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REGULAR ARTICLE

Analysis of fungal endophytes associated with rice roots from irrigated and upland ecosystems in Kenya

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Abstract
Background and aim Fungal endophytes are commonly associated with plants, and are considered an important component of crop production. They can influence plant growth and tolerance to biotic and abiotic stresses. The aim of this study was to analyse and identify endophytic fungi associated with rice roots in irrigated and upland ecosystems in Kenya, as an inventory for a future search for biological control and growth promoting agents.
Methods Fungi were isolated from the roots and selected based on culture characteristics. All selected isolates were sequenced using primers targeting the internal transcribed spacer (ITS) region, intergenic spacer (IGS) region and the gene encoding the translation elongation factor (TEF-1α). The species were determined by comparing their sequences with those of well characterised or type strains. Phylogenetic relationships among the species were used to identify their taxonomic groups, and distribution in the agroecosystems, especially for the *Fusarium* spp.
Results Based on sequencing of the ITS region, 75 fungal isolates were identified as *Fusarium*-like, while the remaining 98 isolates were found to belong to different species representing other genera than *Fusarium*. A further analysis of the *Fusarium* spp., using concatenated IGS and TEF-1α sequences showed that these isolates belong to the *Fusarium oxysporum* (FOSC) and *Gibberella fujikuro*i (GFSC) species complexes. Within the FOSC isolates, a clear divergence was observed between isolates from irrigated and upland ecosystems, while in the GFSC this phenomenon was not observed. When the total number of species was considered, 27 species were identified in the irrigated ecosystems, while only 18 species were found in the upland ecosystems.
Conclusions More fungal species were found in the irrigated ecosystems than in the upland ecosystems. We propose that flooding may affect the assembly of endophytic fungi in rice roots, however, other factors such as rice cultivars, geographical locations and soil types could also be important.

Keywords Endophytic fungi · Paddy fields · *Phoma epicoccina* · *Fusarium* spp · *Talaromyces* spp · *Curvularia* spp

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Introduction

Fungal endophytes are microorganisms that internally colonise plant organs without causing symptoms, and they are broadly classified into clavicipitaceous and non-clavicipitaceous groups according to phylogeny and life history traits (Rodriguez et al. 2009). The clavicipitaceous endophytes infect economically important grass species in the genera *Festuca* and *Lolium* and have been studied for more than 100 years (Clay 1990; di Menna et al. 2012). More recently, many other fungi, taxonomically distinct (non-clavicipitaceous) and diverse, have been found to associate endophytically not only with grasses, but also with several other monocot and dicot plants (Rodriguez et al. 2009; Higgins et al. 2011; Sánchez-Márquez et al. 2012).

The functional roles of endophytic fungi on plant communities are diverse and can vary depending on many factors, such as host, interaction with other microorganisms and environmental conditions. Several studies have shown the potential benefits of endophytic fungi for crops, especially concerning the stimulation of biomass production and the enhancement of resistance to herbivores, pathogens and abiotic stresses (Hamilton and Bauerle 2012; Kleczewski et al. 2012; Aimé et al. 2013; Martinuz et al. 2013). However, fungal endophytes can be deleterious in some cases. Decrease of plant growth and facilitation of pathogen infection have been reported. In addition, endophytes can be latent pathogens (Sánchez-Márquez et al. 2012).

In spite of the potential impact of endophytes on crop performance, and considering the extent of studies on endophytic fungi on other grasses (e.g., Sánchez-Márquez et al. 2008; Ghimire et al. 2010; Higgins et al. 2011, 2014), it is remarkable that this group of fungi has received little attention in rice. Over the past two decades, only a few publications are available about their communities in rice. These fungi have been studied in few countries, more specifically in Italy, China, Malaysia and India (Fisher and Petrini 1992; Tian et al. 2004; Naik et al. 2009; Vallino et al. 2009; Zakaria et al. 2010). Most of these studies have used morphology to characterise the fungal endophytes, but literature has shown that few taxonomic features can distinguish species especially those occurring in complexes (Jiménez-Fernández et al. 2010). Moreover, no studies have investigated root microbiota of rice in different ecosystems. Therefore, a more comprehensive study, which takes advantage of molecular tools, is required not only to describe the endophytic fungal communities in different rice ecosystems, but also to investigate their phylogenetic relationships.

In Kenya, as in other countries, rice faces many biotic and abiotic stresses that cause serious yield reductions. Among the biotic factors, diseases caused by *Magnaporthe oryzae*, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and nematodes are serious, while salinity, drought, radiation and nutrition are primary abiotic stress factors (e.g., Dean et al. 2012; Mansfield et al. 2012; Kyndt et al. 2014; Nhamo et al. 2014). As the demand for rice is expected to increase by 50 million tonnes in the future (Mohanty 2009), new technologies will be required to improve and sustain its production. The knowledge on rice ecosystems, including the characterization and management of above and belowground microbial communities is of great relevance towards achieving this goal (Lenné and Wood 2011). Rice endophytic fungi may benefit their host by protecting them from biotic and abiotic stresses as well as promoting their growth (Le et al. 2009; Redman et al. 2011; Su et al. 2013).

The two rice ecosystems found in Kenya are irrigated and upland. However, 95 % of rice is grown under irrigation and only 5 % is rain-fed (upland), grown mainly by smallholder farmers across the country (Muhunyu 2012). Continuous monocropping of one or more rice cycles per year is a general practise in the irrigated ecosystems, while the upland ecosystems are typified by short-rotations of rice with other crops. The irrigated ecosystems are continuously wet (completely submerged) from transplanting until harvesting, whereas the upland ecosystems (dryland) are characterised by fluctuating dry and wet conditions throughout the growing seasons (Grist 1986). Only few plant species such as rice are adapted to grow in both ecosystems, and for that reason, this crop is an excellent model to study the dynamics of plant-endophyte interactions in different ecosystems.

The objective of the present study was to analyse endophytic fungi of rice roots in irrigated and upland ecosystems in Kenya. The long-term goal is to identify endophytic strains that can promote rice growth or induce its resistance to plant-parasitic nematodes.

Materials and methods

Sampling: ecosystems, rice cultivars and soil characteristics

Samples were collected from irrigated and upland ecosystems (Table 1). Twenty-nine and seventeen fields were sampled in the irrigated and upland ecosystems, respectively. The four rice cultivars commonly grown in these ecosystems include Basmati 370 (Kenya Pishori), IR279380-1, Supa and Nerica 4 (New Rice for Africa). Basmati 370 and IR279380-1 grow best under irrigation and are high yielding lowland cultivars within the *Oryza sativa* species (Asian rice) (Ndiiri et al. 2012). Supa, also within the *O. sativa* species, is a local cultivar grown mainly at the coastal region (Kwale); it is a moderately yielding cultivar. Nerica 4, a crossbreed between *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice), is an upland cultivar, which is drought-resistant and produces high yields (Atera et al. 2011).

Isolation of root endophytic fungi

Roots from three healthy rice plants at the vegetative stage were sampled from each field in both irrigated and upland ecosystems. The samples were transported to the laboratory in coolboxes and processed within 24 h of sampling (Rosa et al. 2010). The roots were gently washed under running tap water and then surface-sterilised by immersion in 75 % ethanol (*v*/*v*) for 30 s and 1 % sodium hypochlorite (*w*/*v*) for 10 min (Yuan et al. 2010). They were rinsed with sterile distilled water and dried in between sterile paper towels before being cut into short (~6 mm) segments (Yuan et al. 2010). Thirty segments per sample were plated on potato dextrose agar (PDA) medium containing 150 mg L^{-1} tetracycline. The plates were incubated at 25 °C in darkness and observed periodically for developing hyphae, which were sub-cultured onto fresh PDA media until pure cultures were obtained.

Identification of endophytic fungi

The isolates were first selected according to phenotypic characteristics such as colony appearance, mycelium colour and growth rate on PDA medium (Gazis and Chaverri 2010). All selected isolates were sequenced. Total genomic DNA was extracted from two-week-old cultures, using the ZR Fungal/Bacteria DNA MiniPrep™ kit (Zymo Research, USA), following the manufacturer's instructions. The internal transcribed spacer (ITS) region, which is a general barcode for fungi, was primarily used to identify all the isolates (Schoch et al. 2012). Isolates identified as *Fusarium* spp. using the ITS-sequences, were further characterised by amplifying their intergenic spacer (IGS) region and the gene encoding the translation elongation factor (TEF-1α). The IGS region has been recommended for the identification of *Fusarium oxysporum* (Edel et al. 1995), while TEF-1α gives high resolution at species level for most *Fusarium* spp. (Geiser et al. 2004).

The ITS region was amplified using ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3) and ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′) primers (White et al. 1990). The 20 μl PCR mixture contained Bioneer pre-mix (Bioneer Corporation, South Korea), sterile-distilled water, 0.125 μM of each primer and 4.5 ng/μl DNA template. PCR reactions were performed as follows: 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 53 °C for 45 s, 72 °C for 30 s, and finally 72 °C for 10 min. The IGS region was amplified using PNFo-F (5′-CCCGCCTGGCGCGTCCGACTC-3′) and PN22-R (5′-CAAGCATATGACTACTGGC-3′) primers (Edel et al. 1995). The 30 μl PCR mixture contained distilled water, 1xPCR buffer, 0.25 mM $MgCl_2$, 0.5 mM dNTPs, 0.75 μM of each primer, 0.125 U/μl Taq DNA polymerase (Fermentas, USA) and 3 ng/μl DNA template. PCR reactions were performed as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 35 s, 52 °C for 35 s, 72 °C for 45 s, and finally 72 °C for 5 min. The primers EF1 (5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′-GGA(G/A)GTACCAGT(G/C)ATCA TGTT-3′) were used to amplify the TEF-1α region (Geiser et al. 2004). The 25-μl PCR mixture contained distilled water, 5xPCR buffer (Bioline, cat BIO-21106), 0.4 μM of each primer, 0.15 U/μl MyTaq DNA polymerase (Bioline, cat BIO-21106) and 5 ng/μl DNA template. The PCR products were purified using the GeneJET™ PCR purification kit (Fermentas, USA) and directly sequenced by the Sanger method at ILRI-BeCA Hub (Nairobi, Kenya) or at LGC genomics (Berlin, Germany) using the ITS4-R, PN22-R and EF1 primers for the ITS, IGS and TEF-1α sequences, respectively.

Sequences were edited and assembled using the CLC Main Workbench (version 6.9.1) software (CLC bio-Qiagen, Denmark). Blast searches were performed

Table 1 Description of rice ecosystems sampled in Kenya: locations, geographical coordinates, climatic conditions, soil characteristics and rice cultivars

Ecosystem	Sites	Geographical Co-ordinates	Annual Rainfall (mm)	Average Temperature (°C)	pH	% Carbon	% Nitrogen	Phosphorus ($mgPkg^{-1}$)	Rice cultivars	Sampling date
Irrigated	Mwea	00′39.357S, 037′17.477E	950[a]	24[a]	5.88	2.15	0.12	6.36	Basmati	09.05.2012
	Ahero	00′09.721S, 034′55.124E	1400[b]	25[b]	5.83	3.72	0.21	29.97	IR279380-1	13.03.2012
Upland	Siaya	00′03.226N, 034′17.136E	1200[c]	21[c]	5.13	2.08	0.14	4.90	Nerica	04.09.2012
	Busia	00′27.523N, 034′06.888E	1500[c]	28[c]	5.12	2.52	0.12	6.38	Nerica	05.09.2012
	Kwale	04′11.195S,039′22.801E	700[d]	30[d]	5.10	0.82	0.03	6.23	Supa	08.09.2012

Analysis of soil pH was conducted according to Otinga et al. (2013). Organic carbon and nitrogen contents were determined following the protocol of Okalebo et al. (2002), while extractable soil phosphorus was determined according to Olsen et al. (1954)

[a] Kihoro et al. 2013

[b] Atieli et al. 2009

[c] Wambugu et al. 2012

[d] Ohaga et al. 2007

against the NCBI nucleotide databases. Isolates identified as *Fusarium* spp. were further analysed in the *Fusarium*-ID and *Fusarium* MLST *Fusarium*-specific databases (Aoki et al. 2014). Because there are many ITS reference sequences in public databases that are wrongly characterised, identification of isolates with this marker relied on sequences of type (well-characterised sequences from peer-reviewed publications) strains and phylogeny (Ko Ko et al. 2011). The nucleotide sequences obtained in this study with <99 % identity to type (well-characterised) strain-sequences were deposited at the NCBI database (Supplementary Table 1 and 2). Accession numbers of sequences with 100 % identity to our sequences were adopted. *Fusarium* spp. only identified using the ITS-region were classified as *Fusarium*-like.

Phylogenetic analysis

Phylogenetic relationships among the species were analysed using MEGA 5.05 (Tamura et al. 2011), where the neighbour-joining method was used to draw the phylogenetic trees and to estimate the evolutionary distances. Bootstrap analysis was run using 1000 replicates to estimate the robustness of the phylogenetic clusters. Cantharellales and Mucorales, also collected in this survey, were used as out-group for non-*Fusarium* species. ITS sequences of the most homologous type or well-characterised strains were included during these analyses. For isolates identified as *Fusarium* spp. an unrooted phylogenetic tree was constructed using concatenated IGS and TEF-1α sequences.

Results

Rice endophytic fungi from irrigated and upland ecosystems

A total of 173 fungal isolates were obtained: 88 from irrigated and 85 from upland ecosystems. Based on sequencing of the internal transcribed spacer of the rDNA, 75 fungal isolates were identified as *Fusarium*-like, while the remaining 98 isolates were found to belong to different species representing other genera than *Fusarium*. When the total number of species was considered, 27 species were identified in the irrigated ecosystems, while only 18 species were found in the upland ecosystems. *Fusarium* spp. dominated the upland ecosystems, while an assortment of species including *Fusarium* spp., *Phoma epicoccina*, *Curvularia* spp. and *Talaromyces* spp. were found in the irrigated ecosystems. Six species i.e., *Phoma epicoccina* (Syn. *Epicoccum nigrum*, *Epicoccum purpurescens*), *Fusarium sp*., *Fusarium nygamai*, *Fusarium oxysporum*, *Penicillium thomii* and *Trichoderma harzianum* were present in both ecosystems. Next to *Fusarium* spp., the other

common taxa in the irrigated ecosystems were *Curvularia* and *Talaromyces* (Supplementary Table 1).

Phylogenetic relationships among fungal endophytes

A phylogenetic relationship of species representing other genera than *Fusarium* is shown in Fig. 1. The most frequently isolated species were ascomycetes, accounting for 25 species from the irrigated ecosystem and 16 species from the upland ecosystem. Two basidiomycetes were isolated only from the irrigated ecosystem whereas two zygomycetes were isolated from the upland ecosystem. Species from the irrigated ecosystem were found to belong to seven orders, whereas those from the upland ecosystem occurred in six orders. Eurotiales, Hypocreales and Pleosporales occurred in both ecosystems. Cantharellales, Capnodiales, Magnaporthales and Sordariales were present only in the irrigated ecosystem. The orders Botryosphaeriales, Mucorales and Trichosphaeriales were only represented in the upland ecosystem. Members of Pleosporales [eight species (29.6 %)] and Eurotiales [seven species (25.9 %)] were dominant in the irrigated ecosystem, while Hypocreales [seven species (38.9 %)] dominated the upland ecosystems (Fig. 2). A phylogenetic analysis of the *Fusarium* spp. using concatenated IGS and TEF-1α sequences showed that the isolated *Fusarium* specimens belonged to the *Fusarium oxysporum* (FOSC) and *Gibberella fujikuroi* (GFSC) species complexes. Within the FOSC isolates, a clear divergence was observed between isolates from irrigated and upland ecosystems, while in GFSC, this phenomenon was not observed. Five clades were identified; two of them were formed within the FOSC representing isolates from either upland or irrigated ecosystems, while the remaining three clades (*F. nygamai*, *Fusarium* spp. 1 and *Fusarium* spp. 2) occurred within the GFSC (Fig. 3).

Discussion

This study has isolated and identified the endophytic fungal community of rice roots from irrigated and upland ecosystems in Kenya, where a total number of 39 different fungal species were identified. When compared with previous studies in other countries, this amount of species indicates the existence of a highly diverse community of fungal endophytes associated with rice in Kenya. In Italy, 31 fungal endophytes were isolated from leaves, sheaths and roots of rice cultivars grown in wet and dry conditions (Fisher and Petrini 1992). A survey performed in Bhadra River Project Area, India, has detected 19 species of endophytes in leaves and roots of rice (Naik et al. 2009). The differences in communities among studies may not only reflect the influence of rice varieties and environmental conditions (Fisher and Petrini 1992; Tian et al. 2004), but also be the result of heterogeneous methodological approaches used in plant sampling, surface sterilization, fungal isolation and identification (Porras-Alfaro and Bayman 2011).

Remarkably, this study found more different fungal taxa in the irrigated than in the upland ecosystems. Continuous monocropping in the irrigated fields for instance may increase propagules of soilborne fungi. In addition, flooding may increase the frequency of infection of endophytic fungi (De Battista 2005; Manici and Caputo 2009). Nevertheless, this difference may also be caused by site-specific factors in each agroecosystem; particularly the cropping systems, water regimes, rice cultivars, geographical locations and soil types (Fisher and Petrini 1992; Carroll 1995; Tian et al. 2004; Naik et al. 2009).

In agreement with previous studies on grass endophytes (Sánchez-Márquez et al. 2012 and references therein), ascomycetes were highly represented in our rice endophyte collection, followed by basidiomycetes and zygomycetes. Within the ascomycetes, most species belonged to Pleosporales, Eurotiales and Hypocreales. A clear difference in species between ecosystems has been detected in this study: in the irrigated rice, most species belonged to the Pleosporales and Eurotiales, while in the upland rice; members of the order Hypocreales were highly represented. The taxa *Epicoccum* and *Talaromyces* (formerly *Biverticillium*, a subgenus of *Penicillium*; Samson et al. 2011) were dominant within the Pleosporales and Eurotiales, respectively. Among the Hypocreales, *Fusarium* species were the most common root endophytes of upland rice. Previously, these species were described in dryland rice and other plant species (Fisher and Petrini 1992; Maciá-Vicente et al. 2008). However, we also found these species in rice roots from irrigated fields, which indicates that they are also present in wet conditions, although at a

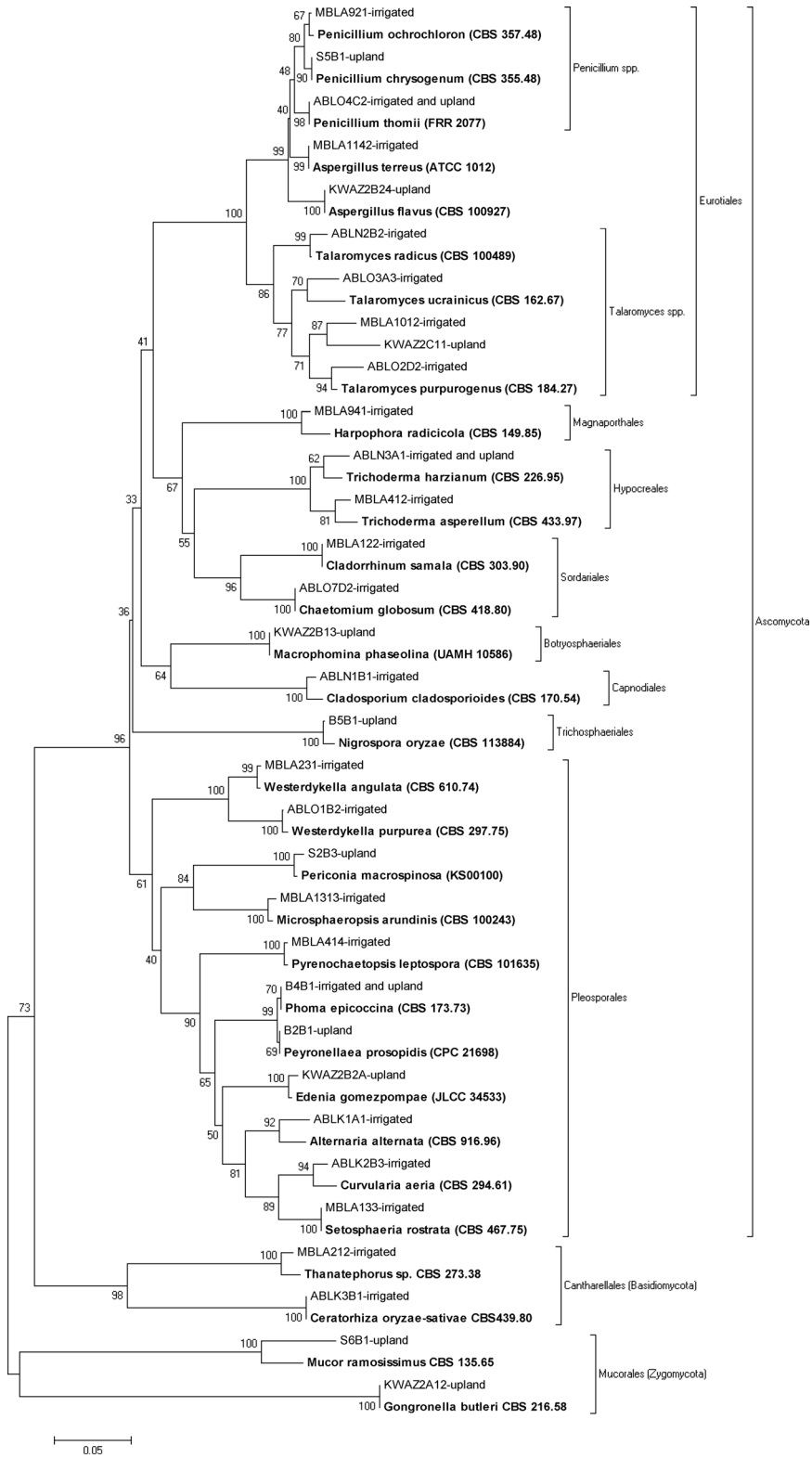

Fig. 1 Phylogenetic relationships of the fungal endophytes and their reference sequences (*in bold*). The tree was constructed using the neighbour-joining method based on ITS-sequences and rooted with Cantharellales (basidiomycetes) and Mucorales (zygomycetes) species. Bootstrap values in percentage (based on 1000 replicates) are shown on the nodes. Species were classified into orders according to Thongkantha et al. (2009); Chomnunti et al. (2011); Zhang et al. (2012); Hyde et al. (2013) and MB: MycoBank, (http://www.mycobank.org/)

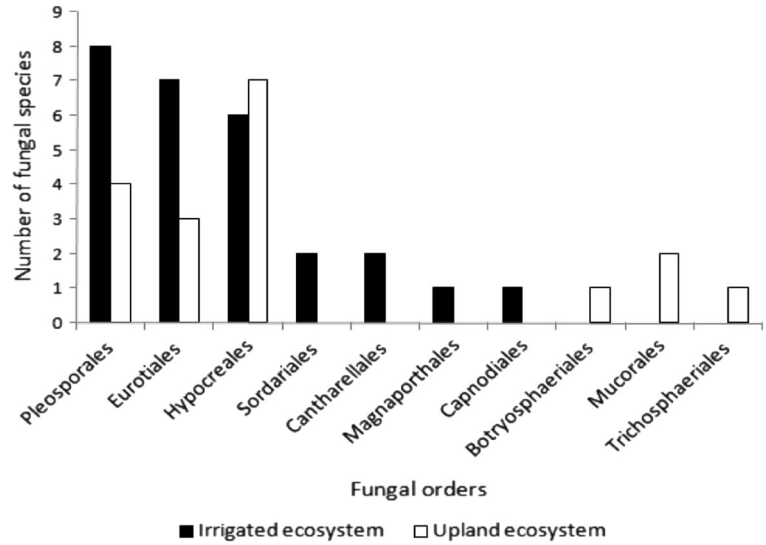

Fig. 2 Distribution of fungal species within individual orders

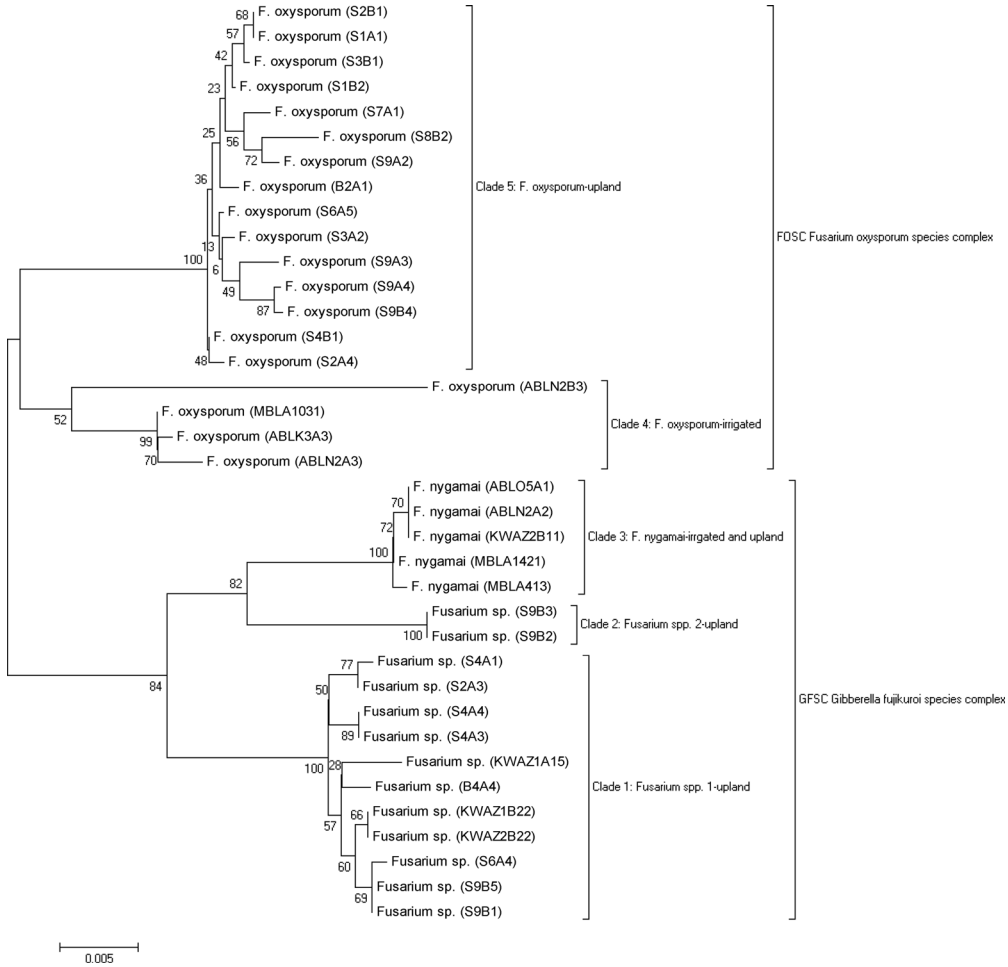

Fig. 3 Tree of endophytic *Fusarium* isolates based on IGS and TEF-1α sequences. Bootstrap values in percentage (based on 1000 replicates) are shown on the nodes

lower frequency. A phylogenetic analysis of the *Fusarium* spp. has shown that isolates from irrigated and upland ecosystems are different. The differences were detected even in isolates of the same species, supporting further the idea that ecosystems may play fundamental roles in species assemblages.

In the past, *F. oxysporum*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *Chaetomium globosum*, *Penicillium chrysogenum* and *Phoma epicoccina* (syn. *Epicoccum nigrum*; *Epicoccum purpurescens*) were isolated, not only from rice roots but also from other tissues and organs (Fisher and Petrini 1992; Naik et al. 2009). Some species such as *Alternaria alternata*, *E. purpurescens*, *F. equiseti* and *Nigrospora oryzae* are seed-borne endophytes of rice (Fisher and Petrini 1992). Our report of *Talaromyces purpurogenus* and *Westerdykella angulata* is the first one from rice roots. Many species in this study have also been described in other plant species as endophytes, saprobes or pathogens (Sánchez-Márquez et al. 2012). *Fusarium oxysporum* for instance is a serious pathogen of many crops (Fisher and Petrini 1992; Dean et al. 2012 and references therein), but evidence also shows that some strains are non-pathogenic with endophytic life styles and may be beneficial to plants (Sikora et al. 2008 and references therein). An isolate of *E. nigrum* (strain P16) has been found to promote growth of sugarcane (Fávaro et al. 2012). ATCC 96794, another strain of *E. nigrum* was found to control brown rot disease of stone fruits (De Cal et al. 2009), yet emerging information has shown that *E. nigrum* can cause leaf spot disease in *Lablab purpureus* (Mahadevakumar et al. 2014). *Alternaria alternata* is an opportunistic pathogen, but can occur as endophyte or saprobe on several crops (Fisher and Petrini 1992; Guo et al. 2004 and references therein).

We have isolated and identified endophytic fungi from rice roots collected from agroecosystems in Kenya. Most of these species were previously identified in rice and other plant species as endophytes, saprobes or pathogens. Our study has provided evidence that fungal communities colonising rice roots may be ecosystem-driven. Flooding may mediate the assembly of endophytic communities in rice, however, other factors such as rice cultivars, geographical locations and soil types should be investigated further. *Fusarium* spp. preferentially colonised roots of upland rice, while irrigated rice supported different fungal communities including *Fusarium* spp., *Phoma epicoccina*, *Curvularia* spp. and *Talaromyces* spp.

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Conflict of interest The authors declare that they have no conflict of interest.

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