Full Length Research Paper

Characterization of the common bean host and *Pseudocercospora griseola*, the causative agent of angular leaf spot disease in Tanzania

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Angular leafspot (ALS), caused by the fungus, *Pseudocercospora griseola,* is one of the most important disease of common bean in Tanzania. Breeding for resistance to this disease is complicated by the variable nature of the pathogen. In Tanzania, a thorough analysis of the variability of this pathogen is lacking which limits breeding for durable resistance. This work aimed at characterizing *P. griseola* in relation to its host in Tanzania. A sample collection of both *P. griseola* and common bean was conducted in the 2013 and 2014 growing seasons from nine regions. Single spore isolation was performed for *P. griseola* isolates and DNA was extracted from both *P. griseola* mycelium and bean leaves from which the pathogen was collected. For characterization of the gene pool origin of the host, Phaseolin DNA marker was evaluated and for the pathogen, the Internal Transcribed Spacer region (ITS) and the Actin gene sequences were evaluated. Phylogenetic analysis showed the presence of 69.7% Andean and 30.3% Mesoamerican strains of *P. griseola* in Tanzania. The common bean host genotypes showed a similar distribution with 84.2% Andean and 15.8% Mesoamerican. In both cases, Andean strains of the pathogen and Andean bean genotypes outnumbered Mesoamerican. In relation to the common bean genotypes, Andean genotypes were more susceptible to ALS as compared to Mesoamerican genotypes. There were few strains that were of Andean origin but were pathogenic on Mesoamerican common bean genotypes, a group that has previously been termed Afro-Andean. Geographically, most of the regions of Tanzania had only Andean strains except for Kagera where 60% were Mesoamerican strains, and in Arusha and Tanga, where 50 and 33% were Mesoamerican, respectively. Only three regions, Kagera, Mbeya and Rukwa, were found to grow Mesoamerican beans. The findings of this study are important in setting basic objectives for breeding for angular leaf spot disease in Tanzania.

Key words: Actin gene, angular leaf spot (ALS), Internal Transcribed Spacer region (ITS), Phaseolin protein, *Phaseolus vulgaris.*

INTRODUCTION

Angular leaf spot (ALS) of common bean (*Phaseolus vulgaris.* L.) caused by the fungus, *Pseudocercospora griseola* (Sacc) Crous and U. Braun (Crous et al., 2006) is an important disease both in the tropics and in the sub-tropical tropics where beans are grown. The pathogen causes significant yield losses of up to 80% of common bean in Africa and in other parts of the world (Muthomi et al., 2011). This disease primarily affects aerial parts of the plant and is more destructive in warm, humid production zones (Crous et al., 2006). The disease causes premature defoliation (Correa-Victoria et al., 1989) and it also causes the reduction of seed quality, in cases where the disease invades the seeds, and thus affects the marketability of bean seed across production zones of the world (Pastor-Corrales et al., 1998).

As with other pathogens, the ALS disease is best controlled by the use of resistant cultivars since once the host resistance technology has been developed, it is packaged in the seed and is easy to disseminate and deploy (Crows et al., 2006; Mahuku et al., 2009). Despite the existence of genetic resistance, the variable nature of the pathogen makes achieving durable resistance a challenge (Mahuku et al., 2002; Pastor-Corrales et al., 1998). Therefore, knowledge on the races of the pathogen to be overcome in a region is crucial when durable resistance is sought, as well as the monitoring of the evolution of the pathogen over time (Mahuku et al., 2002). This pathogen monitoring is even more important when the pathogen shows high levels of variability as is the case for the ALS pathogen. Different methods have been applied in attempting to characterize the ALS pathogen including virulence testing, where isolates are classified according to the reaction they cause to a set of differential cultivars, and using molecular markers where the isolates are distinguished based on their genetic composition. The use of differential cultivars, however, has a major limitation due to variability in environmental conditions (Kolmer et al., 1995; Sebastian et al., 2006). The binomial characterization of the pathogen using the set of differentials can be supplemented with the molecular characterization of the pathogen which detects the variability of the pathogen population regardless of its host and environment (Ddamulira et al., 2014). Different molecular methods have been used to characterize this pathogen including RAPD markers, ISSR markers and Box primers (Abadio et al., 2012; Ddamulira et al., 2014). Sequences from the Internal Transcribed Spacer region (ITS) and Actin genes were used in a similar study evaluating the taxonomic status of *P. griseola* (Crous et al., 2006). Studies on the variability of *P. griseola* isolates revealed the existence of two major groups of the pathogen, Andean and Mesoamerican, which correspond to and have co-evolved with the Andean and Mesoamerican gene pools of common bean (Guzman et al., 1995; Pastor-Corrales et al., 1998; Crous et al., 2006). Mesoamerican strains of this pathogen are considered more virulent as compared to Andean strains and they tend to affect both Mesoamerican and Andean beans while Andean strains are less virulent, affecting mostly Andean genotypes (Pastor Corrales et al., 1998). Apart from these two distinct sets of host and pathogen based on geographical origin, another group was found peculiar to Africa designated as Afro–Andean. This group has characteristics typical of the isolates of Andean origin but it was found to be pathogenic on Mesoamerican common bean which is unusual (Mahuku et al., 2002; CIAT, 1997).

The objective of this work was to characterize the diversity of *P. griseola* in nine regions of Tanzania using ITS and Actin gene sequences of the fungus in relation to the gene pool origin of the common bean host and their distribution across the regions.

MATERIALS AND METHODS

Isolate and genotype collection

P. griseola isolates and host tissue were collected from the nine common bean production regions of Tanzania (Figure 1). The samples were collected from farmers' fields and some were collected from Agriculture Research Stations including: Uyole in Mbeya, Seliani in Arusha, Maruku in Kagera and Sokoine University of Agriculture (SUA) in Morogoro. Common bean infected leaves were collected from plants showing typical Angular Leaf Spot symptoms and were preserved in blotter paper and brought to SUA for isolation.

Young common bean leaves were collected from the same plants where ALS isolates were collected and put in 1.5 mL tubes (Eppendorf tubes).

Isolation of *P. griseola*

Single spore isolation was completed in the pathology laboratory at Sokoine University of Agriculture following the CIAT guidelines (Castellanos et al., 2015). Following isolation, pure cultures were transferred to V8 medium without antibiotics and the cultures were incubated at 24°C for 15 to 20 days. After this incubation period, the mycelium was scraped directly from the media for DNA extraction. The isolates were then maintained following the CIAT guidelines (Castellanos et al., 2015).

DNA extraction and PCR amplification

DNA from the *P. griseola* mycelia and from the common bean host

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Figure 1. A map of Tanzania showing the nine regions (shaded) where *P. griseola* isolates and common bean samples were collected.

were extracted using TES extraction buffer (Mahuku, 2004). Fungal PCR amplification was completed using two primer sets, the ITS 4 and ITS 5 primer pairs, to amplify the Internal Transcribed spacer (ITS) region (White et al., 1990), and the Actin gene with the ACT 512F and ACT 783R primers (Carbone and Kohn, 1999). The PCR conditions used to amplify the ITS region and the Actin gene were 94°C for 4 min for initial denaturation followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and elongation at 72°C for 1 min followed by another cycle of final elongation at 72°C for 4 min. DNA from the common bean samples were amplified using the phaseolin DNA primers Phaseolin1R and Phaseolin1F and the PCR conditions were initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 s and elongation at 72°C for 30 s, and a final extension for 7 min at 72°C.

Electrophoresis and gel documentation

PCR products from *P. griseola* samples were separated by electrophoresis (110 V for one hour and 30 min) and 3 µL of PCR product were run on a 1% agarose gel (Fisher Scientific, NJ) using 1X TBE buffer and 10 µL of gel red (Biotum ®, Hayward, CA). PCR products were visualized using a ...UV - transilluminator... and photographed using UVP® (Program version 6.5.2a 2007 program) (Upland, CA).

PCR products from common bean samples were separated using a 6% horizontal polyacrylamide gel (hPAGE technique) with electrophoresis at 120 V for 3 h, post-stained with ethidium bromide, and using 1X TAE as the running buffer. The gel visualization and documentation was completed using a UV – transilluminator and the gel photo was captured with a Power Shot ...A650IS digital... camera (Canon, USA).

Sequencing and phylogenetic analysis of the *P. griseola* isolates

PCR products for the ITS region and the Actin gene were purified, after confirming the expected band size (600-900 bp for ITS; 200 bp for Actin in an agarose gel), using a QIAquick PCR purification Kit (Qiagen, Valencia, CA). DNA quantification using a Nanodrop (ND-1000 UV/Vis Spectrophotometer, Wilmington, DE) was followed by dilution of the samples to 20–30 ng/ul for Actin and 30-50 ng/ul for ITS in preparation for sanger sequencing (SeqWright Genomic Services, Texas).

Two sequences for both the Actin gene and the Ribosomal RNA gene (ITS), CPC 10468 and CPC 10463, representing the Andean and Mesoamerican gene pools of *P. griseola* (Crows et al., 2006), respectively, were obtained from the NCBI database and included in the analysis as controls. Also, two other sequences for *Passalora loranthi,* strain CBS122466 and *Passalora eucalypti* strain CBS111318, from the NCBI database were used as out groups in the phylogenetic analysis to root the tree. These out groups (*P. loranthi* and *P. eucalypti*) are the sister clades to *P. griseola* (Crows et al., 2006).

Sequence data were first edited using BioEdit v7.2.5 and Sequencer to establish contigs (consensus sequence for each isolate) and the MEGA6 program was used to remove the primer sequence of each contig. The NCBI database was used to perform BLAST searches for each sequence to validate that the sequences belonged to *P. griseola*. Sequence for both the Actin gene and ITS region that were of high quality, were selected for phylogenetic analysis. The multiple sequence alignments were conducted with SATé (Katoh and Standley, 2013) using MAFFT for the alignment and RAxML for the tree estimator using 10 iterations. Four partitions were determined as input for Partition Finder V1.1.1 (Lanfear et al., 2014) to select the best molecular evolution model for each
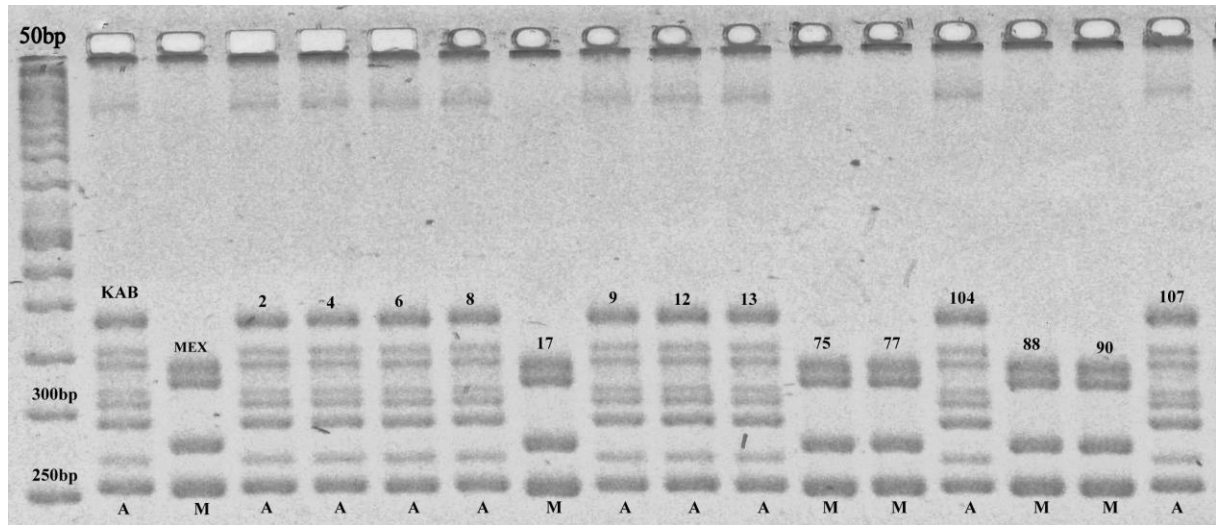
Figure 2. Polyacrylamide gel for Phaseolin protein marker where A = Andean, M = Mesoamerican; LD = molecular weight marker (50bp); Kab and Mex = controls (Kablanketi and Mex 54); and Numbers 2 - 107 = genotype samples.

partition. The best molecular evolution model for each partition were K80+G model for 1st and 2nd codon position of the Actin gene and the ITS region and the HKY model for the 3d codon position of the Actin gene.

Bayesian Inference (BI) was performed to infer phylogeny. BI analysis was implemented in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) with 4 runs and 4 chains for 25 million generations specified with the 2 molecular evolution models previously obtained, sampling every 10,000 generations. Convergence and stationery were visualized in Tracer V.1.4 (Rambaut and Drummond, 2007).

Common bean Phaseolin protein evaluation

The common bean genotypes were evaluated for the Phaseolin marker by scoring the banding pattern on the gel. The data were used to group the genotypes as either Mesoamerican or Andean.

RESULTS AND DISCUSSION

Host genotype characterization

The bean genotypes from which ALS isolates were collected were found to be of Mesoamerican and Andean origin although the gene pool composition differed from region to region of Tanzania. A total of 76 common bean genotypes were characterized using the phaseolin DNA marker (Figure 2) and of these 64 (84%) were of Andean origin and the other 12 (16%) were of Mesoamerican origin.

These results suggest that most of the common bean genotypes that are cultivated in Tanzania are of Andean origin with few of Mesoamerican origin from this relatively small sample. The distribution of these two gene pools across bean growing regions show that 75% of the total Mesoamerican bean samples were collected from the

Kagera region, while very few were collected from Rukwa (17%) and Mbeya (8%) (Figure 3). These results from Tanzania confirm previous findings indicating that both Andean and Mesoamerican beans are grown within some African countries (Wortmann et al., 1998; Mahuku et al., 2002). In Tanzania, it has been found that farmers grow both Andean and Mesoamerican beans and in most cases they mix the two together in the same plot of land as a strategy of risk management since some cultivars fail and others are tolerant to biotic and abiotic factors (Blair et al., 2010).

In terms of regional preferences, the Kagera region of Tanzania leads in cultivation of the Mesoamerican bean type. The regional preference for small (Mesoamerican) or large (Andean) types may be associated with how the common beans are consumed. In the lake zone, beans are mostly consumed when cooked with banana (Matoke) and in this dish, the preference is for small seeded beans. In other regions of Tanzania, beans are consumed mostly with rice and maize meal for which the preference is large seeded beans in these dishes. In studies in countries neighboring Tanzania, the proportion of production of Andean and Mesoamerican bean in Uganda (Okii et al., 2014) is similar to that of Tanzania, while in Rwanda, common beans of Mesoamerican origin outnumbered the Andean ones (Blair et al., 2010).

In the samples of common bean collected in the nine regions of Tanzania, samples of Andean origin outnumbered those of Mesoamerican origin in every region. Thus, farmers prefer to cultivate Andean genotypes as opposed to Mesoamerican genotypes (Figure 3).

Pathogen characterization

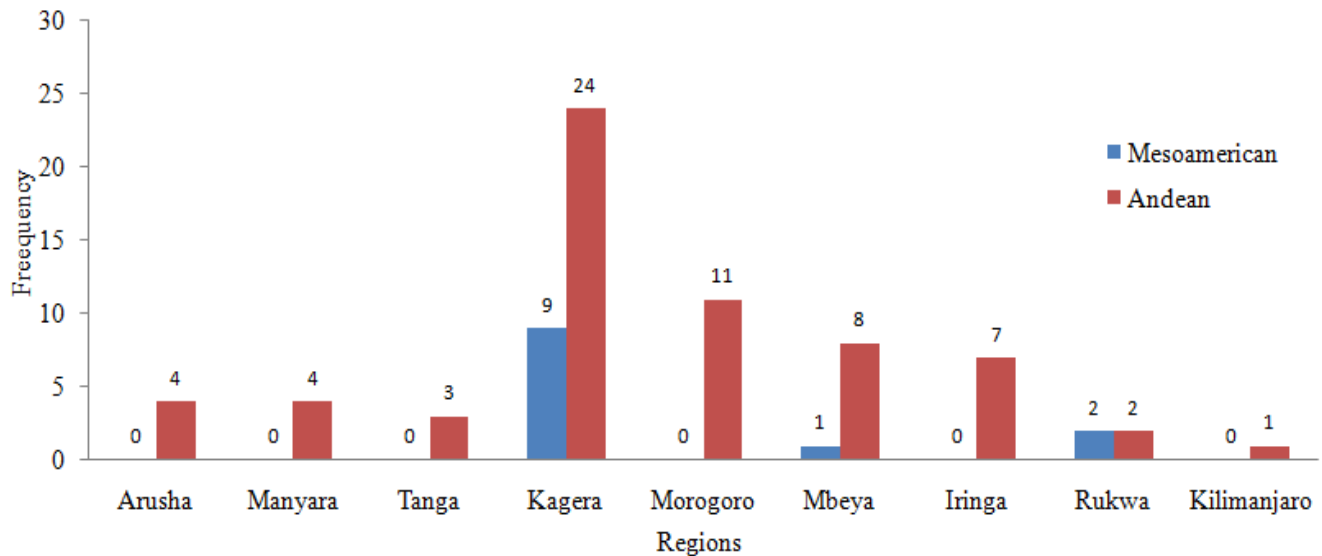The results from the sequence analysis of the Actin gene

Figure 3. Frequency of Andean and Mesoamerican common bean genotypes across bean growing regions as determined by the Phaseolin marker.

and the ITS region showed that the ITS region captures more sequence variation in the pathogen as compared to the Actin gene. Phylogenetic analysis of the sequences from the Actin gene and the ITS region depict the presence of two distinct clusters that group with the Andean and Mesoamerican control sequences (Figure 4). These results were expected due to the presence of the two gene pools of the pathogen as reported by Pastor-Corrales et al. (1998). Furthermore, most of the isolates grouped with the Andean control isolate (60%) as compared to the Mesoamerican control isolate (40%). These results show correspondence between the occurrence of the common bean genotypes, as shown by the phaseolin marker results, with the pathogen strains of the same gene pool origin, thus coinciding with the concept of co-evolution of this pathogen with its host (Guzman et al., 1995).

The distribution of isolates also showed the same trend as the distribution of the common bean genotypes where most of the Mesoamerican strains were collected from the Kagera region with very few from other regions of Tanzania. Thus, in the Kagera region, 60.6% of the isolates were Mesoamerican, while 39.4% were Andean. In Arusha, 50% were Andean and in Tanga 66.7% were Andean (Table 1). When grouping of isolates was organized based on altitude or geographical location of the site of collection, the distribution of the gene pools of the pathogen was not correlated with altitude (data not shown) and somewhat do with GPS position where most of the Mesoamerican are from the lake zone. Somewhat different findings were reported in Uganda (Ddamulira et al., 2014) and in other common bean production regions (Sebastian et al., 2006; Sartorato, 2004) where gene pool grouping due to place of origin of the isolate was not

observed.

Relationships between genotype and pathogen

The relationship between the pathogen and the genotype from which the isolate was collected shows that Andean isolates infected mostly Andean genotypes (92%) and far less Mesoamerican genotypes (8%). On the other hand, Mesoamerican isolates infected mostly Andean genotypes (65%) and fewer Mesoamerican (35%) genotypes. Generally, Andean genotypes are more susceptible to ALS as compared to Mesoamerican genotypes; thus, making Mesoamerican genotypes more resistant to both Andean and Mesoamerican isolates of *P. griseola*. These results may indicate that there are more genes for resistance to ALS in the Mesoamerican gene pool. Because Andean isolates that were virulent on Mesoamerican genotypes were found, this suggests that in Tanzania, both common bean genetic backgrounds need to be improved so as to attain durable resistance. Similar findings have been reported from pathogenic characterization using differential cultivars (Ddamulira et al., 2014); however, in this study, no differential cultivars were used and instead virulence grouping was employed as described by Mahuku et al. (2002) in which the affected genotypes were characterized. Further, in this study, there is interaction between Andean isolates with Mesoamerican genotypes where some Andean isolates were shown to be virulent on Mesoamerican genotypes (Figure 5). Using RAPD markers, the Afro-Andean group was included within Andean isolates (Mahuku et al., 2002). This implies that, there is still within group evolution over time which indicates the continued need to
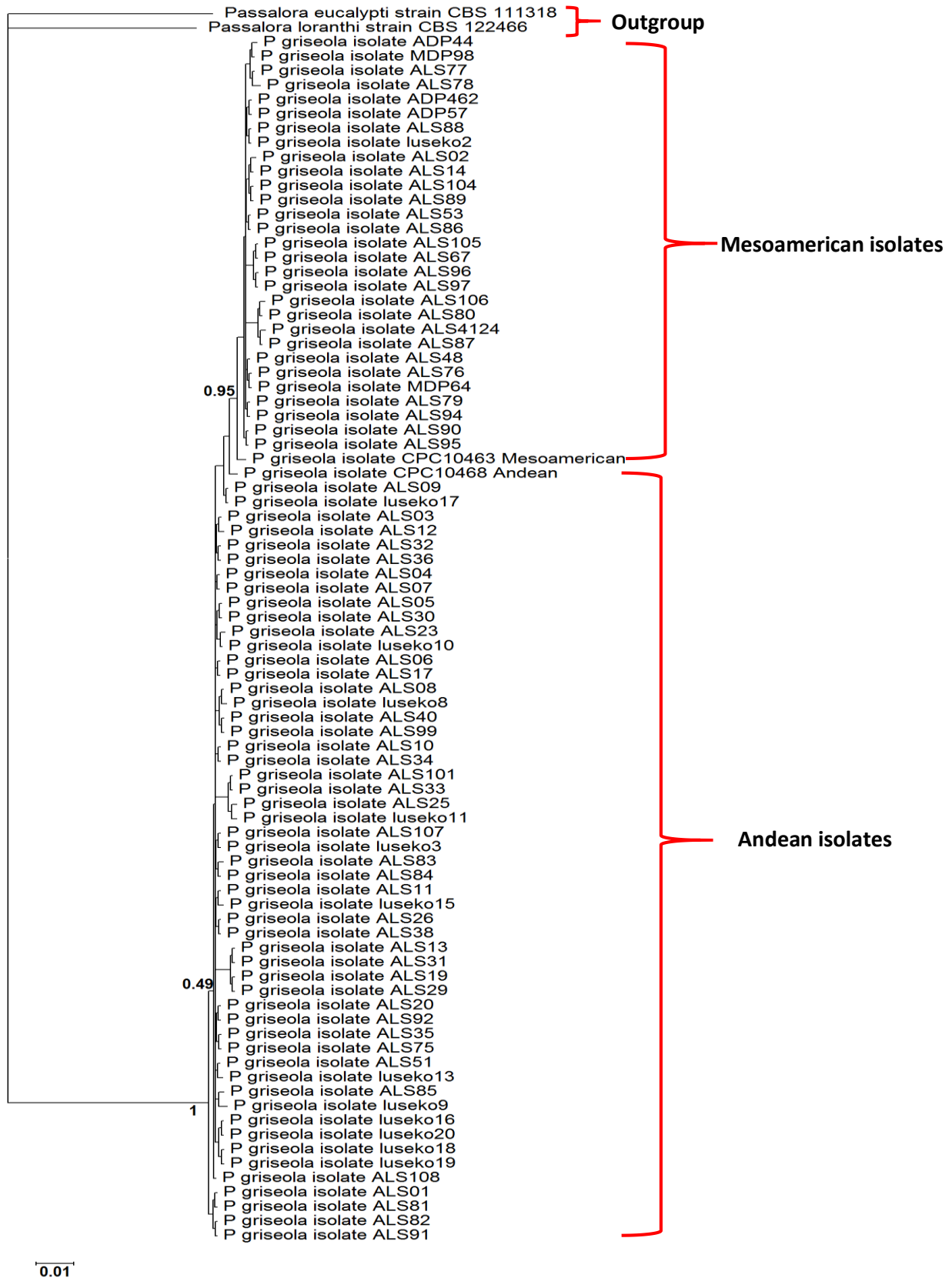
Figure 4. Concensus tree results from Bayesian analysis using concatenated ITS and Actin sequences showing the proportion of Tanzanian Andean and Mesoamerican ALS isolates as grouped with control sequences.

Table 1. Distribution of Andean and Mesoamerican isolates, showing number and percent of isolates per location, of *P. griseola* as revealed by sequence analysis of the ITS region and the Actin gene.

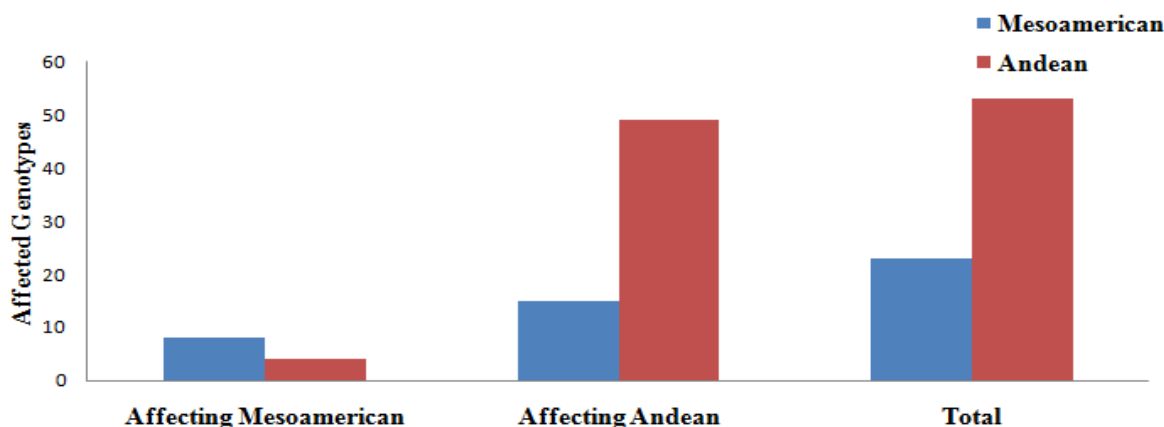| S/N	Region	Andean (%)	Mesoamerican (%)	Total
1	Arusha	2 (50%)	2 (50)	4
2	Kilimanjaro	1 (100%)	0 (0)	1
3	Manyara	4 (100%)	0 (0)	4
4	Morogoro	10 (100%)	0 (0)	10
5	Tanga	2 (66.7%)	1 (33.3)	3
6	Iringa	8 (100%)	0 (0)	8
7	Mbeya	9 (100%)	0 (0)	9
8	Rukwa	4 (100%)	0 (0)	4
9	Kagera	13 (39.4%)	20 (60.6)	33

Figure 5. Number of strains of *P. griseola* from each gene pool that were collected on Mesoamerican or Andean common bean host genotype.

evaluate pathogen variability over time in order to verify possible outbreaks of new strains.

Conclusions

This study reveals the presence of both Andean and Mesoamerican common bean genotypes in the 9 major production regions of Tanzania and that Andean beans are the most preferred gene pool across all bean growing regions. This study also verified the presence of Andean and Mesoamerican strains of *P. griseola* and their distribution, showing that Andean isolates of *P. griseola* outnumbered the Mesoamerican isolates. In the Kagera region (the Lake zone) as opposed to other regions, Mesoamerican isolates outnumbered Andean isolates. Andean isolates were found to affect most of the Andean common bean genotypes and less of the Mesoamerican common bean genotypes. Mesoamerican isolates were found to affect most of the Andean common bean

genotypes and less of the Mesoamerican common bean genotypes. The results from this study sets a foundation for breeding for resistance to angular leaf spot disease in Tanzania by evaluating the presence of both Andean and Mesoamerican genotypes and *P. griseola* strains and their interaction and distribution across growing regions.

Conflict of Interests

The authors have not declared any conflict of interests.

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