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GENETIC DIVERSITY OF *COLLETOTRICHUM SUBLINEOLUM* ISOLATES FROM A SINGLE FIELD IN SOUTHERN ETHIOPIA AND EVIDENCE FOR THE EXISTENCE OF MAT2 GENOTYPES IN DIFFERENT PARTS OF THE COUNTRY

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ABSTRACT: An AFLP analysis was carried out to study the genetic diversity of *Colletotrichum sublineolum* isolates collected from a single field in southern Ethiopia. The AFLP analysis revealed the presence of genetic variation among the tested isolates. Dice similarity coefficient ranged from 0.69 to 0.96 averaging at 0.87. Cluster and principal coordinate analyses categorized the isolates into nine and six major groups, respectively. The presence of diverse isolates even in a single field should be given due consideration in future breeding programs. Such a diversity calls for a need to stack diverse resistance genes using the gene pyramiding technique to come up with durable sources of resistance against *C. sublineolum*. A mating type genes identification study was also conducted with the help of PCR on 23 single spore isolates of *C. sublineolum* collected from different parts of Ethiopia. The degenerate primers SKCM1 and NcHMG were used to amplify the mating type (MAT) genes, MAT1 and MAT2, respectively. MAT2 genes were amplified from 15 of the 23 isolates while no amplification product was obtained using SKCM1. This may suggest that Ethiopian *C. sublineolum* populations might be composed of only MAT2 genotypes. However, further work that includes large number of isolates from several sorghum growing regions is needed to come up with a conclusive result on the mating system of the pathogen populations in Ethiopia.

Key words/phrases: AFLP, degenerate primers, mating type, PCR

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is an important cereal crop around the world. It ranks fifth amongst the world's cereals (FAO, 2009) and is one of the four leading crops in Ethiopia (CSA, 2009). The production and productivity of sorghum across the globe is hampered by several abiotic and biotic constraints. Among biotic problems, anthracnose caused by *C. sublineolum* (P. Henn) is one of the most destructive diseases affecting all above ground parts of sorghum (Thakur and Mathur, 2000; Casela *et al*., 2001). The disease is distinguishable by the characteristic circular to elliptical red spots with few to numerous acervuli on lamina (Marley *et al*., 2004). Sorghum anthracnose occurs in all sorghum growing regions of the globe, including Ethiopia (Pande *et al*., 1993; Marley *et al*., 2001; Ngugi *et al*., 2002; Alemayehu Chala *et al*., 2010a & b) causing yield loss of up to 50% or even more (Harris *et al*., 1964; Powell *et al*., 1977; Thomas *et al*., 1996).

Anthracnose development in sorghum fields is influenced by weather factors, inoculum density, pathogenicity of the strains, host genotypes and cultural practices. The use of resistant varieties seems to be the most viable option in managing sorghum anthracnose. Variations in resistance to anthracnose are reported among sorghum genotypes in different parts of the world (Neya and Normand, 1998; Hess *et al*., 2002; Erpelding and Prom, 2006).

Although a lot of effort has been made in searching for resistance to anthracnose, the high variability of the pathogen and differential reaction of sorghum genotypes under different environmental conditions (Alemayehu Chala *et al*., 2011; Alemayehu Chala and Tronsmo, 2012) call for a greater effort to continuously look for potential sources of resistance from diverse gene pools. Effective and sustainable control strategies are dependent on knowledge of the diversity of the pathogen, and the sources and mechanisms of the pathogen variability. Knowledge on pathogen diversity is also essential to predict the sustainability of management practices (McDonald and Linde, 2002). Earlier works have documented the existence of genetic diversity among *C. sublineolum* populations in different parts of

the world (Browning *et al*., 1999; Rosewich *et al*., 1998; Souza-Paccola *et al*., 2003; Prom *et al*., 2012). Alemayehu Chala *et al*. (2011) have also reported the presence of geographically isolated and diverse populations of *C. sublineolum* in Ethiopia, which are not affected by gene flow. They also suggested that *C. sublineolum* might be a species complex made up of several cryptic species. However, no work has been reported thus far on the genetic diversity of *C. sublineolum* at a field level although Ethiopian farmers are known to grow diverse landraces of sorghum that promote pathogen diversity. Such knowledge is of paramount importance to decide on breeding strategies aimed at developing resistant cultivars of sorghum.

Sexual reproduction in many fungi is controlled by mating type genes, which commonly have two idiomorphs *i.e.,* MAT1 and MAT2 that regulate sexual compatibility and sexual reproduction (Turgeon, 1998). As explained by Zanette *et al.* (2009), understanding the mating type systems will help to elucidate the mechanisms of sexual dimorphism in this important pathogen. The objectives of the current research were, therefore: i) to assess the genetic diversity of *C. sublineolum* at farm level, and ii) to characterize Ethiopian *C. sublineolum* isolates based on mating type.

MATERIALS AND METHODS

Analysis of genetic diversity of C. sublineolum

Isolate collection and isolation of C. sublineolum

Sorghum leaves showing symptoms of anthracnose were collected from a sorghum field in Wolayta, southern Ethiopia (6°59.098′ N latitude and 37°52.645′ E longitude, altitude of 1947 masl), and stored at room temperature in paper bags. The leaves were then cut into pieces, surface sterilized using 0.5% sodium hypochlorite (NaOCl) solution for 90 seconds and rinsed three times in sterile distilled water. The cut and surface-sterilized leaves containing lesions were then placed on oat meal agar (OMA) and incubated at 25°C under continuous fluorescent light for seven days. After 5–7 days, typical *C. sublineolum* sporulation was observed on the OMA plates and the conidia were examined under the microscope to ascertain their identity. Pure cultures of single spore isolates were grown on fresh potato dextrose agar (PDA) amended with 50 ppm of streptomycin and stored at 4°C as stock culture for AFLP analysis.

DNA extraction

For DNA extraction, 22 single spore isolates were transferred from the stock cultures and grown on freshly prepared PDA plates. Approximately 100 mg of fresh mycelium per isolate was crushed in liquid nitrogen using sterilized mortar and pestle. Then, the fine powder was transferred to 2 ml microcentrifuge tubes and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. The quality of extracted DNA was controlled on 0.8% agarose gels and the DNA was stored at 20°C prior to the actual AFLP analysis.

AFLP analysis

AFLP was conducted following the method developed by Vos *et al*. (1995) and further described by Alemayehu Chala *et al*. (2010a). The modifications included the use of fluorescently labeled primers instead of radioactive labeling Description of the adapters and primers used in this study is given in Table 1.

Table 1. Nucleotide sequences of adapters and primers used in the AFLP analysis.

Adapters/Primer	Sequences	Function
E*co*RI	5´CTCGTAGACTGCGTACC3´ CATCTGACGCATGGTTAA5´	Adapter
M*se*I	5´GACGATGAGTCCTGAG3´ TACTCAGGACTCAT5´	Adapter
E*co*RI	5´GACTGCGTACCAATTC3´	Nonselective primer
M*se*I	5´GATGAGTCCTGAGTAA3´	Nonselective primer
Selective primer combination	Primer sequences E*co*RI (5´→3´)	M*se*I (5´→3´)
E12 X M16	GAC-TGC-GTA-CCA-ATT-CAC	GAT-GAG-TCC-TGA-GTA-ACC
E19 X M15	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACA
E19 X M16	GAC-TGC-GTA-CCA-ATT-CGA	GAT-GAG-TCC-TGA-GTA-ACC
E20 X M17	GAC-TGC-GTA-CCA-ATT-CGC	GAT-GAG-TCC-TGA-GTA-ACG
E21 X M16	GAC-TGC-GTA-CCA-ATT-CGG	GAT-GAG-TCC-TGA-GTA-ACC
E21 X M17	GAC-TGC-GTA-CCA-ATT-CGG	GAT-GAG-TCC-TGA-GTA-ACG

Restriction and ligation

Approximately 200 ng DNA from each isolate was double digested with 5 units (U) of E*co*RI and M*se*I endonuclease restriction enzymes each for 2–3 hours at 37°C in a reaction volume of 30 µl containing 5x restriction-ligation (RL) buffer (50 mM Tris-acetate acid, 50 mM magnesium acetate, 250 mM potassium acetate, 25 mM DTT and 250 ng/µl BSA with pH 7.5). Thirty µl of completely digested DNA samples were ligated to 5 pmole E*co*RI and 50 pmole M*se*I adapters overnight at room temperature in a 40 µl reaction volume that contained 10 mM ATP, 5x RL buffer and T_4 DNA ligase (Fermentas, Glen Burnie, Maryland). Ligated samples were then diluted fivefold.

Preamplification

Preamplification PCR was carried out with none selective primers (E*co*RIo and M*se*Io) in a 25 µl total reaction volume containing 5 µl diluted ligation mix, 50 ng/µl of each primer, 10x PCR buffer (100 mM Tris-HCL, 500 mM KCl and 15 mM $MgCl_2$), 2.5 mM dNTP and Taq DNA polymerase (5 U/µl). The PCR program was set as follows: 94°C for 2 min for initial denaturation; 45 cycles of 94°C for 30 sec, 56°C for 30 s, 72°C for 90 sec; and a final cycle of 72°C for 10 min. Non selective PCR products were checked on 1% agarose gel electrophoresis and diluted 10 times for use in the selective PCR.

Selective amplification

Six combinations of M*se*I and E*co*RI primers with three selective nucleotides at their 3´ ends were used for selective amplification (Table 1). The E*co*RI primers were labeled with the fluorescent dye FAM (6-carboxyfluorescein). The selective amplification reaction mix contained 15 µl of 2.5 mM dNTP, 10x PCR buffer, 5 U/µl Taq DNA polymerase, and 6 ng/µl M*se*I and 6pmole E*co*RI primers to which 5 µl diluted none selective PCR product was added as a template. The PCR amplification has the following thermal cycles: One cycle of 94°C for 30 sec, 65°C for 30 sec and 72°C for 60 s; annealing temperature that was lowered by 0.7°C for each of the following 12 cycles, followed by 23 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec. Final primer extension was set at 72°C for 7 min.

Data scoring and analysis

Selective amplification products were separated in an AB3730 automated capillary sequencer (Applied Biosystems Inc., Foster City, California) following the manufacturer's protocol. The presence (1) and absence (0) of AFLP bands was scored using Gene-Mapper software version 4.0 (Applied Biosystems Inc., Foster City, California), checked visually, and only clear and unambiguous bands were entered into a binary data matrix for further analysis. The binary matrices were then used to calculate genetic similarities between the isolates based on Dice similarity coefficient (Dice, 1945); the unweighted pair-group method with arithmetic average (UPGMA) was used to construct genetic similarity tree (Rohlf, 1993) using the NTSYS-pc software, version 2.0 (Exeter Biological Software, Setauket, NY). To further elucidate the genetic relationship among the tested isolates, principal coordinate analysis was conducted using the software GenAlEx6 (Peakall and Smouse, 2006).

Mating type genes identification

Twenty three single spore *C. sublineolum* isolates collected from different parts of Ethiopia were used for mating type genes identification study. Isolate collection, pathogen isolation and DNA extraction were made the same way as described in the genetic diversity study except that isolates used for the mating type genes identification were collected from different locations (Table 2).

Table 2. Geographic origin of *C. sublineolum* isolates used for mating type genes identification.

Isolates	Geographic origin	Altitude (m)
1–8	North	1476–1516
9–6	South	1858–1947
17–23	East	2027–2077

Polymerase chain reactions (PCR)

The degenerate primers SKCM1 and NcHMG were used for PCR to identify the mating type genes present and characterize the isolates based on their mating type genes. The PCR reactions were carried out following the method described by Zanette *et al.* (2009) with slight modifications including the addition of 2.5 instead of 0.25 mM dNTP, decrease in annealing temperature from 1 min to 30 sec for NCHMG primer and loading of 10µL PCR products on 1.4% agarose gels instead of loading 15µL on 1.3% agarose gels.

The reactions were carried out in a 25 µl total reaction volume containing 20ng of DNA, 1 mM

of $MgCl_2$, 2 mM of each primer, 10x PCR buffer (100 mM Tris-HCL, 500 mM KCl and 15 mM $MgCl_2$), 10x BSA, 2.5 mM dNTP and 1 U of Taq DNA polymerase. The PCR program for primers NcHMG (5 'CG CC (CT) (CT) CC (CT) CC (CT) AA (CT) GCTA (CT) AT 3' and 5 'CGGG (AG) TT (AG) TA (AG) CG (AG) TA (GA) T (AG) GG 3 '), was set as follows: 95°C for 2 min for initial denaturation; 30 cycles of 94°C for 1 min, 50°C for 30 sec, 72°C for 1 min; and a final cycle of 72°C for 10 min. For amplifications of SkCM1 primers (5'GCAGATCTCC GCACTGGAGC3' and 5'GCAGATCTGTCGTCGATGGT 3') the PCR conditions were: 95°C for 2 min for initial denaturation; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min; and a final cycle of 72°C for 10 min. PCR products were checked by loading 10 µL of each reaction on 1.4% agarose gels.

RESULTS

Genetic diversity of C. sublineolum

The six primer combinations used in this study generated a total of 119 clearly scorable bands out of which 78 (65.5%) were polymorphic. Dice similarity coefficient for the 22 isolates tested in this study varied from 0.69 to 0.96 with a mean of 0.87 suggesting a moderate level of diversity (Table 3). Cluster analysis based on UPGMA and Dice similarity coefficient categorized the isolates into nine groups, which were further divided into 14 sub-groups (Fig. 1). The first cluster consisted of three isolates in two sub-clusters. Cluster 2 contained six isolates in three sub-groups while the third cluster contained four isolates divided into two sub-groups. Clusters 4, 5 and 6, consisted of 1, 2 and 3 isolates, respectively. Cluster 7, 8 and 9 contained a single isolate each. Isolates 5, 18 and 20 were found to be the most distantly related isolates compared to the other isolates forming their own individual groups.

Principal coordinates analysis (PCO) also revealed the population subdivision among the *C. sublineolum* isolates and categorized them into six groups (Fig. 2). The first three principal coordinates accounted for 36.7, 23.7 and 13.1% of the total variation, respectively. The PCO grouped 14 of the isolates that formed the first four clusters of the UPGMA tree into one group within the first quadrant. The second group was made of two isolates (12 and 14), which formed the fifth cluster of the UPGMA tree. Isolates 5, 18 and 20 made their own individual groups within the second and fourth quadrants, further confirming their genetic distance from the other isolates. Isolates 19, 21 and 22 formed another distinct group within the third quadrant.

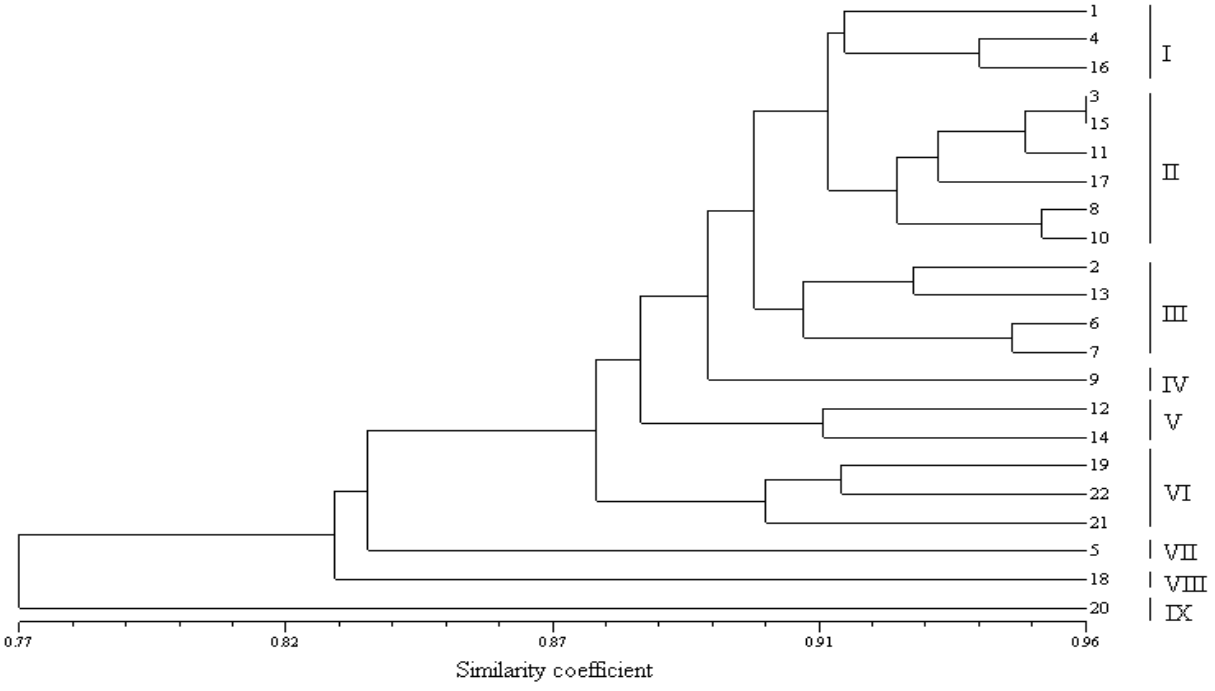

Figure 1. Dendogram showing the genetic diversity of 22 *C. sublineolum* isolates based on Dice similarity matrix of AFLP bands.

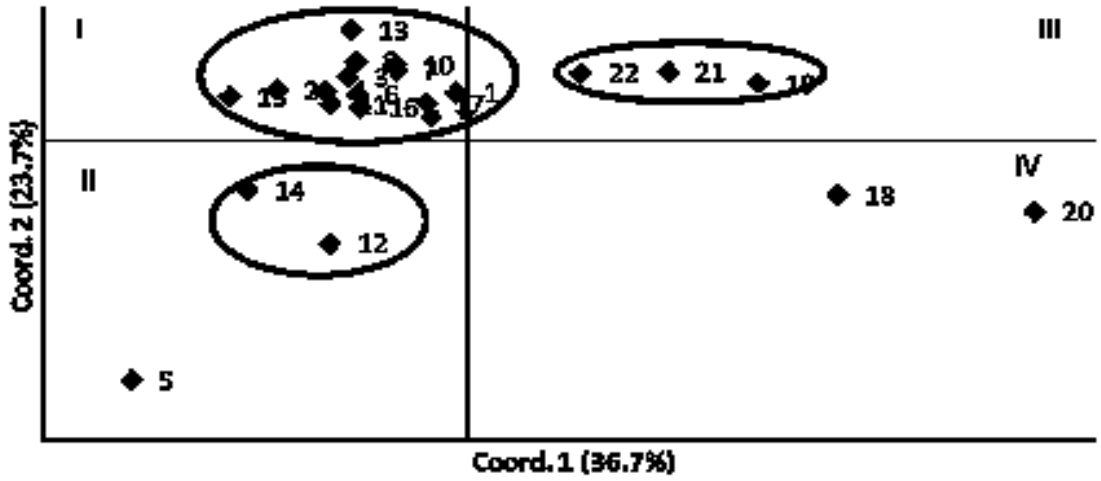

Figure 2. Principal coordinate analysis of 22 *C. sublineolum* isolates based on AFLP fingerprints.

Identification of mating type genes

Two primers *i.e.*, SKCM1 and NcHMG were used for amplification of mating type genes MAT1 and MAT2, respectively. The degenerate primer NcHMG resulted in the amplification of the MAT2 gene in 15 of the 23 test isolates (Fig. 3). The PCR with the NcHMG primer amplified a ~ 600bp fragment on isolates 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 23. But no amplification product was obtained when the degenerate primer SKCM1 (MAT1) was used. No amplification for both idiomorphs was verified in eight isolates *i.e.,* 5, 13, 17, 18, 19, 20, 21, 22.

DISCUSSION

Knowledge on pathogen diversity is of paramount importance to achieve affordable, effective and long lasting disease management strategies. Previous studies have reported great diversity among *C. sublineolum/graminicola* isolates causing anthracnose of sorghum in different parts of the world (Browning *et al*., 1999; Rosewich *et al*., 1998; Alemayehu Chala *et al*., 2011; Prom *et al*., 2012). Results of the current study also revealed the existence of genetic variation among *C. sublineolum* isolates within a single field in Ethiopia as confirmed by both cluster analysis and PCO (Figs 1 and 2). Results of the PCO analysis were more or less in line with that of the UPGMA clustering but the former reduced the overall grouping from nine to six. Four isolates (9, 5, 18 and 20) were distinctly categorized forming their own individual grouping in the UPGMA tree of the cluster analysis and three of these isolates (5, 18 and 20) were also clearly separated from the others in the PCO axes spreading over the second and fourth quadrants.

PCR of the current work using the NcHMG primer resulted in the amplification of MAT2 gene in 15 of the 23 tested isolates. The 15 isolates for which the MAT2 gene was amplified represented different geographic origins (Table 2). This suggests presence of MAT2 genotypes throughout the country. Zanette *et al*. (2009) also reported the amplification of MAT2 genes in 8 of 23 test isolates from Brazil using the same primer. Nevertheless, the fragment size obtained in the current work was consistently larger (~600bp) than those reported by Arie *et al*. (1997) and Zanette *et al*. (2009). On the other hand, Steenkamp *et al.* (2000) have revealed the amplification of larger fragment ~ 800 bp for MAT2 region of another fungus, *Gibberella fujikuroi*. This may suggest that the MAT2 region is not conserved and that it may change with fungal species or even biotypes. The second primer, SKCM1, did not amplify the MAT1 gene in any of the 23 test isolates suggesting the lack of MAT1 genotypes in the country.

Eight isolates did not yield any of the idiomorphs when tested with NcHMG and SKCM1 primers. This might be because they do not have the MAT gene as suggested by Zanette *et al*. (2009), or mating type idiomorphs in these isolates or even others code for a protein other than HMG box region as reported by Nelson (1996) for the yeast, *S. cerevisiae*.

Table 3. Dice Similarity coefficient among 22 isolates of C. *Sublineolum* from a single field in southern Ethiopia.

	Isolates																				
Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	-																				
2	0.88																				
3	0.91	0.92	-																		
4	0.92	0.91	0.92	-																	
5	0.83	0.86	0.84	0.85	-																
6	0.90	0.91	0.90	0.91	0.85	-															
7	0.88	0.90	0.89	0.88	0.81	0.95	-														
8	0.92	0.92	0.92	0.91	0.82	0.91	0.90	-													
9	0.90	0.90	0.87	0.91	0.82	0.89	0.87	0.94	-												
10	0.89	0.92	0.93	0.88	0.83	0.90	0.91	0.95	0.90	-											
11	0.93	0.92	0.95	0.92	0.86	0.90	0.89	0.93	0.90	0.95	-										
12	0.88	0.87	0.88	0.88	0.88	0.91	0.88	0.88	0.91	0.88	0.92	-									
13	0.88	0.93	0.90	0.88	0.79	0.92	0.92	0.92	0.88	0.92	0.92	0.87	-								
14	0.88	0.89	0.87	0.88	0.84	0.89	0.85	0.89	0.87	0.86	0.88	0.91	0.87	-							
15	0.91	0.95	0.96	0.94	0.86	0.91	0.88	0.92	0.90	0.92	0.95	0.90	0.92	0.90	-						
16	0.91	0.90	0.93	0.94	0.85	0.92	0.89	0.93	0.89	0.90	0.92	0.88	0.88	0.90	0.93	-					
17	0.92	0.90	0.947	0.92	0.86	0.89	0.88	0.91	0.86	0.94	0.94	0.88	0.87	0.84	0.93	0.91	-				
18	0.84	0.80	0.85	0.82	0.76	0.82	0.79	0.82	0.85	0.82	0.84	0.84	0.82	0.80	0.83	0.82	0.83	-			
19	0.87	0.87	0.85	0.86	0.77	0.89	0.86	0.86	0.87	0.89	0.87	0.85	0.86	0.81	0.86	0.86	0.88	0.87	-		
20	0.79	0.76	0.75	0.76	0.69	0.77	0.76	0.78	0.77	0.78	0.77	0.76	0.73	0.74	0.74	0.79	0.78	0.77	0.84	-	
21	0.89	0.87	0.90	0.88	0.78	0.87	0.86	0.89	0.88	0.86	0.86	0.84	0.83	0.81	0.87	0.89	0.90	0.87	0.90	0.80	-
22	0.91	0.89	0.88	0.90	0.81	0.90	0.89	0.89	0.90	0.89	0.89	0.85	0.89	0.85	0.88	0.90	0.90	0.83	0.92	0.82	0.91

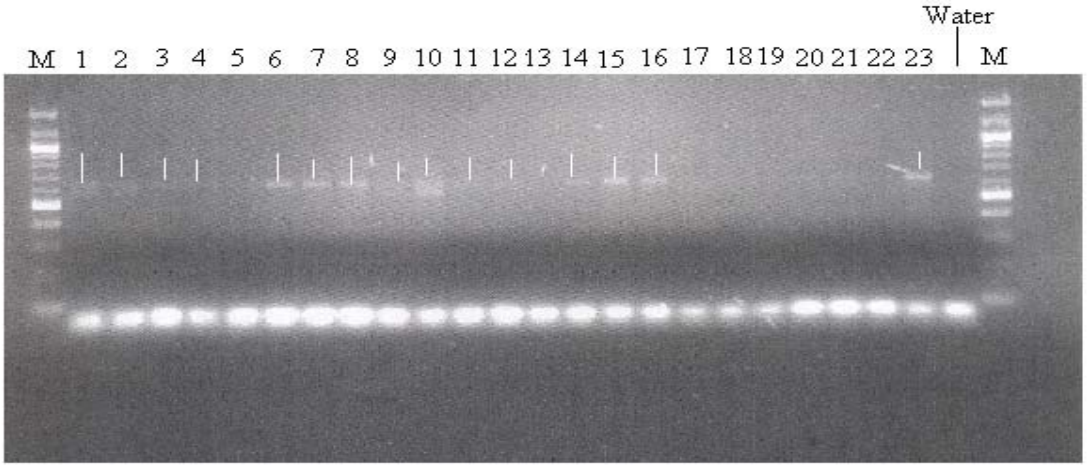

Figure 3. Amplification products of primer NcHMG (MAT2) on 1.4% agarose gel. (White bars indicate amplification products).

Apart from the previous study by Alemayehu Chala *et al*. (2011) that revealed the presence of very diverse *C. sublineolum* populations in different parts of the country, this is the first report on the genetic diversity of *C. sublineolum* within a single field in Ethiopia. It also represents the first attempt to characterize Ethiopian *C. sublineolum* isolates based on mating type genes.

Results suggest the existence of diverse isolates even in a single field that should be given due consideration in future breeding programs. Similar studies should be carried out by collecting isolates from different sorghum fields representing various agro-ecological zones to come up with a comprehensive view of how the pathogen population varies within each sorghum field. Results from the current work and that of Alemayehu Chala *et al*. (2011) also suggest the need for gene pyramiding aimed at stacking various resistance genes to come up with a durable source of resistance. Deployment of cultivars with horizontal resistance may also help in this regard. The mating type gene identification study indicates the lack of MAT1 genotypes and pathogen strains that have both MAT1 and MAT2 idiomorphs. This might prove the presence of asexually reproducing and yet very diverse pathogen populations affected by geographic isolation, host diversity and little gene flow as reported by Alemayehu Chala *et al*. (2010a). Nevertheless, further works that include large number of isolates from different sorghum growing regions of the country are needed to elucidate this conclusion and better understand the mating system of *C. sublineolum* in Ethiopia.

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