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Evaluation of Genetic Variability of Kenyan, German and Austrian Isolates of *Exserohilum turcicum* using Amplified Fragment Length Polymorphism DNA Marker

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Abstract: Eighty nine *Exserohilum turcicum* isolates comprising 56 Kenyan, 26 German and 7 Austrian isolates were isolated from diseased maize plants and cultured on complete liquid medium to generate mycelium for DNA extraction. DNA extraction was done following the CTAB method, DNA purified using spermidin and fingerprinting conducted using Amplified fragment length polymorphism (AFLP) procedure. NTSYSpc, pop gene and Arlequin programs were used to analyze the data and to generate the dendograms. The number of amplified bands and polymorphism varied with the different primer combinations with primer combinations E-ACA/T-CCA, E-ACA/T-CAC, E-ACA/T-CGA, E-ACA/T-CTA revealing a high (79%) level of polymorphism. Cluster analysis of the 607 polymorphic bands from these primer combinations using UPGMA algorithms generated dendograms with 7 main AFLP groups with isolates from different localities grouping together with only two outliers. Pair wise similarity matrix derived with SIMQUAL program showed a wide variation in the AFLP fingerprint of the *E. turcicum* isolates. Nei's genetic distance matrix showed that the three populations of *E. turcicum* isolates differed genotypically with the Kenyan isolates being more genetically related to Austrian isolates (genetic identity of 0.9998) whereas the isolates from Germany and Austria were more diverse (genetic identity of 0.9978). This study showed that AFLP marker is useful in the study of genetic variation of *E. turcicum* and the pathogen has a high level of genetic diversity.

Key words: Banding pattern, clustering, dendograms, genetic distance, primers

INTRODUCTION

Exserohilum turcicum the causal agent of Northern corn leaf blight has a high variability in terms of cultural, virulence and genetic traits. Single conidial isolates from the same single conidial culture differs in color, type of mycelium, rate of growth and sporulation in culture (Ingram and Williams, 1988; Mwangi, 1998). Race typing work using differentials have revealed existence of several races of the pathogen and variability has been reported even within the races (Ferguson and Carson, 2004). Isolates from different locations shows differences in parasitic fitness as indicated by infection efficiency, sporulation and lesion size (Levy, 1991).

Variability in plant pathogen populations can be studied through virulence and molecular analysis. Virulence analysis have limitations such as being less informative, requirement of genetically defined host differentials, time consuming and being laborious. The use of molecular markers involves the detection of DNA sequence polymorphism and it helps to analyze a large number of genetic loci from the genome of an organism with high levels of efficiency and sensitivity (Michelmore and Hulbert, 1987; Freymark *et al*., 1994; Madan *et al*., 1997; Weikert-Oliveira *et al*., 2002; Wagara, 2004). DNA markers such as Random Amplified Length Polymorphism (RAPD), DNA Amplification Fingerprinting (DAF) and arbitrary primed Polymerase Chain Reaction (PCR) are all based on the amplification of random genomic DNA fragments by arbitrarily selected PCR primers. Other methods such as AFLP and restriction fragment length polymorphism (RFLP) are based on the detection of genomic restriction fragments by PCR amplification (Vos *et al*., 1995; Kinyua, 2004).

The AFLP is based on selective Polymerase Chain Reaction (PCR) amplification of restriction fragments from a total digest of genomic DNA. The technique utilizes the reliability of RFLP combined with the power of PCR (Kinyua, 2004). In AFLP technique, genomic DNA is isolated and digested simultaneously with two restriction endonucleases, a six- base cutter and a four-base cutter. This generates the required substrates for ligation and

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subsequent amplification (Wagara, 2004). Selective amplification of a subset of genomic restriction fragments in a two-step amplification strategy results in consistently cleaner and more reproducible fragments. This also helps in managing the complexity of AFLP fingerprint (Kiprop, 2001). The DNA fragments are analyzed by denaturing polyacrylamide gel electrophoresis to generate fingerprints. The use of a high-resolution sequencing gel allows a large number of amplified DNA fragments to be separated for detection (Lin *et al*., 1996).

AFLP have advantage over other markers in that it is not subject to natural selection, assesses variability over a large number of independent loci thus revealing variation in many parts of the genome and gives reproducible results (Majer *et al*., 1996; Kiprop, 2001). Its main disadvantage is that, alleles are not easily recognized and this could result in an overestimation of variation (Majer *et al*., 1996). AFLP has been used to determine genetic variation between and within pathogen species such as *Cladosporium fulvum*, *Pyrenopeziza brassicae*, *Psariopsis griseola*, *Fusarium udum* (Kiprop, 2001; Kinyua, 2004; Wagara, 2004). Not much work has been done to characterize the variability of *E. turcicum* through the use of molecular techniques. The objective of the study was to evaluate the genetic variability of the pathogen populations using AFLP as a molecular marker.

MATERIALS AND METHODS

This study was carried out in the year 2007-2008 at the Department of Crop Sciences, University of Göttingen, Germany.

Collection of pathogen isolates: Infected leaf samples from maize plants were collected from different parts of Kenya, Germany and Austria and 56, 26 and 7 isolates, respectively isolated from each of this locality. Isolation was done by cutting leaf discs (1 mm^2) along advancing margins of the disease and surface sterilizing with 2.5% sodium hypochlorite for three minutes. The tissues were rinsed in 3 changes of sterile distilled water and then plated on Potato Dextrose Agar medium (potato extract 4 g, glucose 20 g, agar 15 g and water 1 L) in 9 mm diameter petri dishes. Incubation was done at room temperature (22±2°C) for 7 days and pure cultures obtained by sub-culturing onto fresh PDA medium. Monosporic cultures were obtained by preparing a dilute conidial suspension (1000 conidia per mL) and seeding this on water agar (agar 20 g, water 1 L). After 12 h of incubation at room temperature, individual conidia were located using a compound microscope at x40 and single conidium transferred onto clean PDA medium using sterile mounted needles.

Production of mycelia in shaken liquid culture: Monosporic cultures of *E. turcicum* isolates were cultured on complete medium [(Minimal medium: Solution A; 10 g $Ca(NO_3)_2.4H_2O$ in 100 mL water 10 mL, Solution B; 2 g KH_2PO_4, 2.5 g $MgSO_4.7H_2O$, 1.5 g NaCl in 100 mL of water. 10 mL, Glucose 10 g, Water 1 L, Micronutrients solution 500 μL) 1 L, yeast extract 1 g, Casein hydrolysate 1 g] by seeding with 1.0×10^6 conidial suspension and incubated at 24°C in a mechanical shaker at a speed of 120 revolutions per minute (rpm). Incubation was done for 3 days after which the mycelia were harvested by vacuum filtration using a Buchner funnel. The mycelium was dried between paper towels and freeze dried for four hours until it was completely dry. These mycelia were then put in plastic petri-dishes, para filmed and kept at 4°C and used for DNA extraction.

DNA extraction: The protocol by Moller *et al*. (1993) was used. Two hundred milligrams of freeze dried mycelia were transferred into a pre-cooled mortar and liquid nitrogen added and pulverization done using a pestle. Sterile sand was added during pulverization to promote homogenization of the pulverized mycelia. The ground mycelia was transferred into 50 mL Falcon tubes containing 10 mL of TES buffer {0.1 M Tris (pH 8.0), 10 mM EDTA, 2% SDS} supplemented with 4 mg of proteinase K (0.2 mL of a 20 mg mL stock solution). This was incubated for 45 min at 45°C while mixing the components in regular intervals of 10 min. Addition of 3.9 mL of pre-warmed (65°C) 5M NaCl was done and mixing done carefully. This was followed by addition of 1.4 mL of pre-warmed (65°C) 10% CTAB which had been dissolved in 0.7 M NaCl and mixing done carefully. Incubation was done at 65°C for 10 min after which the tubes were placed on ice allowing the components to turn cold. This was followed by addition of 10 mL of chloroform:isoamyl alcohol (24:1) and mixing done thoroughly after which incubation was done on ice for 30 min followed by centrifugation for 30 min at 5000 rpm at 4°C.

The upper phase was transferred to a new falcon tube containing 10 mL of isopropanol and mixed thoroughly. This was incubated for 30 min at room temperature followed by centrifugation for 20 min at 5000 rpm at room temperature. The supernatant was discarded taking care not to lose the pellet. The pellet was washed twice by adding 1 mL of 70% ethanol, discarding ethanol in between. The pellet was dried for 30 min using the speed -vac. To the dried pellet, 100 μL of TE buffer supplemented with 250 ng heat treated RNAse was added and the pellet re-suspended overnight. The re-suspended pellet was transferred onto 1.5 mL Eppendorf tubes and 3 μL run on 0.8% agarose gel at 1 volt per cm for 90 min using 250-500 ng Lambda Eco 911 as a standard. The

amount of the DNA for each test isolate was quantified by comparing with the standard marker. The gel was stained with 0.05% ethidium bromide and visualized under UV light. The rest of the re-suspended DNA was stored at -20°C until further use in restriction experiments.

Determination of quality and concentration of DNA: Quality and concentration of the DNA from the various *E. turcicum* isolates was determined by gel electrophoresis. DNA was resolved by electrophoresis in a 0.8% agarose gel prepared by dissolving 0.8 g of agarose in 100 mL of TBE 1xbuffer. The mixture was heated in a microwave until it dissolved completely and then cooled to about 60°C and then poured onto a horizontal gel tray fitted with a comb on one side and allowed to set for at least half an hour. The gel was placed into an electrophoresis tank and 1x TBE buffer poured until the gel was completely submerged. Total genomic DNA (2 μL) was mixed with 1 μL loading dye and 3 μL of reagent water and loaded onto the gel wells. Five hundred nanograms of Lambda Eco 911 were loaded onto one of the wells to serve as a ladder or marker. The gel was run at 60 volts for 90 min and then stained in 0.05% ethidium bromide bath for 10 min. The gel was destained for 10 min in demineralized water then illuminated with uv light and photographed. The intensity of the DNA bands of the samples were compared with the intensity of λ DNA and concentration of DNA determined in nanograms. Clear distinct bands were indications of high quality genomic DNA whereas smears showed degraded or sheared DNA.

DNA purification using spermidin: Addition of 0.1 volume of spermidin trihydrochloride to the DNA sample was done and incubation done for 10 min at room temperature. This was centrifuged for 60 sec at 14000 rpm and the pellet obtained washed twice with 500 μL of 70% ethanol. One volume of exchange buffer (0.3 M Na-Acetate, 10 mM Mg-Acetate) was added before incubation at 50°C to dissolve the pellet. Three volumes of 100% ethanol was added and incubation done for 3 min at room temperature. Centrifugation was done for 10 min at 14000 rpm. The pellet was washed twice in 70% ethanol and then re-suspended on TE buffer. The DNA was run on 0.8% agarose gel to check for quality and determine the concentration.

AFLP analysis of *E. turcicum* isolates

Restriction digestion of genomic DNA: Total genomic DNA (750 ng) was digested with restriction enzymes EcoR I and Tru II in 30 μL final volume. The components of restriction digestion were prepared by adding 4 μL of 4x reaction buffer, 750 ng of sample DNA in 15 μL TE buffer, 2 μL of Tru II, 0.5 μL of EcoR I and 1 μL of casein and topped with distilled water to 30 μL. In the first restriction reaction, the components without enzyme Tru II were incubated at 37°C for 90 min and then Tru II added and incubation done at 65°C for 16 h. After the incubation, 10 μL was transferred into a new tube and stored in a freezer. For the rest of the 20 μL, the reaction was stopped by adding 2 μL of gel loading buffer and completeness of digestion confirmed by running the restriction products on 0.8% gel at 60 volts for 180 min.

Ligation of adapters: The restriction enzymes after restriction reaction were inactivated by incubating at 96°C for 10 min. The restriction fragments were ligated to EcoR I and Tru II adapters to generate template DNA for amplification. The adapter ligation solution (10 μL) and 1 μL T4 DNA ligase were added to the digested DNA mixed gently at room temperature and incubated for 2 h at 20°C. The DNA Ligase enzyme was deactivated by incubating at 65°C for 10 min followed by a 1:10 dilution of the ligated mixture with TE buffer.

Pre-amplification reactions of ligated DNA: The template DNA was amplified with a Tru II primer and an EcorR I primer each containing one selective nucleotide. The following were added into a 0.5 mL microcentrifuge tube: 2 μL of ligated DNA, 1 μL of 10x PCR buffer with 1 μL of both primers, 0.5 μL of dNTP, 0.6 μL of Magnesium chloride, 0.05 μL of BSA, 1 μL of Taq polymerase (5 units μL^{-1}) and 4.4 μL of distilled water. The contents were gently mixed and briefly centrifuged and DNA amplified through 20 PCR cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min in a Biometra thermocycler. A 1:10 dilution of the PCR products was done using TE buffer.

Selective amplification of pre-amplified DNA: The pre-amplification products were diluted at the rate of 1:10 using TE buffer and used as templates for selective amplification. The EcoR I primers were Cy5 labelled and TruII primers containing two selective nucleotide were used in the selective amplification. Selective amplification was performed using 2 μL of the pre-amplified diluted template DNA, 1 μL of 10x reaction buffer, 0.15 μL of labeled EcoR I primer, 1 μL of dNTPs, 0.6 μL of $MgCl_2$, 0.05 μL of BSA,1.7 μL of unlabelled Tru II primer, 0.15 μL of Taq polymerase and 4.05 μL of distilled water. The components were mixed gently and the DNA amplified in a 35 cycle touchdown PCR. The PCR amplification temperature profile was 12 cycles at 94°C for 30 sec, 65°C, for 30 sec and 72°C for 1 min decreasing the annealing

temperatures for each of the 11 successive cycles at 0.7°C. The last 23 cycles were performed at 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min. The primer combinations used were E-ACA/T-CCA, E-ACA/T-CAC, E-ACA/T-CGA, E-ACA/T-CTA (Table 1). The primer combinations that had revealed a high number of bands after polyacrylamide gel electrophoresis were selected.

Electrophoresis of PCR products using capillary sequencer: In sample preparation, 2 μL of Cy5-labelled PCR products, 5 μL of Dy680-labelled PCR products, 5 μL of Dy750 labelled PCR products, 1 μL of IRD800 labelled size Standard 600 (Beckman Coulter Ltd) were put in each well and sample loading solution (Beckman Coulter Ltd.) topped up to 30 μL per sample plate slot. Approximately 10 μL of mineral oil was added per well and run on capillary sequencer. The running parameters were; denatured linear polyacrylamide capillary electrophoresis, running temperature of 50°C, denaturation step at 90° for 120 sec. Sample injection was at 2.0 kV, 30 sec separation: 4.8 kV, 60 min.

Analysis of AFLP fingerprints: Bands were assigned a number in relation to their migration distance within the gel. Only strong and reproducible bands were scored and bands with the highest molecular weight were assigned number one and so on until the band with the lowest molecular weight was assigned. It was assumed that, the bands of the same molecular weight in different individuals were identical in sequence. For each individual, the presence or absence of each band was determined and designated 1 if present or 0 if absent in order to obtain binary banding data. Similarity matrices from binary banding data of each of the five primer combinations were derived with the Similarity for Qualitative Data Program (SIMQUAL) in the Numerical Taxonomy and Multivariate analysis System for personal computer (NTSYS-pc) version 2.0. Estimates for similarity were based on Jaccards coefficient, Nei and Li's similarity index and simple matching coefficient. Matrices of similarity were analysed using UPGMA (unweighted pair group method with arithmetic averages) clustering method. Dendograms were generated with the tree option of NTSYS-pc and goodness of fit calculated using COPH and MXCOPH programs.

Table 1: Primer sequence used in pre-amplification and amplification

Primer	Sequence (5′-3′)
Cy5 (E_ACA	(Cy5) GACTGCGTACCAATTCACA
T_C	GATGAGTCCTGAGTAAC
T-CCA	GATGAGTCCTGAGTAACCA
T_CAC	GATGAGTCCTGAGTAACAC
T_CGA	GATGAGTCCTGAGTAACGA
T_CTA	GATGAGTCCTGAGTAACTA
T-CCC	GATGAGTCCTGAGTAACCC

RESULTS

Extraction of DNA: The incubation period of fungal liquid cultures beyond four days yielded high amount of mycelia biomass but it was not possible to fully restrict the DNA in the AFLP restriction step. The amount of mycelium harvested from the 3 day-old cultures from complete medium ranged from 250 to 2000 μg and the 200 μg of mycelium used for the DNA extraction yielded between 20 and 250 DNA μL^{-1}. The use of undigested Lambda Eco 911 as a standard provided a good estimate of the concentration of DNA present in the various *E. turcicum* isolates. DNA purification by use of spermidin enhanced the restriction of DNA.

AFLP analysis: A high level of polymorphism was revealed by the four primer combinations namely E-ACA/T-CCA, E-ACA/T-CAC, E-ACA/T-CGA, E-ACA/T-CTA used to evaluate the *E. turcicum* isolates. The first primer combination had a total of 197 bands amplified whereas the other three primer combinations had a total of 255 bands each amplified. A total of 765 bands were amplified by the three primer combinations (E-ACA/T-CAC, E-ACA/T-CGA, E-ACA/T-CTA) and 607 (79.35%) were polymorphic (Table 2). Primer combination E-ACA/T-CGA revealed the highest level of polymorphism (83.92%) whereas primer combination (E-ACA/T-CAC) had the lowest level of polymorphism. Dendogram derived from three primer combinations was used to classify the 86 *E. turcicum* isolates into nine AFLP groups (Table 3). AFLP group I comprised of 12

Table 2: Primers, amplified bands and polymorphic bands from AFLP analysis of 86 isolates of *Exserohilum turcicum*

Primer combination (EcoR I/Tru II)	No. of isolates with bands	No. of bands	Polymorphic bands	Polymorphism (%)
E-ACA/T-CAC	86	255	191	74.9
E-ACA/T-CGA	86	255	214	83.92
E-ACA/T-CTA	84	255	202	79.22
Total		765	607	79.35

Table 3: AFLP groups of *Exserohilum turcicum* isolates obtained using dendograms of three primer combinations

AFLP group	No. of isolates	*E. turcicum* isolates
I	12	G17, RZ 250, G41, G29, G2, S55, G16, G31, S64, RACE 0, G30, G11
II	19	ENG4, G48, ENG2, S53, S1, PHANTOM, G9, ETDZ, G35, G10, G40, S2, G18, MMSZ, ORT, KGMGCZ, G44, G5, G51
III	11	G13, G46, G4, G14, SORTE 5, SORTE 3, SORTE 2, P5, S62, G34, P6,
IV	25	B5, G8, B1, ENG11, B3, LUGAN, SORTE 1A, G42, G26, G21, S31, G15, SORTE 4, G19, G49, G47, G45, G50, G33, ENG5, B4, G12, G32, G24
V	10	G20, G43, G25, G1, MMCZ, ENG8, G22, BENICIA, G38, ENG7
VI	3	G36, RZ230, RZ MAESTRO
VII	3	G27, DELITOP, G39
VIII	1	G3
IX	1	B2

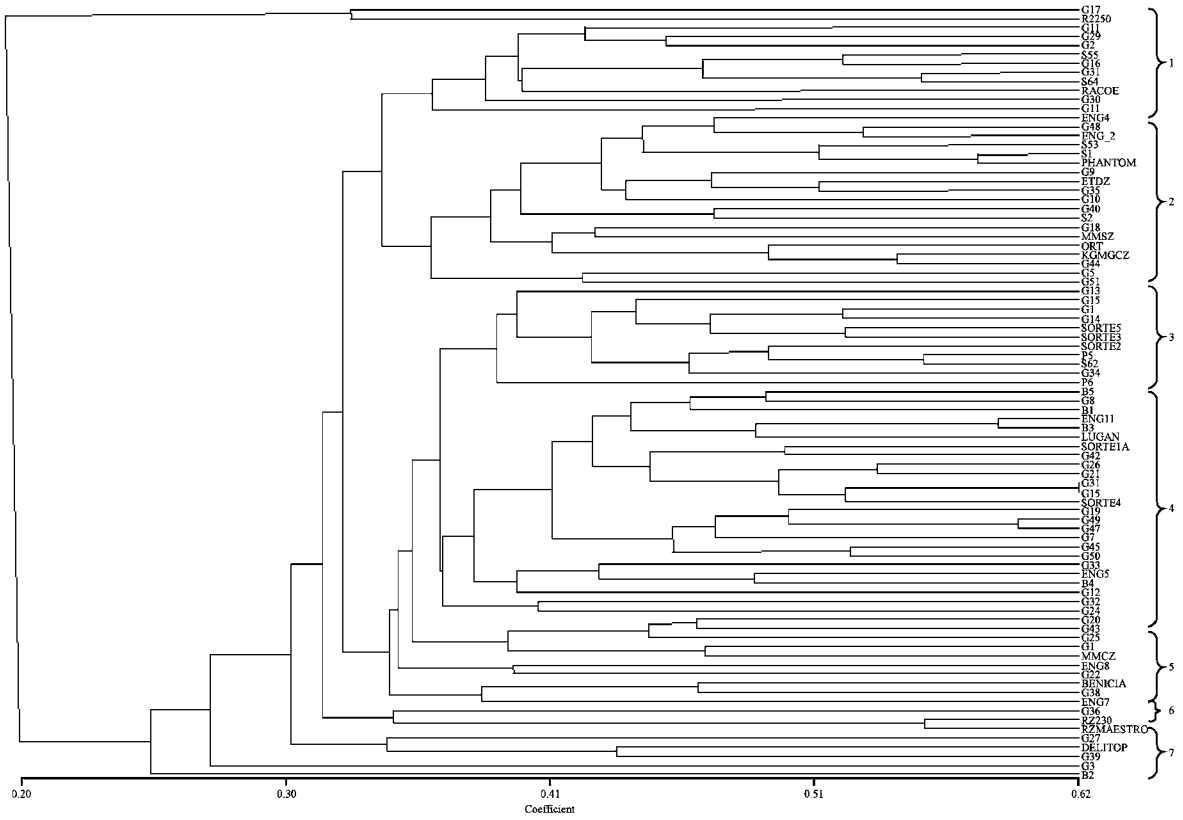

Fig. 1: Dendogram derived from AFLP analysis of *Exserohilum turcicum* isolates from three primer combinations (E-ACA/T-CAC, E-ACA/T-CGA, E-ACA/T-CTA) using NTSYSpc 2.1 Program

isolates whereas AFLP groups II to VII had 19, 11, 25, 10, 3 and 3 isolates respectively. AFLP group VIII and IX had one isolate each. Apart from AFLP group VII and IX where each of the group was represented by one isolate each, all the other groups comprised of isolates from the different countries. Likewise, isolates from the different districts and AEZs were represented in the major AFLP groups indicating that the isolates were genetically related irrespective of the geographical area of origin. AFLP group 4 which had the majority (29%) of all the isolates, comprised of 56% of Kenyan isolates, 36 and 8% of German and Austrian isolates respectively. It was only in the AFLP groups 5 and 6 where the Austrian isolates were not represented though these two groups had few (3) isolates each.

Cluster analysis of the 607 polymorphic bands of primer combinations (E-ACA/T-CAC, E-ACA/T-CGA, E-ACA/T-CTA) using UPGMA algorithms generated dendograms with seven main clusters (Fig. 1). Dendograms from the three primer combinations generated 7 main clusters whereas dendograms obtained from binary data of primer combination E-ACA/T-CGA and E-ACA/T-CTA generated 6 clusters for each. Pair wise similarity matrix) derived with SIMQUAL program of NTSSYS-pc Software showed a wide variation in the AFLP fingerprint of the *E. turcicum* isolates. Although there was a pattern of clustering among some isolates in the different clusters, isolates from the different countries generally scattered across the dendograms (Fig. 1) Cluster I isolates were similar at 36.6%, cluster 2 isolates were similar at 39.6%, cluster 3 were similar at 40%, cluster 4 were similar at 41%, cluster 5 were similar at 40%, cluster 6 were similar at 34.5%, cluster 7 were similar at 34%, while cluster 8 and 9 were represented by single isolates each.

Analysis of the binary banding data from all the 4 primer combinations using bootstrap value of 1000 showed that the 86 *E. turcicum* isolates were genetically related as shown by Neis gene diversity index values. However, among the clusters there were isolates that were more closely related than others (Table 4).

Genetic differentiation among the *E. turcicum* populations: There was considerable genetic diversity among the *E. turcicum* isolates from Kenya, Germany and Austria. A dendogram constructed with the UPGMA method based on Neis genetic distance matrix showed that the three populations of *E. turcicum* isolates differed

genotypically with the Kenyan isolates being more genetically related to Austrian isolates (Fig. 2, Table 5) whereas the isolates from German and Austrian were more diverse.

Isolates from Kiambu and Machakos and Kiambu and Uasin Gishu on the other hand were genetically similar (I = 1.00) whereas isolates from Embu and Trans zoia were more diverse (D = 0.0082). Isolates from Kirinyaga and Uasin Gishu and isolates between Machakos and Nakuru were very close with genetic identities of 0.9986 and 0.9998, respectively (Table 6, Fig. 3).

A dendogram based on Nei's unbiased genetic distance matrix showed that the seven populations based on the districts of origin clustered into three major groups with group 1 comprising of isolates from Transzoia, group 2 comprising of isolates from Uasin Gishu, Kiambu, Nakuru, Machakos and Kirinyaga districts whereas group 3 comprised of isolates from Embu district (Fig. 3).

Comparisons of isolates in different AEZs: The *E. turcicum* populations from the various AEZs showed a very high genetic identity. Isolates from LH3 and UM2, UH3 and UM2, UH3 and UM4 and UH3 and LH3 were genetically identical (I = 1.00). This was further reinforced by the low diversity values that were recorded for all the isolates between the different AEZs (Table 7). A dendogram based on Nei's unbiased genetic distance matrix showed that the eight populations based on AEZs clustered into three groups (Fig. 4). Group one comprised of isolates from AEZs UM2, LH3, LH1, UM4 and LH2, group two comprised isolates from AEZ UH3 and group three comprised of isolates from AEZ UM1.

Table 4: Number of polymorphic loci, mean of gene diversity (H) and genetic diversity (Ho) of Kenyan, German and Austrian isolates

Population	No. of polymorphic loci	Polymorphism (%)	Gene diversity (H)	Genetic diversity (Ho)
Kenyan	561	73.33	0.0901	0.1610
German	492	4.31	0.1021	0.1774
Austrian	198	25.88	0.0623	0.1012

Table 5: Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal) for three populations of *E. turcicum* isolates based on Nei (1987)

Populations	Kenyan	German	Austrian
Kenyan	***	0.9987	0.9998
German	0.0013	***	0.9978
Austrian	0.0002	0.0022	***

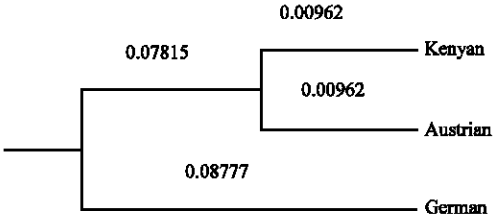

Fig. 2: Dendogram based on Nei's genetic distance between three populations of *E. turcicum* derived from NEIGHBOURprocedure of PHYLIP version 3.5

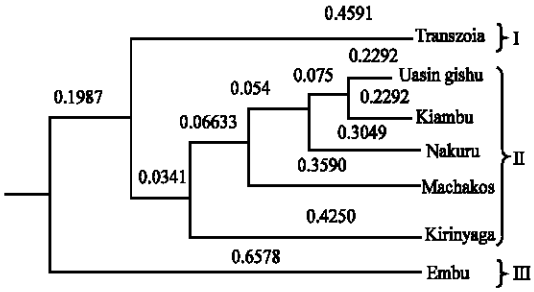

Fig. 3: Dendogram based on Nei's genetic distance between seven populations of *E. turcicum* derived from NEIGHBOUR procedure of PHYLIP version 3.5

Table 6: Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal) for *E. turcicum* isolates from different districts in Kenya based on Nei (1987)

Districts	Trans Zoia	Uasin Gishu	Kiambu	Nakuru	Kirinyaga	Machakos	Embu
Trans Zoia	****	0.9986	0.9973	0.9960	0.9983	0.9967	0.9918
Uasin Gishu	0.0014	*****	1.0000	1.0003	0.9998	1.0004	0.9944
Kiambu	0.0027	-0.0011	****	0.9987	0.9987	1.0000	0.9957
Nakuru	0.0040	-0.0003	0.0013	****	0.9978	0.9998	0.9938
Kirinyaga	0.0017	0.0002	0.0013	0.0022	****	0.9993	0.9953
Machakos	0.0033	-0.0004	-0.0003	0.0002	0.0007	****	0.9970
Embu	0.0082	0.0056	0.0043	0.0062	0.0047	0.0030	****

Table 7: Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal) for *E. turcicum* isolates from different AEZs in Kenya based on Nei (1987)

	UM2	LH1	UM4	LH3	UM3	LH2	UH3	UM1
UM2	****	0.9992	0.9998	1.0007	1.0010	0.9974	0.9868	0.9871
LH1	0.0008	****	0.9996	0.9971	0.9995	0.9976	0.9896	0.9830
UM4	0.0002	0.0004	****	0.9986	1.0007	0.9989	0.9877	0.9873
LH3	-0.0007	0.0030	0.0014	****	1.0008	0.9980	0.9816	0.9834
UM3	-0.0010	0.0005	-0.0007	-0.0008	****	0.9993	0.9873	0.9853
LH2	0.0026	0.0024	0.0011	0.0020	0.0007	****	0.9849	0.9887
UH3	0.0132	0.0105	0.0123	0.0185	0.0127	0.0152	****	0.9736
UM1	0.0130	0.0172	0.0128	0.0167	0.0148	0.0113	0.0268	****

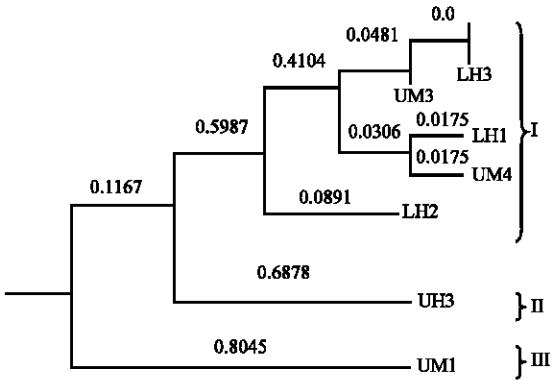

Fig. 4: Dendogram based on Nei's genetic distance between *Exserohilum turcicum* isolates from different AEZs derived from NEIGHBOUR procedure of PHYLIP version 3.5

DISCUSSION

The complete medium was found to be ideal for culturing *E. turcicum* isolates for DNA extraction as compared to potato dextrose broth. Similar findings were reported by Yoder (1988), who reported that growing *E. turcicum* isolates should not exceed three days to avoid accumulation of polysaccharides that interferes with restriction reactions. The CTAB method was used successfully to extract DNA from *E. turcicum* isolates and this method has been used to extract DNA from other fungi (Moller *et al*., 1993). Addition of casein during the DNA restriction reaction was found beneficial as it helped to ensure complete digestion of DNA by restriction enzymes. This has also been reported by Boom *et al.* (1999) and casein has been found to enhance restriction enzyme activity. The DNA purification procedures also helped to improve the restriction digestion of DNA during the AFLP analysis and similar findings have been reported by Arun *et al.* (2002). The presence of RNA in the extracted DNA as seen in the agarose gels necessitated the addition of RNAse (20 mg mL^{-1}) in order to degrade the RNA. Addition of RNAse to degrade RNA is a common procedure since the presence of RNA interferes with subsequent PCR and restriction reactions.

AFLP technique has been reported to be more efficient in rapid generation of genotype data for large number of individuals including fungi (Majer *et al*., 1996). The number of amplified bands and polymorphism varied with the individual primer combinations with some showing high level of polymorphism and others revealing low levels of polymorphism. This is consistent with findings by Majer *et al.* (1996), who reported that the level of polymorphism varies with the primer combinations in AFLP analysis. The number of fragments amplified in the AFLP analysis depends on composition of C and G in the selective nucleotide. The more the Cs and Gs used as selective nucleotide the less the amplification process and the fewer the DNA fragments amplified. In this study, two selective nucleotides at the 3' end of both primers were found to be appropriate in generating suitable AFLP fingerprints. Vos *et al.* (1995) also reported that primer selectivity is good for primers with one or two selective nucleotides in simple genomes such as in fungi.

The AFLP markers that were used in this study were able to distinguish isolates from the same localities into different genotypic groups implying that the technique has a superior discriminative power to differentiate related fungal species. Jansen *et al.* (1996) demonstrated that the AFLP markers are capable of differentiating highly related strains of Xanthomonas. This study confirmed that differences in *E. turcicum* biotypes exists at molecular level and AFLP is a useful tool in rapid and efficient characterization of the pathogen.

Considering that *E. turcicum* reproduces asexually, the source of the high genetic diversity reported in this study can be attributed to mutation and migration. This variation is driven by forces of selection and genetic drift further amplifying the genetic variation as is also reported by Zeigler *et al.* (1995). The cultivation of different maize varieties may have led to a higher selection pressure due to the host pathogen interaction and environmental constraints resulting in increased diversity in the populations structure of the pathogen. The study identified AFLP groups based on molecular profiles and in screening for resistance, these groups should be represented to obtain germplasm with a broad spectrum of resistance.

CONCLUSIONS

Presence of metabolites that interfere with DNA isolation procedures and downstream applications such as DNA restriction amplification and cloning necessitates the need to purify the DNA to make it more amenable to PCR amplification and restriction digestion. This study showed that *E. turcicum* has a high level of genetic diversity and AFLP markers are useful in the study of genetic variation of *E. turcicum* isolates. This diversity exists among the various *E. turcicum* isolates regardless of their geographical locality. Despite the geographical separation between isolates obtained from Kenya, Germany and Austria, there were a lot of similarities in terms of groupings evidenced by the clusters obtained using NTSYSpc 2.1 Program. The high genetic diversity

has an implication in that, breeding programmes should target lines with a broad genetic base to obtain durable resistance.

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