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Use of Repetitive Extragenic Palindromic (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC) and BOX sequences to fingerprint *Exserohilum turcicum* isolates

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ABSTRACT

Objective: To examine the distribution of dispersed repetitive DNA (Enterobacterial Repetitive Intergenic Consensus (ERIC), BOX, and Repetitive Extragenic Palindromic (REP) sequences in the genome of *Exserohilum turcicum* using conserved primers.

Methodology and results: DNA amplification was done using standard PCR procedures and the resultant PCR products analyzed by agarose and polyacrylamide gel electrophoresis. The different markers produced characteristic banding patterns and the similarity matrices from binary banding data was derived with the similarity for qualitative data program (SIMQUAL). Cluster analysis showed close genetic similarities between Kenyan, German and Austrian isolates of *E. turcicum*. The average gene diversity from BOX sequences was 0.28 whereas for ERIC and REP sequences, it was 0.14 and 0.05, respectively.

Conclusion and application of findings: The study showed that discrimination of closely related strains can be achieved using REP, BOX and ERIC PCR fingerprinting. We recommend these markers as a rapid tool for the genetic characterization of large populations of *E. turcicum*.

INTRODUCTION

Northern leaf blight of maize caused by *Exserohilum turcicum* is a limit to corn production globally (Agrios, 2005) especially in areas characterized by moderate (20^0C-28^0C) (Cuq *et al*., 1993). Losses of up to 70% have been reported when heavy infection occurs before tasseling since this causes extensive defoliation, which affects the grain filling process (Carson, 1995). Besides yield losses, the disease reduces sugar content and the germinative capacity of maize seeds (Cardwell *et al*., 1997), and predisposes the crop to stalk rots (Gowda *et al*., 1992).

E. turcicum has high variability in terms of cultural, virulence and genetic characteristics (Yeshitila, 2003). Variability has been reported within the nine races with electrophoretic isozyme phenotypes having been identified (Ferguson & Carson, 2004). Molecular tools such as RFLP and RAPD have been used to characterize *E. turcicum* isolates and these have targeted internal transcribed spacer (ITS) region of the rDNA (Rita *et al*., 2002). Apart from these two fingerprinting techniques, other molecular tools such as amplified fragment length polymorphism involving the use of PCR have been applied in other

organisms such as *Fusarium*, *Colletotrichum* to discriminate genetic variation between strains. Families of short intergenic repeated sequences have been reported in some prokaryotes and these sequences contain highly conserved central inverted repeats (Bruijn, 1992). Repetitive DNA sequences is a common feature of all eukaryotic species and these have been exploited in designing PCR primer pairs to amplify inter-repeat sequences to produce species or strain specific gel electrophoresis patterns (Edel *et al*., 1995). For example, this approach has been used to differentiate *Aspergillus* species. These repeated motifs comprise of enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic elements also known as palindromic units and the BOX element (George *et al*., 1997, Versalovic *et al*., 1991).

The consensus DNA sequences in the conserved inverted repeats of REP, BOX and ERIC type elements have been used to design specific oligonucleotide primers that can be used to probe the genomes of bacteria and other organisms (Bruijn, 1992). When subjected to PCR, these sequences produce characteristic patterns after separation on agarose gels, and thus can constitute a method to fingerprint genomes. DNA fingerprinting technique is central to analysis of genetic diversity and population structure of *E. turcicum* and can provide information needed for rational deployment of resistant genes. Resistant varieties based on Ht resistance genes exist but their expression is highly influenced by environmental factors and hence use of molecular techniques to map and deploy resistance will be more reliable. Other molecular techniques such as amplified fragment length polymorphism are labour and cost intensive and thus there is need for an alternative technique with high discriminative power but simple in application.

MATERIALS AND METHODS

Fungal strains: The *E. turcicum* isolates were isolated from naturally diseased corn plants obtained from Kenya, Germany and Austria between months of March and May 2006. Collection of the samples was done from farms located in different agro ecological zones to capture more pathogenic variability as isolates from different localities are known to be more variable due to evolutionally trends. The varieties and other cultural practices were noted. Isolation of monosporic cultures was done on PDA medium, for mycelia production, the monosporic cultures were grown on complete medium and incubated in a mechanical shaker at 120 rpm (24 ^{0}C) for 4 days. The mycelia were harvested by vacuum filtration using Buchner funnel.

DNA extraction: The mycelia were freeze dried and ground in liquid nitrogen. The ground mycelia were transferred to falcon tubes containing 10ml of TES buffer {0.1 M Tris (pH 8.0), 10mM EDTA, 2% SDS} supplemented with 4mg of proteinase K (0.2 ml of a 20mg/ml stock solution). This was incubated for 45 min at 45^{0}C while mixing the components at regular intervals of 10 min. A volume of 3.9 ml of pre-warmed (65 ^{0}C) 5M NaCl was added and mixed carefully, followed by addition of 1.4 ml of pre-warmed (65^{0}C) 10% CTAB which had been dissolved in 0.7 M NaCl, and mixed carefully. The mixture was incubated at 65^{0}C for 10 minutes after which the tubes were placed on ice to cool. This was followed by addition of 10 ml of chloroform:isoamyl alcohol (24:1) and mixing thoroughly, then incubated on ice for 30 minutes followed by centrifugation for 30 minutes at 5000 rpm at 4 ^{0}C.

The upper phase was transferred to a new falcon tube containing 10 ml of isopropanol and mixed thoroughly. This was incubated for 30 minutes at room temperature followed by centrifugation for 20 minutes at 5000 rpm at room temperature 22±2^{0}C. 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 minutes using a speed-vac. To the dried pellet, 100 µl of TE buffer supplemented with 250ng 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 minutes using 250-500ng Lambda *Eco* 911 as a standard. The amount of DNA of each test isolate was quantified by comparing with the standard. 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^{0}c until further use in the PCR reactions.

PCR procedures: For ERIC the two oligonucleotide primer pairs used for PCR amplification had 22 nucleotides in length each and they were obtained from University of Goettingen, Germany. The sequences were:

Primer 1; 5' – ATG TAA GCT CCT GGG GAT TCA C - 3'

Primer 2; 5' –AAG TAA GTG ACT GGG GTG AGC G - 3'.

Reactions were carried out in a 20µl volume containing 100ng of DNA, 2µl of 1x Taq- buffer, 0.25mM each of dATP, dTTP, dGTP and dCTP, 1.5mM $MgCl_2$, 20 pmol each of primers and 1unit of Taq polymerase. A thin layer of paraffin oil was added to prevent evaporation. The DNA amplification was done in a thermal cycler (Biometra T-Gradient, Goettingen, Germany) using the following PCR cycles; The first denaturation step of 7 minutes at 94 ^{0}C, followed by 36 cycles of denaturation (1 min at 96 ^{0}C, annealing (1 min at 52 ^{0}C) and final extension for 3 min at 65 ^{0}C. Amplifications were performed twice to confirm consistency of the method. Two microlitre aliquots of PCR products were analysed by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and photographed over UV transilluminator.

For the REP PCR, the PCR cycle comprised of the first denaturation step of 7 minutes at 94^{0}C, followed by 30 cycles of denaturation (1 min at 94 ^{0}C), annealing 1 minute at 44 ^{0}C and final extension for 8 minutes at 65 ^{0}C. The PCR cycle for Box PCR were similar to those of REP PCR.

Electrophoresis of PCR products: The PCR products were denatured for 2-3 minutes at 85^{0}C and loaded onto 7% polyacrylamide gel ReproGel™ LongRead (Amersham Pharamacia Biotech, Uppsala, Sweden) in an ALFexpress II DNA analyzer. The set up was prepared as originally described by Vos *et al*.,1995 and modified by Laurentin & Karlovsky, 2006.

Analysis of BOX, REP and ERIC 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. 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 (Rohlf, 1993). Estimates for similarity were based on Jaccards 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.

RESULTS

DNA extraction from fungal strains: The amount of mycelia harvested from the 3 day old cultures from complete medium ranged from 250 to 2000ng with DNA yields ranging from 20 and 250ng DNA/µl. There was no correlation of the amount of mycelia and DNA with origin of isolates. 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. The isolates and their origin are as shown in Table 1.

ERIC, REP and BOX fingerprinting: Distinct banding patterns were generated with each fingerprinting technique using the respective oligonucleotide primers and the different PCR conditions. The amplified bands in ERIC ranged in length from 250 to 2500bp whereas for the REP and BOX, they ranged from 150 to 3000bp and 250 - 2000bp, respectively. Reproducible fingerprint profiles were generated with each technique upon repetition of the procedures. The number and intensity of amplified bands varied with each fingerprint technique, with ERIC consistently revealing more intense and more polymorphic bands. Most of the *E. turcicum* isolates showed amplified bands ranging from 8 to 11.

In general, isolates characterized using the three molecular techniques had comparable number of bands with some degree of polymorphism. Clustering using ERIC primers generated 3 main clusters at 64% similarity level, with one outlier (Figure 1). The first cluster for ERIC fingerprinting had 2 German isolates out of the six isolates. Cluster analysis of the isolates using REP primers showed 3 main clusters at 81% similarity level (Figure 2). Clustering using BOX primers generated three main clusters at 85% level of similarity

(Figure 3). Cluster analysis of the PCR banding patterns of the isolates from Kenya, Germany Austria showed close correspondence between the groupings based on ERIC, REP and BOX fingerprint techniques. Using the BOX primers, the percentage of polymorphic loci was 81.82% whereas with REP and ERIC primers it was 11.11 and 63.64%, respectively. The average gene diversity and Shannon's information index when using BOX, ERIC and REP primers using Nei's unbiased measure of genetic identity and genetic distance are as shown in Table 2.

Table 1: *Exserohilum turcicum* isolates used in the various DNA fingerprinting techniques

Isolates	**Country of origin**	***E. turcicum* isolates used in the various PCRs**		
		BOX	**REP**	**ERIC**
ENG 11	Germany	×	×	×
ENG 1	Germany	×	×	×
ENG 6	Germany	×	×	×
B2	Germany	×	-	×
ENG 9	Germany	×	-	×
PHANTOM	Germany	×	-	-
G40	Kenya	×	×	×
G36	Kenya	×	×	×
ETDZ	Kenya	×	×	×
G41	Kenya	×	-	×
G50	Kenya	×	-	×
G51	Kenya	×	-	×
G43	Kenya	×	-	×
S1	Austria	×	-	-
SORTE 2	Austria	×	×	×
S64	Austria	×	-	×
SORTE 3	Austria	×	×	×

Key: × = Isolate included and amplified; - = Isolate included and failed to amplify

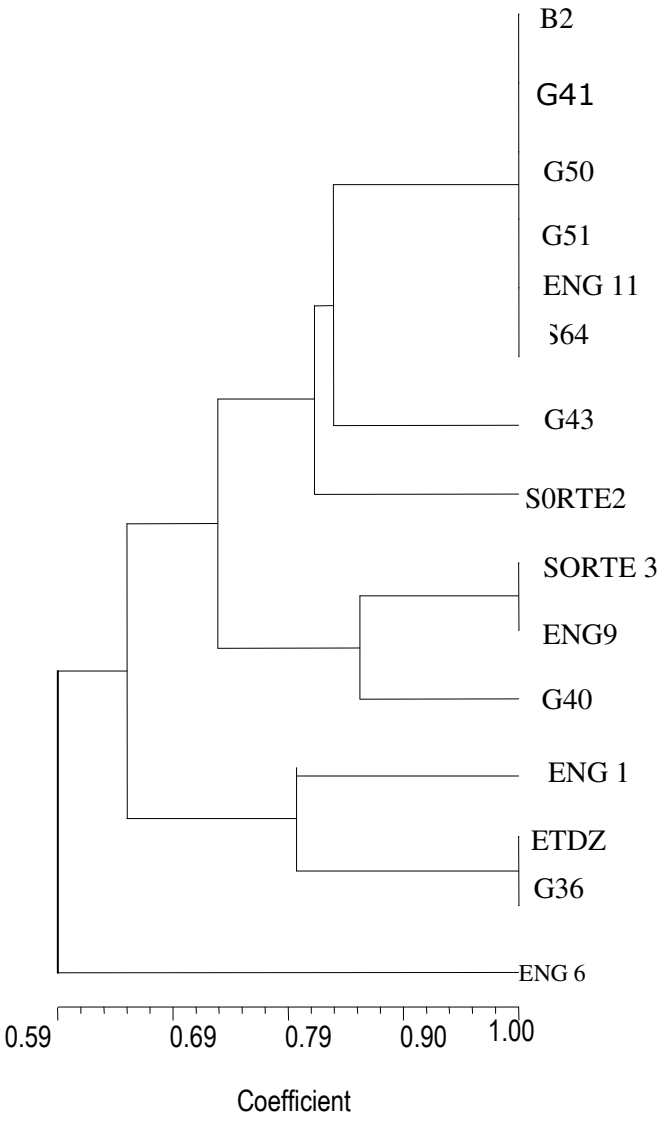

Figure 1: Dendogram based on Nei's genetic distance of *Exserohilum turcicum* populations from Kenya, Germany and Austria derived with NEIGHBOURprocedure of PHYLIP version 3.5 using ERIC primers.

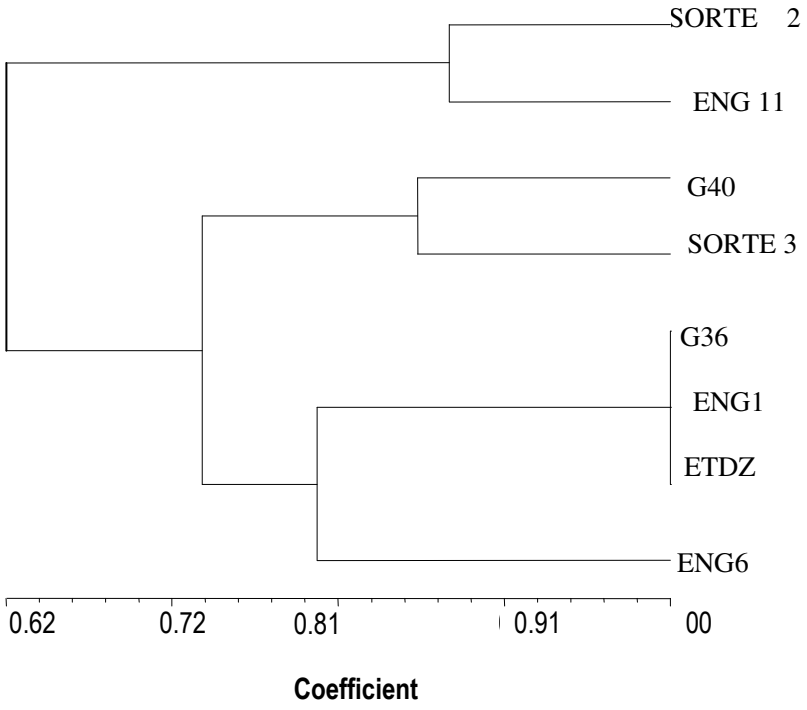

Figure 2: Dendogram based on Nei's genetic distance of *Exserohilum turcicum* populations from from Kenya, Germany and Austria derived with NEIGHBOUR procedure of PHYLIP version 3.5 using REP primers.

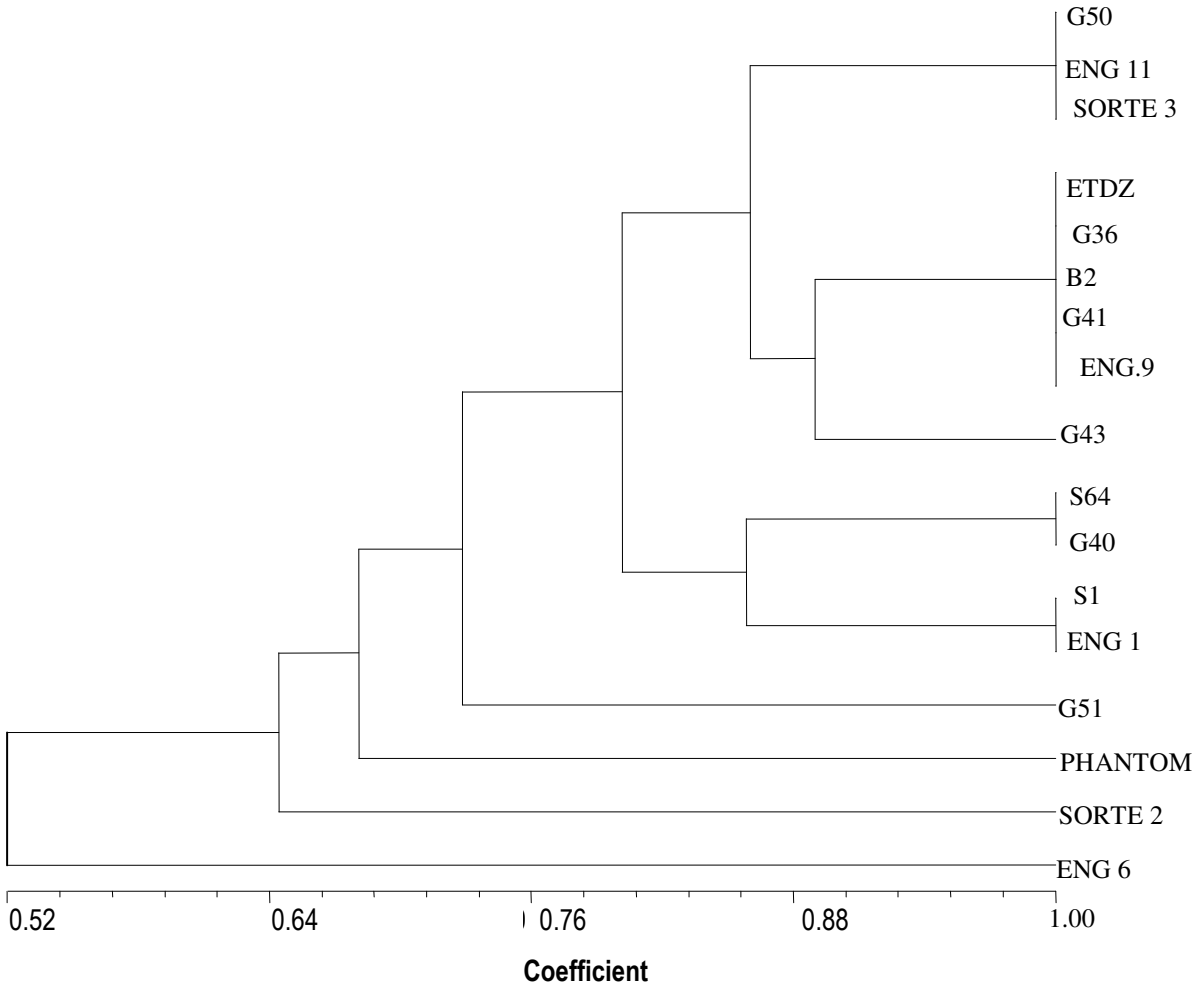

Figure 3: Dendogram based on Nei's genetic distance of *Exserohilum turcicum* populations from Kenya, Germany and Austria derived with NEIGHBOURprocedure of PHYLIdP version 3.5 using BOX primers

Table 2: Percentage polymorphic loci, average gene diversity and Shanon's information index from the three fingerprinting techniques

	% polymorphic loci	Gene diversity index	Shanon's information index
ERIC	63.64	0.140	0.235
REP	11.11	0.046	0.067
BOX	81.82	0.281	0.416

Box primers showed that isolates from Kenya and Austria were more genetically related compared to isolates from Kenya and Germany or those from Austria and Germany (Table 3 and Figure 4). Isolates from Germany and Austria were more genetically diverse with a genetic distance of 0.1192.

Table 3: Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal) for three populations of *Exserohilum turcicum* isolates based on Nei (1987) using BOX primers

	Kenyan isolates	German isolates	Austrian isolates
Kenyan isolates	********	0.9305	0.9437
German isolates	0.072	********	0.8876
Austrian isolates	0.058	0.1192	*********

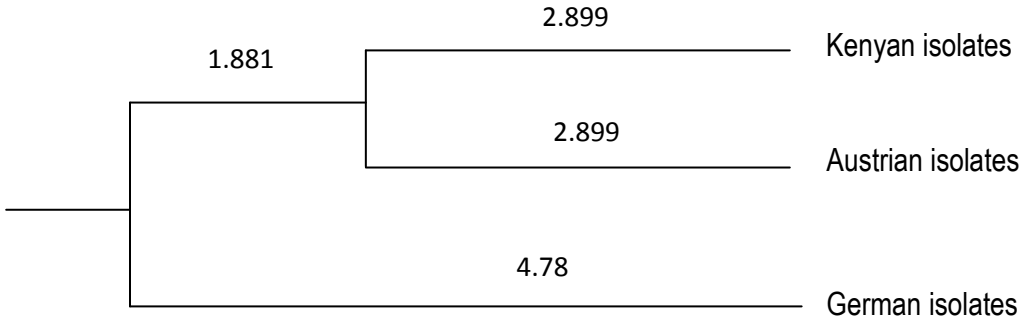

Figure 4: Dendogram based on Nei's genetic distance of three populations of *Exserohilum turcicum* derived from NEIGHBOURprocedure of PHYLIP version 3.5 using BOX primers.

The values for the genetic identity of *E. turcicum* isolates from the three countries using ERIC primers were very close indicating a high level of genetic relatedness among the pathogen populations. This was corroborated by the equally low genetic distances (Table 4 and Fig 5).

Table 4: Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal) for three populations of *Exserohilum turcicum* isolates based on Nei (1987) using ERIC primers

	Kenyan isolates	German isolates	Austrian isolates
Kenyan isolates	********	0.9957	0.9787
German isolates	0.0043	********	0.9724
Austrian isolates	0.0215	0.0280	*********

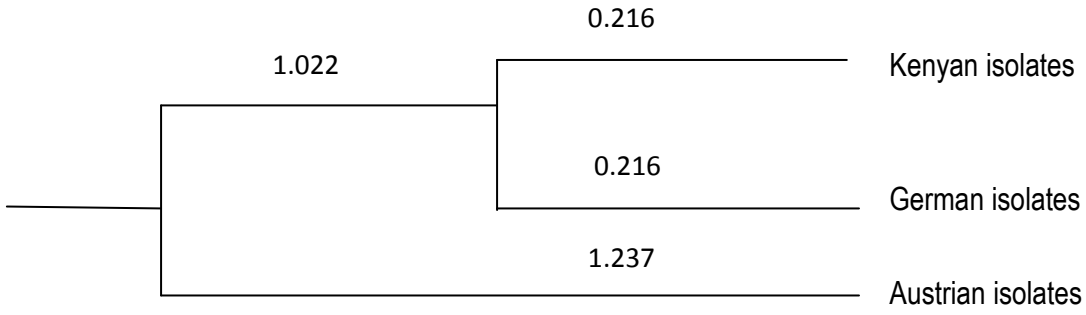

Figure 5: Dendogram based on Nei's genetic distance of three populations *Exserohilum turcicum* derived from NEIGHBOURprocedure of PHYLIP version 3.5 using ERIC primers

The grouping and clustering using REP fingerprints showed that Kenyan and Austrian isolates were very closely related with genetic identity of 0.9959 compared to Kenyan - German and German - Austrian isolates. Conversely, the genetic distances between Kenyan and Austrian isolates was lowest being 0.0041 (Table 5 and Fig. 6).

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) from REP primers.

	Kenyan isolates	German isolates	Austrian isolates
Kenyan isolates	********	0.9775	0.9959
German isolates	0.0227	********	0.9771
Austrian isolates	0.0041	0.0232	*********

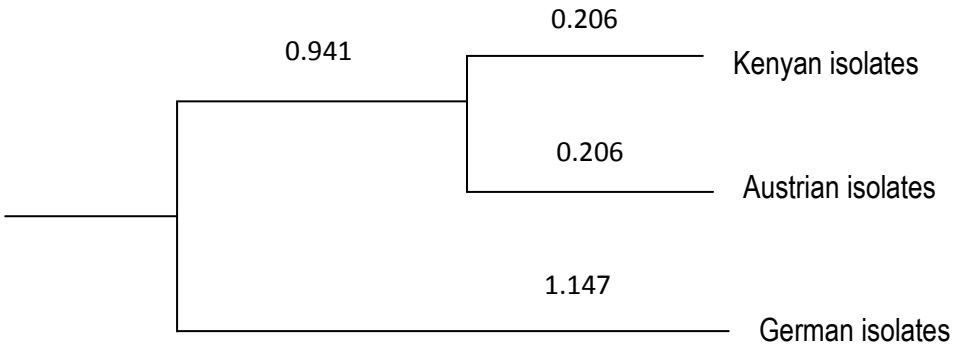

Figure 6: Dendogram based on Nei's genetic distance of three populations of *Exserohilum turcicum* derived from NEIGHBOURprocedure of PHYLIP version 3.5 using REP primers.

DISCUSSION

The CTAB method which has been used successfully by other researchers to extract DNA from fungi and related organisms (Moller *et al*., 1993) was used successfully on *E. turcicum* isolates in the present study. These markers were able to distinguish *E. turcicum* isolates into different genotype groups showing that the techniques had the requisite discriminative power to differentiate closely related fungal strains. Isolates from the different countries were found to cluster together showing that despite the vast geographical separations, the isolates still shared a some genetic similarities that have remained unchanged throughout the evolutionary pathway.

The three fingerprinting techniques generated characteristic banding profiles that can be used in clustering and grouping the pathogen isolates. The banding profiles were different with the different techniques, which can be explained by the fact that these markers make use of dispersed repetitive sequences and there exists some differences in the consensus sequences. PCR amplifications with REP primers yielded less amplification products which could be due to greater sequence conservation.

This study confirmed the assertion by Versalovic *et al*. (1991) that direct amplification and agarose gel electrophoresis of PCR products can provide genomic fingerprint of sufficient complexity to distinguish species and strains. The study also demonstrated that BOX, REP and ERIC like sequences are present in many fungi as observed by Maria *et al*. (2003). PCR methodologies employing REP, BOX and ERIC sequences as PCR primer binding sites can be used to study the distribution of repetitive sequences in different genomes. Oligonucleotides primers matching conserved protein sequence motifs can be used to rapidly amplify unknown DNA sequences thus allowing characterization of new repetitive elements in different species. This permits the use of the technique in the molecular genetic analysis of any microorganism.

The three techniques were found to have the ability to discriminate between the isolates of *E. turcicum* pathogen. Edel *et al*., (1995) demonstrated

that ERIC and PEP elements can be used to characterize different strains of *Fusarium oxysporum*. Our study also revealed that the repetitive elements which are highly conserved in the bacterial kingdom are also present in *E. turcicum*. The distribution of these elements was variable among the different isolates of *E. turcicum* allowing the differentiation of the isolates into clusters or groups. Existence of ERIC like sequences has also been reported in *Aspergillus* spp allowing the ERIC-PCR fingerprinting to be used to differentiate closely related strains (Edel *et al*., 1995).

As opposed to other DNA fingerprinting techniques, the inter-repeat PCR procedures such as ERIC and REP are easier to handle as they involve only rapid minipreparation of DNA, PCR amplification and agarose gel electrophoresis. The genetic distances and genetic diversities as shown by Nei's unbiased measure of genetic diversity and distances revealed that these techniques are well suited for diversity studies of natural populations. PCR based procedures that are well adapted for large scale characterizations of pathogenic strains are required for diversity studies (Edel *et al*., 1995). The inter-repeat PCR procedures give results that compare well to other molecular markers. George *et al*. (1997) demonstrated close correspondence between lineages defined by REP PCR and RFLP analysis. In addition to serving as a fingerprinting tool, the markers can also be used to construct genetic maps. In this study, the REP-PCR was found to generate few bands as reported earlier by George *et al*. (1997) prompting a suggestion of combining other primers to generate more markers.

The number of *E. turcicum* isolates included in ERIC, BOX and REP PCR techniques was not balanced since some of the isolates failed to amplify properly and did not generate scorable bands that could be used in the analysis. This is in line with report by George et al., (1997) and the recommendation is to try more primers. However over 80% of the isolates amplified properly showing that these techniques have potential to be used to distinguish strains of fungal pathogens. Identification of strains or biovars that exists in an area is important in any work on breeding for resistance as it is necessary to include a wide range of strains to ensure durable resistance. It is worth noting that use of resistant varieties is the most effective strategy in the management of northern leaf blight of maize.

CONCLUSIONS

This study revealed the presence of BOX, REP and ERIC like sequences in *E. turcicum* populations thus DNA fingerprinting techniques can be used in genetic studies to elucidate the population structure of the pathogen and generate knowledge to be employed in disease management. DNA fingerprinting by REP, BOX and ERIC techniques has an excellent potential as a tool for tracking the evolution and population dynamics of northern leaf blight pathogen.

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