for 1 h at 121 °C and aerated for 2 days prior to usage. Surface-sterilized and hydro-primed tomato seeds were then sown in seedling trays in 24-well multi-pot trays containing ED 73 substrate. The planting trays were maintained in a growth chamber at 25 ± 3 °C with 16 h diurnal light, 60–70 % humidity. The trays were watered as needed and fertilized weekly with a slow release formulation (N:P:K 14:10:14) at 2 g/l of water. Three weeks after germination, the plants were uprooted and the roots were carefully washed under running tap water. The roots were then inoculated with spore suspensions of the selected fungal isolates as indicated in the next section and replanted in a mixture of field soil and sand.

Spore harvesting and fungal inoculation

A primary selection experiment was performed to determine which of the 40 fungal isolates should be included in the screening for biocontrol potential. In view of the results from the selection experiments, fungal endophytes were narrowed down from 40 to 7 isolates, which showed promise in biocontrol. The criterion used was based on: good and adequate in vitro sporulation, re-isolation potential, nematode suppression and geographical origin of the fungus. Reference strains included *Fusarium oxysporum* 162 (Reference 1) from the University of Bonn which was originally isolated from the cortical tissue of surface sterilized tomato roots cv. Moneymaker in Kenya (Hallmann and Sikora 1994), and a commercial *Trichoderma asperellum* (Reference 2) isolate provided by Real IPM in Thika, Kenya.

For all experiments, freshly prepared spores were used. To initiate fresh cultures, some of the fungal mycelia plugs, stored in mineral oil (Carl-Roth) at −80 °C in micro-vial tubes were transferred onto the center of PDA plates. The plates were incubated, at 28 °C in the dark for 7–14 days. For spore harvesting, 10 ml sterile demineralized water was added and both mycelia and spores were carefully scrapped from the surface of the plate using a sterile microscope glass slide. To separate the spores from the fungal mycelia, the suspension was filtered through 3 layers of Miracloth and collected via a funnel into a 15-ml falcon tube.

For spore counting, relevant dilutions were prepared and spore densities were determined using a haemocytometer (Fuchs-Rosenthal chamber; Hausser Scientific, USA) and adjusted with sterile water to 1×10^7 CFU ml^{-1}. The roots of 3-week-old plants were then submerged individually in separate beakers for 30 min in a spore solution of the respective fungal endophyte or sterile water as a negative control. The plants were arranged in a completely randomized block design. Three weeks after the first fungal inoculation, a few plants were randomly selected to determine fungal colonization by surface sterilization, while the rest were inoculated with 500 juvenile stage two (J2s) of *M. incognita*. Treatments were replicated six times and the experiment was conducted twice.

Nematode extraction and infection assay

The root-knot nematode *M. incognita* used in all experiments originated from cultures provided by Dr. D. Dickson (University of Florida, Gainesville, Florida, USA). Nematodes were continuously propagated on the susceptible tomato cultivar Moneymaker, grown in a large container (150 × 80 × 40 cm) with sandy loam soil in a greenhouse (27 ± 5 °C). Nematode eggs were extracted from a 2-month-old heavily galled tomato roots using a modified extraction technique (Hussey and Barker 1973).

The plants were uprooted, and the roots were rinsed, cut into 1- to 2-cm pieces and macerated in a Warring blender (Ultra Turax T25; Whatman, Dassel, Germany) in 0.6 % (w/v) NaOCl for 15 s at low speed and 20 s at high speed. The suspension was distributed over two 1-l Erlenmeyer flasks and shaken for 2 min to release the eggs from the gelatinous matrix. After shaking, 1 l tap water was added to each Erlenmeyer flask to dilute the NaOCl concentration.

To separate the eggs from all plant particles and to remove the NaOCl, the suspension was thoroughly washed with tap water through a stack of 5 sieves (Retsch, Haan, Germany) with mesh sizes of 200, 180, 100, 50 and 25 μm, respectively. The eggs remaining on the last sieve were rinsed once again with tap water and collected. To verify the success of the extraction, the egg suspension was assessed using a stereomicroscope (Leica, Wetzlar, Germany) at 8x magnification.

To obtain preparasitic second stage juveniles (J2s), eggs were let to hatch in the dark at 24 °C 28 °C for 7–10 days. To separate the J2s from the non-hatched eggs, non-active

Table 3 Number and taxonomic affiliation of OTUs isolated from tomato roots

Isolates code	Geographical origin	Phylum	Class	Order	OTU	Abundance (number of isolates)
KC 1	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma asperellum*	3
KC 2	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma asperellum*	3
KC 3	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	FSSC	3
KC 4	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	FSSC	3
KC 5	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	FOSC	3
KC 6	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	FOSC	3
KC 7	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	FOSC	3
KC 8	Kirinyaga	Ascomycota	Sordariomycetes	Hypocreales	FOSC	3
MKC 9	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	2
MKC 10	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	3
MKC 11	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	3
MKC 12	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	2
MKC 13	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	2
MKC 14	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	1
MKC 15	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	3
MKC 16	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	2
MKC 17	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	FSSC	2
MKC 18	Mombasa	Ascomycota	Sordariomycetes	Hypocreales	*Fusarium nygamai*	1
MKC 19	Lamu	Ascomycota	Sordariomycetes	Hypocreales	FOSC	3
MKC 20	Lamu	Ascomycota	Sordariomycetes	Hypocreales	FOSC	2
MKC 21	Lamu	Ascomycota	Sordariomycetes	Hypocreales	FOSC	3
MKC 22	Lamu	Ascomycota	Sordariomycetes	Hypocreales	*Fusarium*spp.	1
MKC 23	Lamu	Ascomycota	Sordariomycetes	Hypocreales	FSSC	1
MKC 24	Lamu	Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus sclerotiorum*	1
MKC 25	Lamu	Ascomycota	Sordariomycetes	Hypocreales	*Fusarium* spp.	1
MKC 26	Lamu	Ascomycota	Sordariomycetes	Hypocreales	FSSC	1
MKC 27	Lamu	Ascomycota	Sordariomycetes	Hypocreales	*Hypocrea lixii*	1
MKC 28	Kilifi	Ascomycota	Dothideomycetes	Pleosporales	*Curvularia aeria*	1
MKC 29	Kilifi	Ascomycota	Sordariomycetes	Hypocreales	FOSC	1
MKC 30	Kilifi	Ascomycota	Sordariomycetes	Hypocreales	FOSC	3
MKC 31	Kilifi	Ascomycota	Sordariomycetes	Hypocreales	FOSC	2
MKC 32	Kilifi	Ascomycota	Dothideomycetes	Pleosporales	*Stemphylium lycopersici*	1
MKC 33	Kilifi	Ascomycota	Dothideomycetes	Pleosporales	*Stemphylium lycopersici*	1
MKC 34	Kilifi	Ascomycota	Dothideomycetes	Pleosporales	*Stemphylium lycopersici*	1
MKC 35	Kilifi	Ascomycota	Dothideomycetes	Pleosporales	*Stemphylium lycopersici*	1
MKC 36	Kilifi	Ascomycota	Sordariomycetes	Hypocreales	*Fusarium verticillioides*	1
MKC 37	Taveta	Ascomycota	Sordariomycetes	Hypocreales	*Fusarium* spp.	1
MKC 38	Taveta	Ascomycota	Sordariomycetes	Hypocreales	FOSC	2
MKC 39	Taveta	Ascomycota	Dothideomycetes	Pleosporales	*Altenaria solani*	1
MKC 40	Taveta	Ascomycota	Dothideomycetes	Pleosporales	*Cochliobolus* spp.	1
Ref 1	Bonn	Ascomycota	Sordariomycetes	Hypocreales	FOSC 162	1
Ref 2	Real IPM Thika	Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma asperellum* 1	1

FSSC Fusarium solani species complexes, *FOSC Fusarium oxysporum* species complexes

juveniles and remaining plant debris, the entire suspension was carefully passed through an 11-μm sieve that had been placed on a beaker filled with water. After incubating for 1 h in the dark at room temperature, the active J2s that swam through the 11-μm sieve were collected at the bottom of the water-filled glass beaker. The J2s were then concentrated by carefully removing the water. Three 1-cm-deep holes were made with a pipette tip around the stem of each plant and the nematode suspension was inoculated into the holes.

Finally, the number of nematodes penetrating was assessed 14 days after nematode inoculation and the number of galls and egg masses were assessed 8 weeks after nematode inoculation. In the case of nematode penetration, the entire root was taken, while 5 g of roots were taken after 8 weeks for galls and egg mass assessment. For the nematode penetration analysis, tomato roots were stained with acid fuchsin (Sigma-Aldrich) as described by Bybd et al. (1983) with minor modifications. Egg masses were stained with 0.015 % Phloxine B (Sigma-Aldrich) for 20 min and washed with tap water to remove excess stain (Holbrook et al. 1983).

Statistical analysis

Statistical analyses of the biocontrol experiments were performed using SPSS 21 software (IBM SPSS Statistics). The normal distribution and the homogeneity of variance were checked before each analysis and, when both assumptions were met, data was further analyzed via one-way ANOVA. When the data failed to meet one of the assumptions, the data were further log transformed ($\log_{10}x + 1$) and further analyzed via One-way ANOVA. Non-parametric tests (Kruskal-Wallis one-way ANOVA on Ranks) were performed for data which did not satisfy one of the assumptions even after the log transformation. If there was any significant difference between treatments, the Dunnett multiple comparison test was used to check if there was any difference in the means of the different treatments compared to the negative control.

Results

Endophytic mycobiota in tomato roots

Irrespective of the sampling region (central or coastal region), endophytes were recovered from about half of the root sections. After transfer to fresh medium, some isolates did not grow, whereas others showed insufficient- or no sporulation for an adequate identification and were classified as '*mycelia sterilia*' (Guo et al. 1998). None of these isolates were further analyzed. The inability to sporulate makes an endophyte much more difficult and, thus, less interesting for development into a biocontrol product. Among the fungal isolates that were finally recovered and sporulated, 76 strains were recognizable on the basis of their morphological characteristics. To confirm the morphogical identification and to better characterize them, the isolates were subjected to a multi-locus sequence analysis based on ITS, *β-tubulin* and *tef1α* genes. In total, 40 OTUs were identified (Table 2).

All endophytic taxa were included in the phylum Ascomycota, within 3 classes and 3 orders (Table 3). Sixty-eight isolates, i.e. 89 % of the total, belonged to the Sordariomycetes, represented by a single order (Hypocreales), whereas a few isolates belonged to the orders Pleosporales and Eurotiales with a relative frequency of 9 and 1 %, respectively (Fig. 1a). At the genus level, *Fusarium* dominated with 80 %, followed by *Trichoderma* and *Stemphylium* with 9 and 5 %, respectively (Fig. 1b). At the subgeneric level, members of the *F. solani* and *F. oxysporum* species complexes (FSSC and FOSC, respectively) dominated with the highest number of isolates (28 isolates) followed by *Trichoderma asperellum* (6 isolates). Other genera were rarely isolated and constituted one or at most two isolates each: *Alternaria solani, Cochliobolus geniculatus, Curvularia aeria, Aspergillus sclerotiorum, and Hypocrea lixii*. These rare isolates were unique to the coastal counties of Kenya.

Phylogeny

Phylogenetic trees of the 40 isolates were constructed using individual and combined ITS, *β-tubulin* and *tef1α* sequences. Dothideomycetes and Eurotiomycetes also collected in this study were used as outgroups. When available, reference strain sequences were included in the phylogenetic analysis

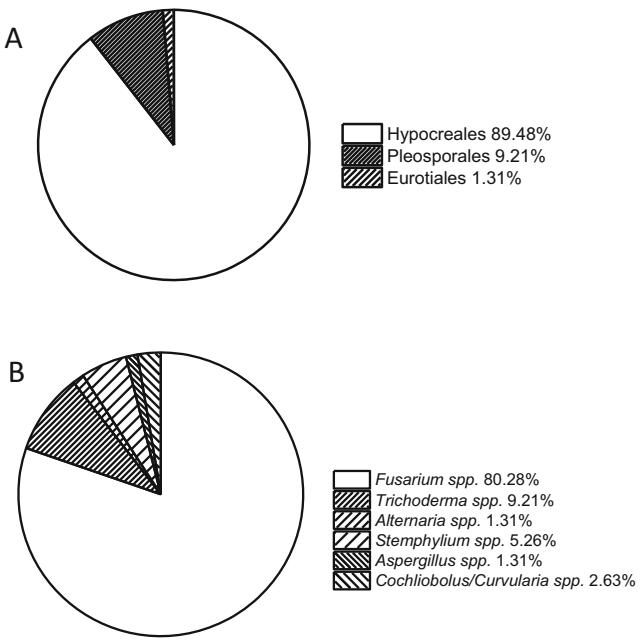

Fig. 1 Relative frequencies of isolated OTUs at the level of orders (**a**) and genus (**b**)

Table 4 Representative fungal isolates from this study and their reference strains

Representative isolate	Reference strains and Best Hit (GenBank)	Similarity (%) with reference strains		
		[a]ITS	[b]*β-tubulin*	[c]*tef1α*
Trichoderma asperellum KC1	[a]*Trichoderma asperellum* CBS 433.97[T] [b]*Hypocrea lixii (*EF027167.1) [c]*Trichoderma spp*. GJS 08137	99 %	98 %	100 %
FSSC MKC9	[ab]*Fusarium solani* NBRC 8505 [c]*Fusarium solani* SZMC 11456	97 %	98 %	99 %
FOSC KC5	[abc]*Fusarium oxysporum* CBS 159.57 [T]	99 %	100 %	99 %
Fusarium spp. MKC37	[a]*Fusarium spp.* NRRL 28852 [b]*Fusarium oxysporum* CBS 159.57 [T] [c]*Fusarium secorum* NRRL 62593	99 %	100 %	99 %
Fusarium spp. MKC22	[a]*Fusarium spp.* NRRL 28852 [b]*Fusarium oxysporum* CBS 159.57 [T] [c]*Fusarium oxysporum* NRRL 46602	99 %	99 %	99 %
Fusarium spp. MKC25	[a]*Fusarium spp.* NRRL 28852 [b]*Fusarium oxysporum* CBS 159.57 [T] [c]*Fusarium spp.* NRRL 26061	99 %	100 %	99 %
Fusarium nygamai	[a]*Fusarium nygamai* CBS 749.97 [T] [b]*Fusarium nygamai* NRRL 13448 [T] [c]*Fusarium nygamai* NRRL 52708	99 %	100 %	99 %
Altenaria solani	[ac]*Alternaria solani* CBS 116651 [b]*Alternaria solani* ATCC 58177 [T]	100 %	99 %	99 %
Cochliobolus spp. MKC40	[a]*Cochliobolus geniculatus* NBRC 100369 [b]*Aspergillus peyronelli* NRRL 4754 [c]*Embellisia abundans* CBS 535.83	100 %	97 %	86 %
Stemphylium lycopersici	[a]*Stemphylium lycopersici* EGS 46–001 [bc]*Stemphylium lycopersici* CNU070067	99 %	100 %	100 %
Curvularia aeria MKC28	[a]*Curvularia aeria* ISHAM-ITS_ID MITS1387 [b]*Aspergillus peyronelli* NRRL 4754 [c]*Embellisia abundans* CBS 535.83	100 %	93 %	84 %
Fusarium verticillioides	[ab]*Fusarium verticillioides* CBS 576.78 [T] [c]*Gibberella moniliformis* CBS 576.78 [T]	99 %	100 %	99 %
Aspergillus sclerotiorum	[a]*Aspergillus sclerotiorum* ATCC 16892 [T] [b]*Aspergillus sclerotiorum* NRRL 415 [T]	99 %	96 %	–
Hypocrea lixii MKC27	[a]*Hypocrea lixii* G.J.S 97-96 [b]*Hypocrea lixii (*EF027167.1) [c]*Hypocrea lixii* SH3204	99 %	99 %	99 %

FSSC Fusarium solani species complexes, *FOSC Fusarium oxysporum* species complexes, *CBS* Centraalbereau voor Schimmelcultures, Fungal and Yeast, Netherlands, *GJS* Gary J. Samuels Culture Collection, *NBRC* Nite biological resource centre, *SZMC* University of Szeged, microbiological collection, Hungary, *NRRL* Northern Regional Research Laboratory, USA, *EGS* Emory Simmons Culture Collection, *ATCC* American Type Culture Collection, *ISHAM* International Society for Human and Animal Mycology

[a] ITS, [b] *β-tubulin*, [c] *tef1α*, [T] ex-type

(Table 4). Sequences of the ITS and 5.8 S region of rDNA, ranged from 480 to 515 bp while those of *β-tubulin* and *tef1α* ranged from 280 to 300 bp and 640 to 660 bp, respectively. The results of the phylogenetic reconstructions for *β-tubulin*, *tef1α* and the three genes combined are depicted in Figs. 2, 3 and 4, respectively. For each gene, a similar topology was observed in ME, ML and BI analyses. However, the trees from Bayesian inference provided slightly better clade support. Therefore, only the tree based on the BI analysis for each gene is presented, including the ME and ML bootstrap values.

The ITS rDNA tree was only informative at the genus level, but did not sufficiently resolve species in many clades well (Supplemental data, Fig. S1). Trees inferred from the *β-tubulin* dataset resulted in a phylogram (Fig. 2) which is topologically similar to that of ITS with a

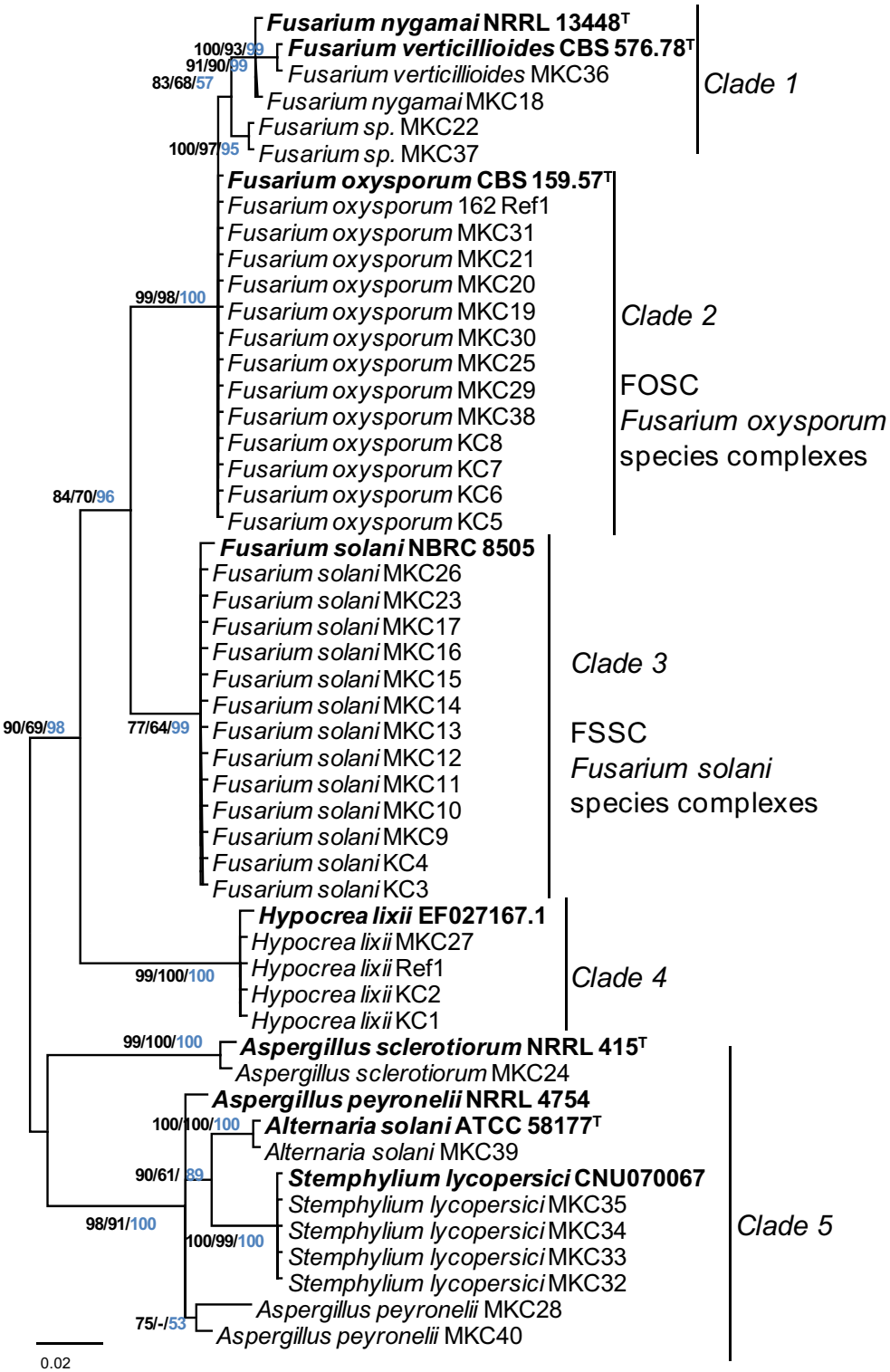

Fig. 2 Phylogram generated from Bayesian inference (MrBayes) based on *β-tubulin* sequence data. The *numbers above or below the branches* indicate bootstrap support (values greater than 50 %) in minimum evolution (Fasttree), maximum likelihood (RaXML) and Bayesian Inference (MrBayes) analyses. Minimum evolution and maximum likelihood bootstrap support values are indicated on the *left*- and *middle* side, while the Bayesian Inference values are illustrated on the *right* side. Clade 5 represents outgroup taxa. Reference sequences are in *bold*

slightly different resolution. In general, five major clades were also resolved in the *β-tubulin* tree with high bootstrap support.

Phylogenetic trees inferred from *tef1α* also comprised five well resolved clades with bootstrap values of 99, 77, 100, 100 and 100 % (Fig. 3). In comparison to ITS and *β-tubulin*, the *tef1α* gene separated all species included in this study at the species level, with high bootstrap support and provided the best resolution. Analysis of the combined ITS, *β-tubulin* and *tef1α* genes (Fig. 4) generated an overall phylogenetic tree with the strongest clade support.

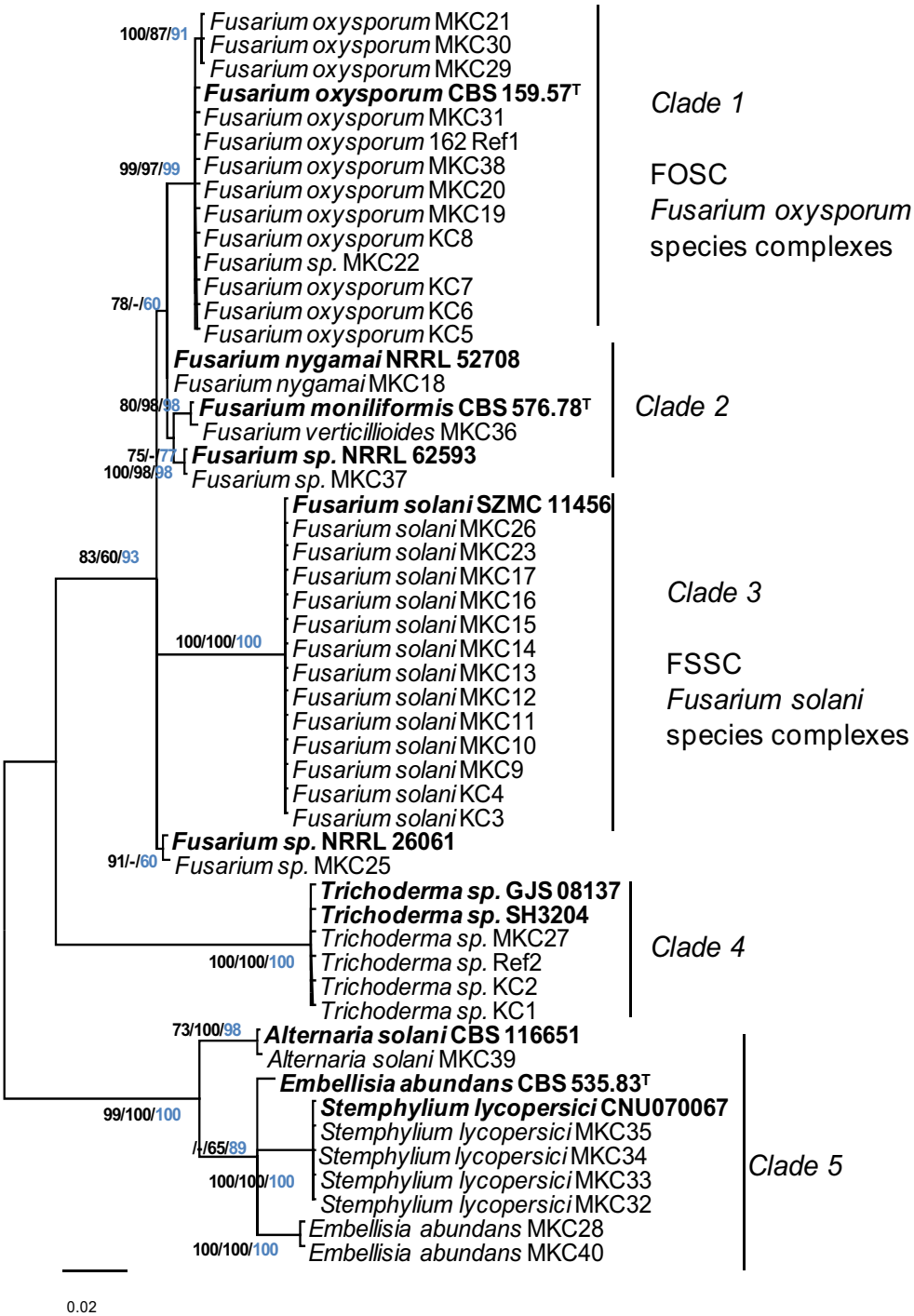

Fig. 3 Phylogram generated from Bayesian inference (MrBayes) based on *tef1*α sequence data. The *numbers above or below the branches* indicate bootstrap support (values greater than 50 %) in minimum evolution (Fasttree), maximum likelihood (RaXML) and Bayesian Inference (MrBayes) analyses. Minimum Evolution and Maximum Likelihood bootstrap support values are indicated on the *left*- and *middle* side, while the Bayesian Inference values are illustrated on the *right* side. Clade 5 represents outgroup taxa. Reference sequences are in *bold*

Biocontrol experiments

All isolates appeared to be nonpathogenic to tomato as no plant disease symptoms or death was recorded. Twelve days after nematode inoculation, penetration per root system was reduced when the plants had been pre-inoculated with endophytes as compared to endophyte free plants (Fig. 5a–c). Nematode penetration rates with some isolates such as *Trichoderma asperellum* KC1, FOSC KC5 and two reference isolates was significantly ($P < 0.05$) lower than in control roots. Similar results were observed in the root galling damage and egg mass production. Female fecundity in plants treated with endophytes was also negatively affected. Some isolates, i.e. *Trichoderma asperellum* KC1 and FOSC isolates KC5 and KC6, provided a greater level of egg mass

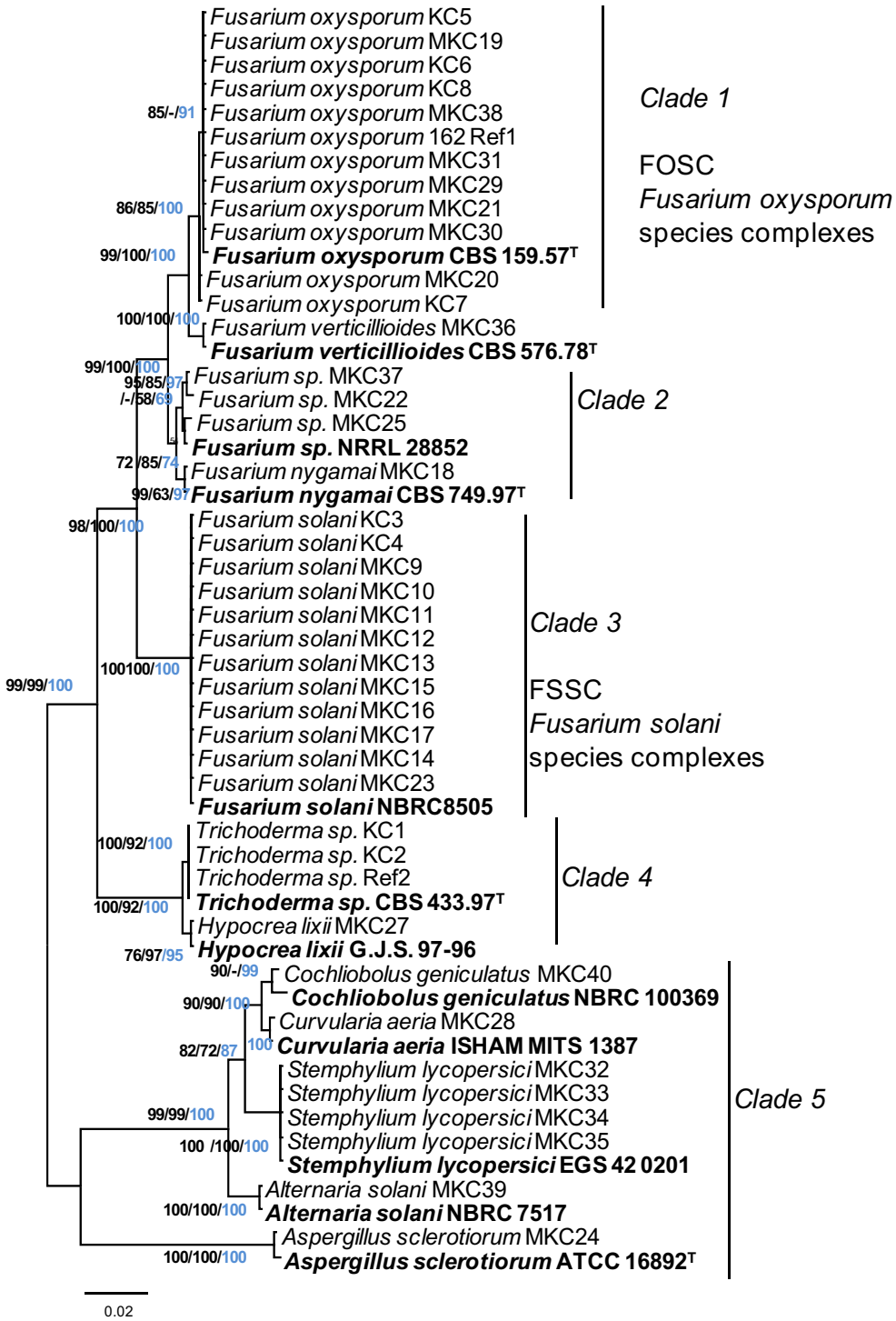

Fig. 4 Multi-gene phylogram generated from Bayesian inference (MrBayes) based on ITS, *ß-tubulin* and *tef1* α sequence data. The *numbers above or below the branches* indicate bootstrap support (values greater than 50 %) in minimum rvolution (Fasttree), maximum likelihood (RaXML) and Bayesian Inference (MrBayes) analyses. Minimum evolution and maximum likelihood bootstrap support values are indicated on the *left* and *middle* side, while the Bayesian Inference values are illustrated on the *right* side. Clade 5 represents outgroup taxa. Reference sequences are in *bold*

suppression. In these three isolates, egg mass production was reduced by 46, 37 and 35 %, respectively. These isolates performed as well as the two reference strains FOSC 162 (Fo162-Ref1), and *T. asperellum* Ref2. The two members of the FSSC that were screened in this study had no effect regarding nematode suppression.

Discussion

Diversity of root endophytes

The diversity of endophytic fungi found in this study in the roots of tomato is in accordance with the general pattern of

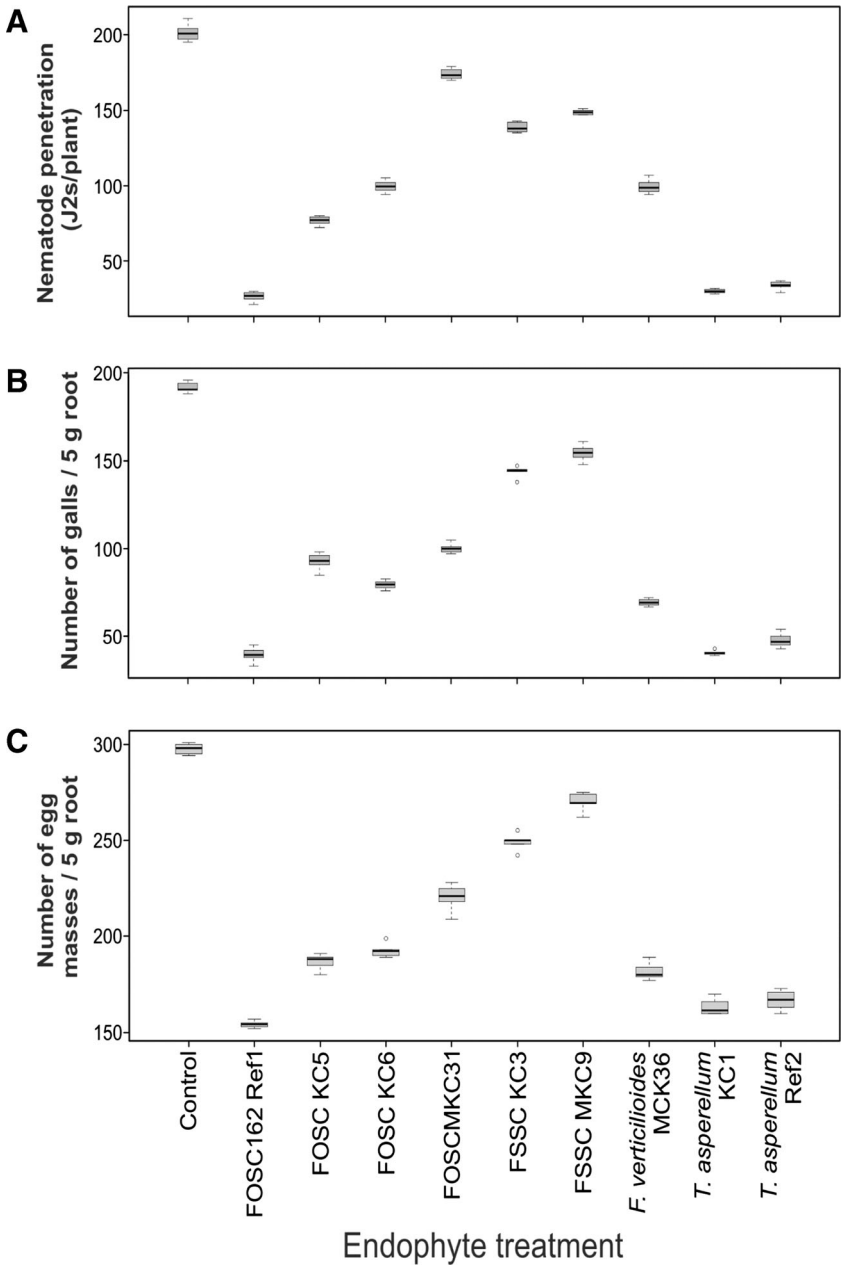

Fig. 5 Effect of various fungal endophytes on nematode penetration (**a**), number of galls (**b**) and number of egg masses (**c**) in tomato cv. Moneymaker. The number of juveniles per root system was determined 12 days after nematode infection while the number of galls and egg masses was evaluated after 8 weeks. *Box plots* were drawn using R program v.3.2.3 (The R Foundation for Statistical Computing) with sample size $n = 6$

endophytic diversity of annual plants of the Solanaceae (Kim et al. 2007; Demers et al. 2014). All isolates belonged to the phylum Ascomycota. Within this phylum, the majority of OTUs belonged to the Hypocreales (Sordariomycetes) followed by Pleosporales (Dothideomycetes) and finally Eurotiales (Eurotiomycetes). Other fungi (Basiodiomycota, 'zygomycetes', Glomeromycota) were not isolated in this study. The predominance of ascomycetes appears characteristic of endophytic mycobiota (Keim et al. 2014; Sánchez Márquez et al. 2012). In our results, members of FSSC and FOSC dominated, which were both represented by 28 isolates out of the total of 76. These results are consistent with other reports in which several endophytic *Fusarium* species were isolated from roots in which tomato was shown to be a potential host. Demers et al. (2014) assessed the diversity of tomato associated with FOSC populations within tomato fields and, similar to the results in this study, found that members of the *F. oxysporum* species complexes (FOSC) were the most common *Fusarium* species detected.

Isolates from the two most common *Fusarium* clades, FOSC and FSSC, are generally considered as being plant pathogens. However, they were isolated from healthy plant roots and no disease symptoms, like leaf wilting or yellowing or necrotic root sections, were observed after

subsequent inoculation of tomato plants in our research. Nonpathogenic, endophytic *F. oxysporum* strains are commonly isolated from plants infected with pathogenic *F. oxysporum*, suggesting that colonization of a single plant by multiple genotypes of different virulence is not unusual (Olivain et al. 2006). It can therefore be assumed that the isolates collected in this study either represent generally nonpathogenic strains or that they represent strains pathogenic to plants other than tomato. Nevertheless, inoculation of the FOSC reference strain, Fo162, on various tomato cultivars and other plants, like melon, pepper, *Arabidopsis* and banana has never resulted in disease development (Dababat et al. 2008; Vu et al. 2006; Martinuz et al. 2015).

The absence of other fungi, e.g., Basidiomycota or zygomycetes, in the current study could be because of the cultivation approach taken, which generally excludes the detection of non-culturable species. Additionally, slower-growing Basidiomycota are usually outcompeted on standard isolation media by Ascomycota and zygomycetes (Lindahl and Boberg 2008). This bias can be corrected by assessing the entire fungal community, for example through environmental metagenomic analysis approaches, which generally yields a significant number of sequence data, thereby enabling the detection of rare and non-culturable organisms (Peršoh 2015). In conclusion, the number of OTUs observed in tomato roots in this study may be biased and most likely represents only a fraction of the total fungal diversity present.

When comparing fungal isolates obtained with regard to the two geographic locations, it was observed that all rare isolates were mostly represented by single isolates, e.g., *Alternaria solani*, *Cochliobolus* sp., *Aspergillus sclerotiorum*, *Stemphylium solani*, and *Curvularia aeria*. Furthermore, these species were only found in the coastal region. The community composition of endophytic fungi can vary greatly and depends on multiple factors such as the host plant (genotype), plant density, nutrient availability, environmental conditions and seasonal moisture regimes can further shape and modify the endophyte community (Gonthier et al. 2006).

The sampling sites at the coastal region are characterized by an extremely warm climate with an average temperature of 26.3 °C and firm clay loam soils. Soil parameters, e.g., soil texture, soil type, soil organic content and climatic conditions such as temperature have been reported to have an impact on the root-associated fungal communities (Peršoh 2015). Based on these parameters, the plant may select which endophytes can colonize the interior part of the roots thereby resulting in some species dominating over others. However, we acknowledge that more areas were sampled in the coastal region than in the central region, which could be a possible reason why more rare species were collected from this region.

Multi-gene phylogeny

The primary focus was to study the phylogenetic utility of ITS and protein coding sequences (*ß-tubulin*, *tef1α*) individually and in combination. Phylogenetic analysis of the ITS gene yielded a slightly lower resolution for some species as compared to the protein coding regions. Similar results were reported by O'Donnell et al. (2000) who stressed that the ITS region was not sufficient enough to resolve phylogenetic relationships among *Fusarium* species due the fact that it has few sites that are variable enough for resolving species boundaries within this genus. Other studies performed by O'Donnell and Cigelnik (1997) and O'Donnell et al. (1998) highlighted that every species within the *Fusarium Fujikuroi* complex possesses two divergent xenologous (homologs evolved by lateral gene transfer among species) or paralogous (homologs evolved by gene duplication) nuclear rDNA ITS2 types rendering phylogenetic analysis using the ITS gene region less informative. To amplify a fungus at the species or even isolate level, other genome sequence regions are necessary that show more variability, e.g., protein coding regions *ß-tubulin*, *tef1α*, RPB1 or RPB2.

The topologies of the trees generated using *ß-tubulin* and *tef1α* were similar but not completely identical. The *tef1α* gene proved to be superior to *ß-tubulin* with respect to its discriminatory power among the *Fusarium* species. Analysis of *ß-tubulin* sequencing trace files revealed double peaks at numerous nucleotide positions consistent with divergent paralogs of this gene. O'Donnell et al. (2000) noted that divergent *ß-tubulin* paralogs in the *F. solani* species complex may be pseudogenes or evidence of interspecific hybridization. *Tef1α* sequences provided better resolution mainly because of the larger size and number of introns.

To sum up, *tef1α* gene appears to be a good molecular marker to discriminate at the species level frequently but not always. These results reinforce those obtained by Maharachchikumbura et al. (2012) who also found that *tef1α* had a higher discriminatory power in comparison to ITS and *ß-tubulin*. Combined sequence analysis of ITS, *β-tubulin* and *tef1α* genes successfully resolved all of the species used in this study with high bootstrap support. Phylogenetic analysis using multiple loci can thus be considered the most straightforward way for sequence-based identification especially of fungi.

Fungal endophytes with biocontrol potential

Based on the phylogenic analysis of the isolates, it is still not possible to do a more targeted search for candidate biocontrol fungi because biocontrol candidates do not form a monophyletic group. However, isolates within one clade showed different levels of biocontrol. This may have been expected, since the mode-of-action by which nematodes are controlled has not

yet been resolved and may be multifactorial and even differ for the various endophytes. Consequently, the currently analyzed set of marker genes is therefore not suitable for pinpointing potential biocontrol candidates. This may seem obvious for the reason that these marker genes are most likely not involved in directly controlling the nematode infections. Nevertheless, since they are important for regulating rRNA metabolism, which is one of the most key processes in cellular biology, the IGS regions are likely to affect not only the direct growth and development but also the ecological competence of the organism (Weider et al. 2005). Variations in these IGS regions may thus reflect adaptation to particular environmental conditions, like competing with other organisms. Until better marker genes have been identified, biological assays will thus be necessary to determine the antagonistic competence of an endophyte toward nematodes.

Numerous publications have highlighted the drawbacks of the pesticide application regimes in intensive urban and peri-urban vegetable production in sub-Saharan Africa (Atcha-Ahowé et al. 2009). Consequently, biological control is gaining interest especially in tropical countries. Results of the current study demonstrated the ability of nonpathogenic fungal isolates to successfully reduce nematode penetration and subsequent galling as well as reproduction. Penetration of tomato cv. Moneymaker roots by *M. incognita* second stage juveniles was significantly lower in endophyte inoculated roots as compared to the control. Our study is in accordance with previous experiments by others (Dababat and Sikora 2007a, b; Martinuz et al. 2012b; Sikora et al. 2008). With respect to reduced female fecundity, Martinuz et al. (2012a) and Sharon et al. (2001) also reported that fewer adult root-knot nematodes had infected tomato roots and also fewer nematodes had reached the female stage 28 days after *M. incognita* and *M. javanica* inoculation in endophyte-treated plants.

There are several modes of action that have been proposed by which endophytes can protect their host, some of which include preemptive colonization, direct antagonism through toxic metabolites, induced systemic resistance and competition. Intensive studies performed by Sikora et al. (2008) emphasized that the establishment of endophytic symbiosis with plants seems to be a general prerequisite for an effective reduction of nematode pathogens. Other modes of action that lead to the overall reduction in *M. incognita* infestation may include the accumulation of certain fungal metabolites or the triggered plant defense responses.

Not all the fungal isolates analyzed were competent with respect to suppressing nematode colonization. Indeed, in comparison with the two reference isolates, FOSC 162 Ref1 and *T. asperellum* Ref2, the newly found isolates do not perform better when nematode control is concerned. Due to this, we would argue that there is no immediate benefit gained over our isolates in comparison to our reference isolates. However, one does not know that beforehand and in fact better performing endophytes may still be around; we just have not found them (yet). On the other hand, the level of control provided by the reference strains FOSC 162 Ref 1 and *T. asperellum* Ref2 maybe the maximum that can be reached, which would also be in itself an interesting finding.

We can conclude that endophytes can play an important role in plant protection against nematode infection. The isolates that showed a promising effect in the suppression can be used for testing local Kenyan tomato varieties in the future. Interestingly, we also confirm that some isolates of *F oxysporum* species complex and *T asperellum* continue to provide a promising biocontrol potential. Additionally, the sequence analysis of relevant genomic regions can distinguish endophytes that can be useful in biocontrol strategies. This is, to the best of our knowledge, the first report concerning a survey of root endophytic mycobiota of tomato in Kenya, multi-gene phylogeny and assessment of biocontrol potential of some selected fungal isolates against the root-knot nematode *M. incognita*.

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Author contributions CB and AS designed the research, conducted the experiments, analyzed the data and wrote the manuscript. GK performed the survey work in Kenya and conducted experiments. AE analyzed the data. GS conducted experiments. AKB, BM and MT contributed with new methods of phylogenetic analyses. MT and FG carefully reviewed the manuscript. All authors read and approved the manuscript.

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