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Morphological and molecular identification of *Pythium* spp. isolated from common beans (*Phaseolus vulgaris*) infected with root rot disease

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Common beans (*Phaseolus vulgaris* L.) is the main leguminous crop grown primarily by small-holder farmers in the East and South African countries. *Pythium* root rot disease is the major production constraints which results in yield losses of 70% to most commercial bean cultivars in eastern Africa. Study focused on ascertaining preliminary information on bean cultivation practices in Tanzania, morphological and molecular characterization and identification of *Pythium* species from infected beans plants and determining the relationship between soil pH and the occurrence and distribution of the *Pythium* spp. Soil samples and infected bean plants were collected by aseptic pathogenic isolation and DNA extraction. Universal primers (ITS1 and ITS4) were used for amplification and followed by sequencing. About 63.0% of farmers practiced sole beans cropping, 31.0% mixed cropping and 6.0% intercropping. Corn, banana, cassava, Irish potatoes and coffee were either mixed or intercropped with beans. Also, 52.4% of farmers use farm saved seeds and 92.9% do not use fertilizer in their bean fields. Eleven species of the *Pythium* spp. were identified: *Pythium aphanidermatum*, *Pythium splendens, Pythium ultimum*, *Pythium attrantheridium*, *Pythium graminicola*, *Pythium oligandrum*, *Pythium dissotocum, Pythium irregurale*, *Pythium camurandrum, Pythium paroecandrum* and *Pythium acanthophoron*. Phylogenetic analysis showed diversity and homogenity among the *Pythium* spp. across the collection area. A high incidence and wide distribution of *Pythium* species were recorded in soils in the 5.03 to 5.95 pH range.

Key words: Incidence, internal transcribed spacer (ITS), leguminous, molecular characterization of pathogen.

INTRODUCTION

Common beans (*Phaseolus vulgaris* L.) is one of the most significant food leguminous crops in the world (CIAT, 2001). It is grown by most small-holder farmers in the eastern African countries for home consumption as

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well as for cash earnings (Hillocks et al., 2006). This region is the most important common bean production area in sub-Saharan Africa and has a high varietal diversity of the crop (Fivawo and Msolla, 2011). Production of common beans in different production areas is hampered by various biotic and abiotic factors which lead to continuous decline of the crop production per unit area (Hillocks et al., 2006). Soil borne diseases caused by either *Fusarium* spp., *Pythium* spp. and or *Rhizoctonia* spp. are biotic constraints to the production of common beans in East African regions; these pathogens act either individually or in a complex manner (Rusuku et al., 1997). Root rot diseases have received little attention until recent years when they became major concerns in East Africa (CIAT, 2003). In Tanzania, limited studies have been conducted regarding this disease and its causative microorganisms. Cultivation of most of the popular commercial common bean genotypes in parts of East Africa is constrained by *Pythium* root rot which results in yield losses of up to 70%. Predictive models have been used to identify new areas where root rots are expected to become a serious problem in Tanzania (Morogoro, Usambara Mountains, parts of Kilimanjaro, Arusha, Mbeya and Kagera), Kenya (Kisii and Nyahururu) and Uganda (Nebi, Apac and parts of Ntungamo) where farmers have already started to encounter root rots, Malawi (the Chitipa Highlands and Shire Highlands), Mozambique (Manica and Lichinga) and Ethiopia (Hararghe) (CIAT, 2003; Wortmann et al., 1998). Therefore, this study aimed at i) ascertaining preliminary information on bean cultivation practices in bean growing parts in Tanzania, ii) in-depth scientific investigation on the incidence and occurrence of *Pythium* root rot disease in relationship with soil pH within some parts of Tanzania.

MATERIALS AND METHODS

Collection of diseased plants and soil samples

From surveyed farmers' fields, six infected bean plants showing symptoms of root rot disease were characterized by poor seedling establishment, damping-off, stunting and premature defoliation, deterioration of leaves, plant wilt and death. Symptomatic plants were uprooted using a shovel (Mwang'ombe et al., 2007), put in paper bags and transported to Sokoine University of Agriculture (SUA) Laboratories, Tanzania. Soil samples were also collected at random in the farm at a depth of 0-10 cm with soil auger. Soil pH was determined in a ratio of 1:2.5 soil : water suspension by the potentiometric method (McLean, 1982).

Isolation of *Pythium* spp. from infected bean plants

Corn meal agar (CMA) growth media (17 g in 1000 ml of distilled water) was autoclaved at 121°C for 15 minu; when media was cooled at 40°C, 3 and 0.15 mL of the antibiotics Rifampicin and Pimaricin were added, respectively. Approximately 0.5 to 2 cm of infected root tissue was cut and rinsed first in 70% ethanol for 30 s then in 2% solution of sodium hypochlorite (NaClO) for 1-2 min and finally rinsed twice in sterilized distilled water. Cut tissues were blotted dry on sterile filter paper, plated on CMA growth media and incubated for 5-7 days at 24°C to allow growth of the pathogen. Based on morphological characteristics of sporangia, oogonia wall, antheridia and oospores are distinctive features of *Pythium* from other root rot pathogens. Purification of *Pythium* culture was carried out by cutting a small piece of the media with mycelia from the edge of a colony and then subcultured onto new growth media. Pure isolates were transferred to potato dextrose agar (PDA) slants and after 14 days were stored at -20°C.

DNA extraction

Prior to extraction, pure isolates of *Pythium* were reactivated by sub-culturing on PDA growth media and incubated at 24°C for 14 days to allow massive production of mycelia. DNA was extracted from mycelia of *Pythium* using the protocol developed by Mahuku (2004).

Polymerase chain reaction (PCR)

The internal transcribed sequence (ITS) region was amplified using universal primers ITS1 and ITS4. A reaction volume of 50 µL containing 23.0µL nuclease free water, 25.0 µL of EconoTaqPLUS GREEN 2X Master, 0.5 µl of each primer (10µM) [ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3')] and 1.0 µL of DNA template (Lucigen Corporation 2505 Parmenter St, Middleton, WI 53562 USA) was used. Amplification conditions were achieved in a BIO RAD My Cycler thermal cycler programmed for initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 1 min. At the end of amplification reaction, a final extension step was accomplished at 72°C for 10 min. PCR products attained were run at 1% agarose gels dissolved in 1× TAE (Tris-Acetate EDTA buffer) concentration as the running solution followed with post staining of ethidium bromide (0.5 µg/ml). Electrophoretic migration was carried out for 1 h electrophoresed at 100 V. The amplified products were visualized and photographed under ultraviolet (UV) light. A 100 bp EZ Load molecular ruler (Bio-Rad Laboratories, Inc. CA, USA) was used to estimate the size of PCR products.

Sequencing, identification and phylogenetic analysis

PCR products with a size of 450 bp and above were sent to Beckman Coulter Company and 43 PCR products were subjected to single pass sequencing (Beckman Coulter Genomics, Inc. Danvers, MA USA). ITS sequences of *Pythium* isolates were compared with ITS sequences of known *Pythium* species available in the GenBank database by performing nucleotide blast search at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/blast.cgi). The MEGA 6 software was used for phylogenetic analysis (Tamura et al., 2013).

Statistical analysis

The data collected from the survey was summarized using descriptive statistics such as means, frequencies, percentages and cross tabulations were used to establish the strength of association

Table 1. Relative percentages of common bean cultivars grown in Lushoto and Mbozi districts.

	Common bean cultivars	Frequency	Percentage	Cumulative percent
1	Rozikoko	21	25.00	25.00
2	Soya	20	23.80	48.80
3	Njano round	20	23.80	72.60
4	Njano ndefu	9	10.60	83.30
5	Nyeupe ndogo	4	4.80	88.10
6	Kablanketi	3	3.60	91.70
7	JKT	2	2.40	94.10
8	Uyole 2003	2	2.40	96.50
9	Mchanganyiko (Mixed cultivars)	1	1.20	97.70
10	Kibumburi	1	1.20	98.90
11	Selian 94	1	1.20	100.00

Table 2. Categories of root rot symptoms in farmers' field.

	Observed symptoms	Frequency	Percentage
1	Wilting, yellowing, water soaked roots and spongy discoloration	34	40.4
2	Dropping yellow leaves, stunted growth and poor germination	30	35.8
3	Death of roots and emerging adventitious roots	16	19.0
4	Water soaked stem extended to hypocotyl	4	4.9

between variables (SPSS Inc., Chertsey, England) (Steel et al., 1997).

RESULTS

Root rot distribution

Root rot disease severity was identified from selected hot spot areas, using 84 samples collected which include 43 samples from Lushoto district (five ward locations; Lushoto, Ubiri, Lukozi, Gare and Kwemashai) and 41 samples from Mbozi district (four ward locations; Ruanda, Igamba, Mlowo and Myovizi).

Production practices of common beans producers within the sampling area

Most of the farmers (63.0%) practiced sole cropping of beans, 31.0% mixed cropping and 6.0% intercropping. Corn (22.7%) was the major crop for either mixed or intercropping systems and other minor crops were banana (4.8%), cassava (2.5%) Irish potatoes (2.5%) and coffee (4.8%). Most farmers (52.4%) kept their own seeds after the season which are used for planting in subsequent cropping season, (32.1%) bought seeds from the local markets, 3.6% got seeds from neighbors, 6.0% from Agro-dealers and 6.0% from Research Centers. This study showed that no fertilizer was applied by 92.9% of the farmers unless planted in association with corn (7.1%) in the same field. Due to marketability and food sources, the following common bean cultivars were found to be preferred by farmers in Mbozi and Lushoto districts; Soya (23.8%), Njano round (23.8%), Rozikoko (25.0%), Njano ndefu (10.6%), Kablanket (3.6%), Uyole 2003 (2.4%) and JKT (2.4%), Kibumburi (1.2), Selian 94 (1.2) and mixed cultivars (1.2%) (Table 1).

Occurrence of root rot symptoms in farmers' field

Deterioration of the leaves, wilting, water soaked roots and spongy with discolored cavities dominated in several fields at a frequency of 40.4%, dropping of yellow leaves, stunted growth, uneven growth and poor germination was reported in 35.8% of samples, death of roots and emergence of adventitious roots above the dead root parts and extended brownish color in 19.0% of the samples, water soaked stem extended to hypocotyl of bean seedlings in 4.8% of the samples (Table 2). About 50.0% of respondents indicated that they had observed the above symptoms in their fields in all the seasons, 35.7% observed these symptoms before the 2012 seasons, 8.3% during the 2013 season, and 6.0% throughout the 2014 season (Table 3).

Table 3. Period of occurrence of symptoms in farmers' field.

No.	Duration symptoms seen	Frequency	Percent
1	Before 2012 season	30	35.7
2	2013 season	7	8.3
3	2014 season	5	6.0
4	All over seasons	42	50.0

Morphological and molecular characterization of isolated *Pythium* species and their distribution

Distinctive features of *Pythium*: oogonial wall, oospores, antheridia and sporangia were observed in purified isolates (Figure 1). Forty three PCR product samples with a size of 450 bp and above showed banding patterns and was sequenced for phylogenetic classification according to their respective molecular sequences of the ribosomal fragments (Figure 2). Eleven different *Pythium* species were identified after single pass sequencing; *Pythium aphanidermatum* (31.25%) and *Pythium splendens* (28.13%) being widely distributed in the entire surveyed area. Other species confirmed include: *Pythium ultimum* (6.25%), *Pythium attrantheridium* (6.25%), *Pythium graminicola* (6.25%), *Pythium oligandrum* (6.25%), *Pythium dissotocum* (3.13%), *Pythium irregurale* (3.13%), *Pythium camurandrum* (3.13%), *Pythium paroecandrum* (3.13%) and *Pythium acanthophoron* (3.13%) (Table 4).

Relationship of Soil pH and disease occurrence

The pH of soil samples collected in the study areas ranged between 5.03 – 6.41 of which 23 isolates were found in pH range of 5.03 – 5.95 and 9 isolates in soil pH range of 6.05 – 6.41. None of the soils had pH higher than 6.5 indicating that, these soils are acidic. Though, *P. splendens* and *P. aphanidermatum* were found in both low and higher ends of the acidic spectrum, *P. splendens* was found in most soils with lower acidic pH values of between 5.03 – 6.12; while *P. aphanidermatum* was found in mostly soils with higher pH values of between 5.41 – 6.41 (Table 4).

Phylogenetic relationship of *Pythium* spp.

Five clusters with ten sub-clusters showed diversity and homogenity of species within geographical location (Figure 3). Cluster specific sequences were dispersed over the ITS regions and contributed to the divergence between clusters and convergence between sub-clusters. In cluster I, convergence was observed between *P. aphanidermatum* and other species, in particular *P. dissotocum, P. acanthophoron* and *P. oligandrum* although they originated from different geographical locations. In cluster II, *P. attrantheridium*, *P. paroecandrum, P. irregurale* and *P. graminicola* were clustered together due to their close relationships. *P. splendens* and *P. aphanidermatum* in cluster III were closely related despite their origin. Likewise, alignments revealed that, *P. aphanidermatum, P. ultimum* and *P. oligandrum* were closely clustered together due to their similar origin in Lushoto district under cluster IV. *P. camurandrum* is the only isolate having distant relationship from other *Pythium* species.

DISCUSSION

According to the survey conducted in this study, similar root rot symptoms were observed as described by previous findings (Abawi et al., 2006; Agrios, 2005; Buruchara et al., 2010). More farmers use farm saved seeds in which survival structures of the pathogen can be stored together with the seeds and when planted they germinate together and lead to infection and development of the disease. Seed rot and pre-emergence damping-off normally reduce germination rates of planted cultivars due to infection caused by *Pythium* species (Xi et al., 1995). Sole cropping of bean was found to be the dominant system of cultivation as compared to mixed cropping and intercropping due to short rainy season. Previous studies in Uganda showed the incidence of *Pythium* root rot disease in mixed cropping systems. Potatoes, sorghum, maize and peas were found to be susceptible and infected by *Pythium* when intercropped with beans and the cultivation of common beans in mixed cropping systems with other crop species. This partly contributed to beans root rot epidemics and sorghum and peas were found to be the alternative hosts of the pathogenic *Pythium* species (Gichuru, 2008). Studies conducted in Japan showed prevalence of *P. paroecandrum*, *P. spinosum* and *P. ultimum* infecting common beans in rotation plots rather than in sole bean cropping plots because of inoculum build up from other alternate crop prone to root rot (Kageyama, 1981).

Soil pH influences some life cycle stages of *Pythium* species particularly during formation of oospores and sporangia (Martin and Loper, 1999). This study showed that more incidences of *Pythium* pathogen were found between soil pH of 5.1 to 5.6 and less incidence occurred between 6.1 to 6.5 pH; these soils are classified as strongly/moderately acid and slightly acidic, respectively (Soil Survey staff, 1993). Soil pH affects composition of the root exudates, which attract soil borne pathogens (Agrios, 2005). The use of liming materials balances the soil pH to neutrality, and the response of liming materials to soil pH increase is because it removes imbalance of nutrients particularly reduction of aluminum and

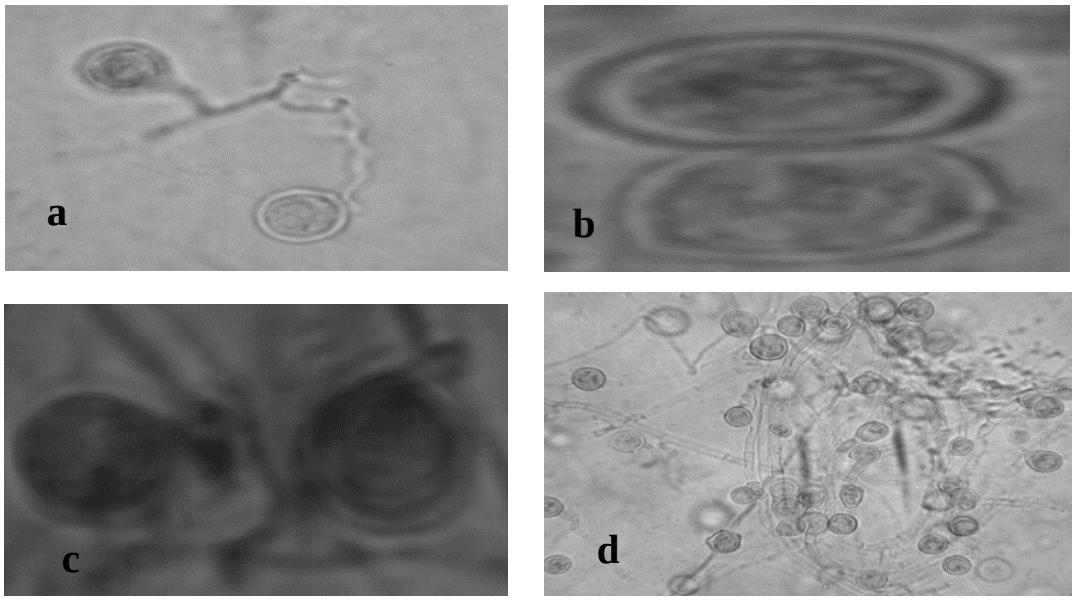

Figure 1. Morphological features of *Pythium* spp. a: Globose sporangia b: Oospores in an oogonium c: antheridial cell in similar configuration d: Scattered sporangia (large bodies with thin walls) and oospores (smaller, rounder bodies with thick wall) (Magnification 100x).

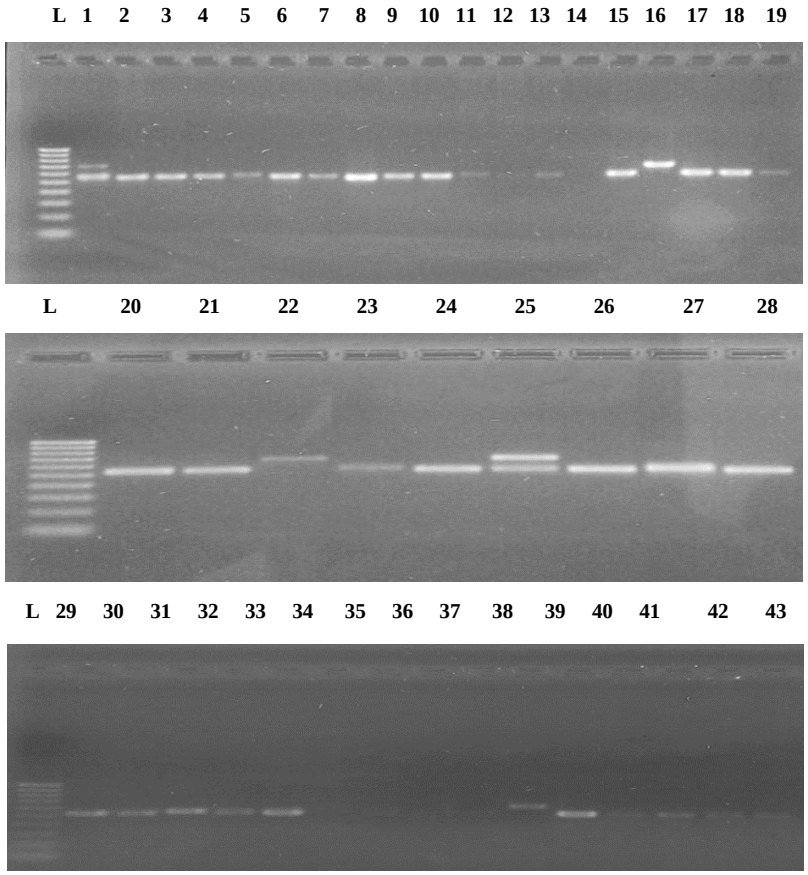

Figure 2. Banding patterns for PCR products electrophoresed at 100 V for 1 h. L = EZ molecular Ruler 100 bp, 1-43 = PCR product samples.

Table 4. *Pythium* species by geographical location and soil pH.

District	Ward	Latitude	Longitude	Altitude (m)	Isolate Codes	*Pythium* spp.	Soil pH
Lushoto	Ubiri	04°50'03.683"	038°19'48.468"	1220	LSH10/UBR	*Pythium aphanidermatum*	6.28
Mbozi	Ruanda	09°00'14.639"	033°06'39.954"	1646	MBZ02/RND	*Pythium splendens*	5.53
Lushoto	Lushoto	04°47'51.312"	038°15'37.853"	1437	LSH06/LS	*Pythium ultimum*	6.05
Mbozi	Ruanda	08°59'12.642"	033°06'22.878"	1639	MBZ01/RND	*P.splendens*	6.17
Lushoto	Lushoto	04°47'56.670"	038°17'22.061"	1401	LSH02/LS	*P.aphanidermatum*	5.74
Lushoto	Lukozi	04°39'52.421"	038°17'32.591"	1805	LSH03/LKZ	*Pythium irregurale*	5.56
Mbozi	Igamba	08°59'26.298"	032°55'56.771"	1634	MBZ10/IGB	*P.splendens*	5.03
Lushoto	Kwai	04°42'00.906"	038°20'15.575"	1615	LSH01/KWA	*Pythium olingandrum*	5.83
Lushoto	Lukozi	04°39'57.054"	038°16'59.574"	1754	LSH02/LKZ	*P.aphanidermatum*	5.90
Mbozi	Mlowo	09°02'36.906"	032°57'52.422"	1582	MBZ04/MLO	*P.splendens*	6.14
Lushoto	Lushoto	04°48'08.010"	038°18'37.996"	1526	LSH19/LS	*P.aphanidermatum*	5.41
Lushoto	Lushoto	04°46'40.601"	038°17'19.445"	1523	LSH14/LS	*P.aphanidermatum*	5.92
Lushoto	Lushoto	04°47'58.421"	038°18'21.840"	1418	LSH17/LS	*P.ultimum*	5.65
Mbozi	Igamba	08°59'20.006"	032°55'07.835"	1630	MBZ07/IGB	*P.splendens*	5.07
Lushoto	Ubiri	04°50'05.568"	038°19'48.155"	1213	LSH09/UBR	*P.aphanidermatum*	6.08
Lushoto	Lushoto	04°47'56.172"	038°18'34.830"	1457	LSH16/LS	*P.splendens*	5.78
Mbozi	Mlowo	09°04'33.034"	032°58'59.405"	1633	MBZ11/MLO	*Pythium graminicola*	6.04
Mbozi	Igamba	08°58'59.915"	032°54'39.630"	1628	MBZO4/IGB	*Pythium dissotocum*	5.95
Lushoto	Lushoto	04°46'32.190"	038°17'18.702"	1593	LSH13/LS	*P.splendens*	5.84
Mbozi	Myovizi	09°00'15.810"	033°01'58.020"	1642	MBZ02/MYZ	*Pythium paroecandrum*	5.42
Lushoto	Gare	04°47'17.298"	038°20'54.732"	1414	LSH07/GRE	*Pythium attrantheridium*	5.35
Lushoto	Gare	04°47'56.598"	038°20'40.728"	1423	LSH01/GRE	*P.attrantheridium*	5.31
Lushoto	Gare	04°47'51.551"	038°20'09.810"	1538	LSH10/GRE	*Pythium acanthophoron*	6.26
Mbozi	Mlowo	09°02'21.245"	032°58'32.876"	1623	MBZ01/MLO	*P.graminicola*	5.65
Lushoto	Kwai	04°40'00.906"	038°20'12.575"	1623	LSH03/KWA	*P.aphanidermatum*	5.78
Lushoto	Lushoto	04°46'40.308"	038°17'18.240"	1541	LSH15/LS	*P.aphanidermatum*	5.67
Mbozi	Igamba	08°59'50.628"	032°55'44.483"	1635	MBZ09/IGB	*P.ollingandrum*	5.74
Lushoto	Ubiri	04°50'08.178"	038°19'19.446"	1238	LSH04/UBR	*P.splendens*	5.81
Mbozi	Ruanda	09°01'45.329"	033°06'15.282"	1712	MBZ04/RND	*P.splendens*	6.12
Mbozi	Igamba	08°58'19.362"	032°54'24.263"	1626	MBZ06/IGB	*Pythium camurandrum*	5.89
Lushoto	Gare	04°47'25.644"	038°20'40.476"	1481	LSH05/GRE	*P.aphanidermatum*	5.47
Lushoto	Ubiri	04°50'00.738"	038°19'16.272"	1226	LSH05/UBR	*P.aphanidermatum*	6.41

manganese toxicities which provides calcium ions to counteract its deficiency (Biswas and Mukherjee, 1994; Nekesa et al., 2005). Also, soil pH affects the availability of nutrients to the plant which are needed for strong cell walls and resistance to fungal infestations. For instance, high levels of available calcium in more alkaline soils have been implicated in the resistance to root diseases caused by *Pythium* species (Paulitz, 2002).

Ribosomal DNA sequences of the ITS region identified eleven *Pythium* species; *P. aphanidermatum* and *P. splendens* which were the most widely distributed species in the study locations. Other species confirmed include *P. dissotocum, P. ultimum, P. irregurale, P. camurandrum, P. attrantheridium, P. graminicola, P. paroecandrum, P. acanthophoron* and *P. oligandrum*. Previous studies conducted by Mukalazi (2004) and Nzungize et al. (2011) in Uganda and Rwanda, respectively identified some similar species that cause root rot disease. However, none of these identified eleven species have been previously studied and documented in Tanzania. Therefore, this study is the first to identify these *Pythium* spp. in common bean cultivation in Tanzania.

There was no association between the geographic distribution and identification of *Pythium* species within the collection area. For instance, *P. aphanidermatum* was found in altitudes of 1213, 1526 and 1754 m above sea level which is similar to the study conducted by Nzungize et al. (2011) that *P. vexans* was found in the highest number of districts where common beans were grown and this species was identified in low, intermediates and high altitudes of 900-1400, 1400-1650 and 1650-2300 m,

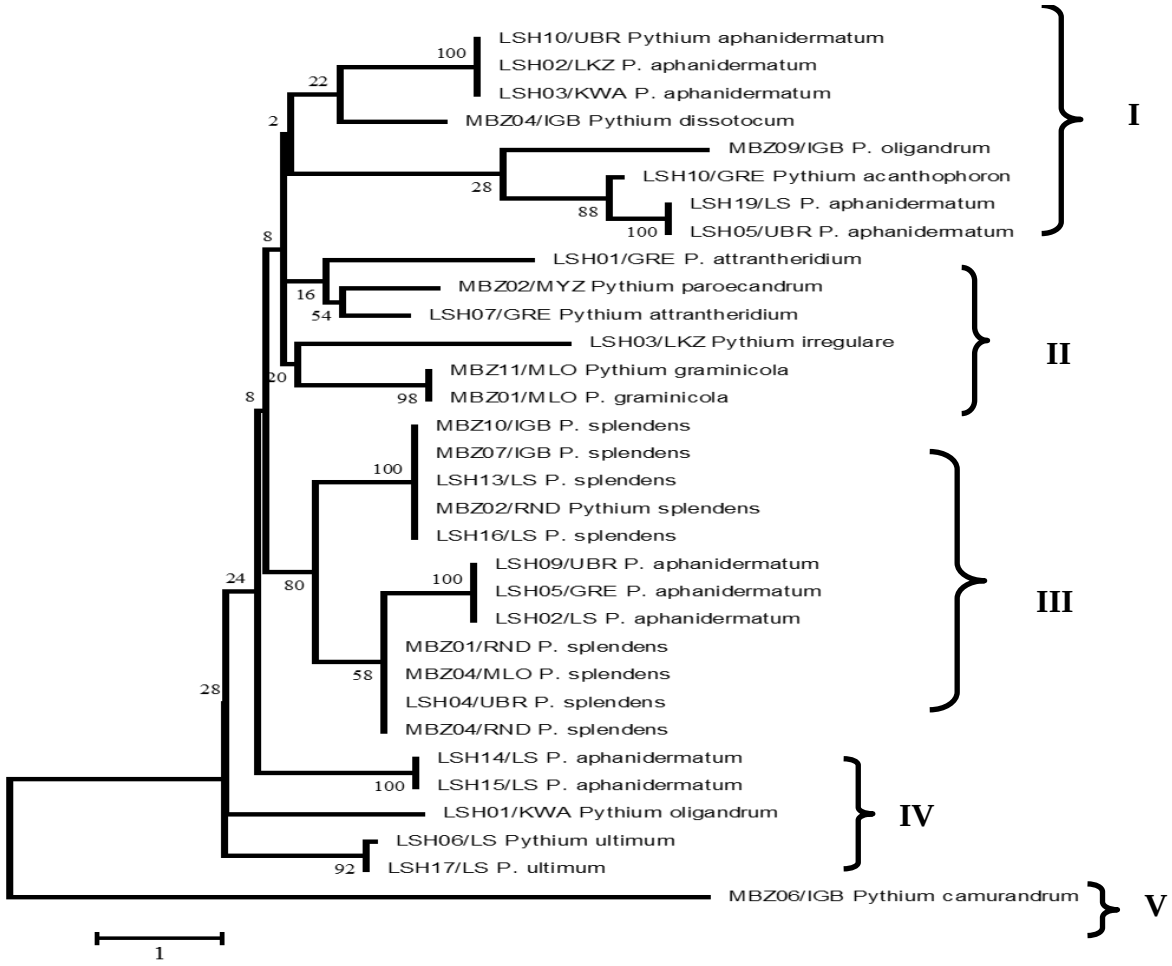

Figure 3.*1* Evolutionary phylogenetic relationship of *Pythium* spp. based on ITS ribosomal DNA sequences aligned by ClustalW and constructed by maximum likelihood tree. First three letters representing district, a numeral represent sampling number within a ward and last letters represent wards (LSH=Lushoto, MBZ=Mbozi, LKZ=Lukozi, KWA=Kwai, LS=Lushoto, IGB=Igamba, UBR= Ubiri, MLO= Mlowo, MYZ= Myovizi, GRE=Gare, RND=Ruanda).

respectively. In previous findings, similar studies identified *P. apahanidermatum* as a causal agent of root rot and crown necrosis of mature bean plants in Oman. This species was identified as the most aggressive and pathogenic species in the genus; it also has a wide host range that causes many economically important root rot disease (Al-Mahmooli et al., 2015; Ben Yephet and Nelson, 1999; Haritha et al., 2010). The most common species of *Pythium* that cause plant diseases of economic importance in Florida are *Pythium myritylum* and *P. aphanidermatum* and other species of *Pythium* that are sometimes associated with dysfunctional plants were *P. splendens* and *P. irregulare*. Also, *P. splendens* was identified from *Eucalyptus grandis* in northern Natal in South Africa and from soybean and corn in Ohio, USA (Dorrance et al., 2004; Kucharek, 2000; Linde et al., 1994). In eastern Washington, several species including *P. attrantheridium, P. irregurale* and *P. paroacandrum* were identified by using a real time PCR using collected soil samples (Li et al., 2014; Schroeder et al., 2006). This study also identified *P. oligandrum* from farmers' field within mono cropping system of common beans (Gichuru, 2008).

The phylogenetic analysis indicated diversity and similarities obtained from the alignment analysis of the ITS sequenced data using species-specific primers of 5.8S rDNA sequences. Specific proportional studies of the nucleotide sequences of rDNA genes provide a significant way of analyzing phylogenetic relationships over a wide range of taxonomic specie levels in fungal and non-fungal groups (Berbee et al., 1995; Harlton et al., 1995).

The spread of *Pythium* spp. occurs mostly through the movement of infested soil and plant materials by irrigation

water, wind, farm equipment or animals. Heavy use of nitrogenous fertilizers and removal from farm products like plant residues after harvesting take alkaline nutrients off, tend to accelerate the rate of soil acidification and make favorable conditions for *Pythium* development. Therefore, farmers are advised to leave plant residues in the field after harvesting and use agricultural liming materials so as to increase soil pH from acidity to neutrality. This study provided information on the occurrence and distribution of *Pythium* root rot disease in two districts of Tanzania where common beans are grown.

Conflict of interests

The authors have not declared any conflict of interest.

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