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GENETIC PROFILING OF *EXSEROHILUM TURCICUM* ISOLATES FROM MAIZE AND SORGHUM

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ABSTRACT

Turcicum leaf blight is one of the most important foliar fungal diseases of maize and sorghum, causing reduced photosynthesis and grain yield losses exceeding 50 per cent. The present study was conducted during 2023–2024 at the Department of Plant Pathology, Agricultural College, Bapatla. DNA was isolated from 24 *Exserohilum turcicum* isolates collected from different mandals of Bapatla, Guntur and Prakasam districts. Amplification with Internal Transcribed Spacer (ITS) primers produced a 600 bp amplicon in all isolates, confirming them as *E. turcicum.* Polymorphism analysis using five restriction endonucleases three hexacutters (EcoR I, BamH I and Hind III) and two tetracutters (Hae III and Taq I) revealed considerable genetic variability. Among the hexacutters, Hind III showed the highest polymorphism (80%) with a PIC value of 0.46, followed by BamH I with 75% polymorphism and a PIC value of 0.45. Among tetracutters, Hae III produced four polymorphic bands with 80% polymorphism, whereas Taq I did not produce any polymorphism. Principal Coordinate Analysis (PCoA) performed using GenAlex 6.5 explained 45.14% of the total genetic variation in the first two principal coordinates, with Component 1 contributing 24.42% and Component 2 contributing 20.73%.
***Keywords*:** *Exserohilum turcicum*, Molecular Characterization, Polymorphism, Principal Coordinate Analysis, Restriction endonucleases.

Introduction

Maize (*Zea mays* L.) is an important coarse cereal and ranks as the third major crop in India after rice and wheat. It is believed to have originated in Mexico and Central America. According to Norman E. Borlaug, maize possesses the highest yield potential among all cereals. While the past two decades witnessed revolutions in rice and wheat production, the coming decades are expected to be the “era of maize.” In India, maize is cultivated on 8.69 million hectares with an annual production of 21.81 million tonnes. Major maize-growing states such as Karnataka, Andhra Pradesh, Maharashtra, Uttar Pradesh, Bihar, West Bengal, Rajasthan, Madhya Pradesh and Punjab together contribute 60 per cent of the total area and 70 per cent of national production. However, the average yield of 25.09 q/ha remains much lower than that of many other maize-producing countries.

India records about 61 diseases affecting maize (Payak and Sharma, 1982), of which 16 have been identified as major diseases under the All India Coordinated Maize Improvement Project. With the introduction of high-yielding hybrids both indigenous and exotic—and the increased use of fertilizers, maize production has increased substantially. However, the crop remains vulnerable to several foliar diseases, stalk rots, downy mildews and ear rots (Payak and Sharma, 1982). Foliar diseases are considered the primary biotic constraints to maize yields globally, and their prevalence varies with region and season (Smith, 1997). Among these, turcicum leaf blight (TLB), also known as Northern corn leaf blight, caused by

Exserohilum turcicum (Pass.) Leonard and Suggs. (syn. *Helminthosporium turcicum* Pass.), is of worldwide significance. TLB severely affects photosynthesis and can cause grain yield losses exceeding 50 per cent (Raymundo and Hooker, 1981). The present study focuses on assessing the genetic variability of *E. turcicum* (Pass.) Leonard and Suggs. under Andhra Pradesh conditions.

Material and Methods

Cultures and growth conditions for DNA isolation: The *E. turcicum* cultures of maize and sorghum were obtained by inoculating PDB with mycelial discs (cut from the periphery of actively growing seven-day old culture) of each isolate followed by incubation for seven days at 27±1^{0}C. The mycelial mat developed on PDB was filtered through sterilized muslin cloth, washed in sterile distilled water and was blot dried over filter paper. The mycelial mat was stored at -40^{0}C for future use (Murray and Thompson, 1980).

Isolation of fungal DNA: Fungal DNA was isolated by using CTAB method as described by Murray and Thompson (1980) with modifications. 100 mg of mycelial mat of respective isolates were taken in a sterilized pre chilled mortar and pestle and ground to a fine powder using liquid nitrogen. Isolation buffer of 1 ml was mixed with powder and dispensed in to centrifuge tube. The samples were incubated in water bath at 65 ^{0}C for 1 hr. The samples were cooled to room temperature, added with equal volume of chloroform: isoamyl alcohol (24:1) mixture and centrifuged (Eppendorf refrigerated centrifuge, India) at 10,000 rpm for 10 min at 24 ^{0}C. Aqueous phase was carefully collected and the samples were centrifuged again at 10,000 rpm for 10 min after adding equal volume of chloroform: isoamyl alcohol (24:1) mixture. Finally, the aqueous layer was collected into another centrifuge tube and 0.6 volumes of isopropanol was added and mixed gently by tilting the tube. DNA in the samples was allowed to precipitate by incubating at -20 ^{0}C. An hour after incubation, the samples were spun in refrigerated centrifuge at 10,000 rpm for 10 min. The supernatant was discarded and the pellet left in the centrifuge tube was washed with 70% ethanol thrice to purify the DNA from other metabolites and was air dried. Ethanol free pellets were dissolved in 20 µl of molecular grade water and stored at -20 ^{0}C for further use.

DNA quantification and concentration: Quantification of DNA was done by visualizing 5 µl DNA on 1.2 % Agarose gel in 1X TAE buffer containing ethidium bromide (10 mg ml^{-1}). The quantity and concentration of the extracted DNA was checked by measuring the absorbance on Nano-Drop ND-1000 spectrophotometer (Nanodrop Technologist) at 260 and 280 nm. Quantification was analyzed by absorbance ratios *i.e.,* 260/280 nm.

PCR amplification of *E. turcicum* isolates using ITS primers: Amplification of Internal Transcribed Spacer (ITS) region of *E. turcicum* isolates of maize and sorghum was done using universal primers previously described by White *et al.* (1990). Forward and reverse primers, *viz.*, ITS 1 (5' TCCGTAGGTGAACCTGCG G 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') respectively, synthesized based on conserved 18S and 28S coding regions of the nuclear rDNA were used. Amplification was carried out in 0.2 ml thin walled PCR tube with 25 µl reaction mixture

Initially, master mix was prepared by adding all the components except template DNA and Taq polymerase. Master mix was transferred to each PCR tube, then template DNA (50 ng/µl) and Taq polymerase were added and spinned at 3000 rpm for 30 sec. Amplification was performed in 0.2 ml thin walled PCR tubes using a thermocycler (Biorad T100 thermocycler, USA). Gradient PCR was kept between 55^{0}C – 62^{0}C to know the annealing temperature at which primers are going to bind to the template DNA and amplify the DNA. At 55^{0}C, the DNA got amplified and yielded an amplicon with product size of 600 bp.

Analysis of PCR products by agarose gel electrophoresis: The amplified product and genomic DNA were analysed by agarose gel electrophoresis as described by Sambrook and Russell (2001). Agarose gel of 1% (w/v) was prepared by dissolving 1.5 g of agarose in 100 ml of 1 X TAE buffer. At lukewarm temperature, 2.5 µl of ethidium bromide (10 mg ml^{-1}) was added and poured into gel casting tray of horizontal electrophoresis unit (Genaxy, India). The DNA samples were loaded after mixing with loading dye and electrophoresis was carried out in 1 X TAE buffer at 80 V till the dye front reached the lower part of the agarose gel. The migration pattern of the DNA fragments in the gel was recorded using gel documentation system (Biorad, USA).

Restriction enzyme analysis of ITS regions of *E. turcicum* isolates: Polymorphism was determined by digesting the amplicon obtained using ITS primers with five different restriction endonucleases *i.e.,* three hexa base pair cutters (*Eco*RI, *BamH*I, *HIND* III) and two tetra base pair cutters (*Hae* III, *Taq* I). A total volume of 15 µl reactions were prepared, containing 2 µl of 10 X buffer, 1 U of restriction enzymes, 2 µl of molecular grade water and 10 µl of PCR products and

spinned at 3000 rpm for 15 sec. The restriction digestion was performed in a water bath at 37 ^{0}C for 1 h. The restriction fragments were separated by electrophoresis on 2.0% agarose gel and were viewed under UV light.

Restriction bands were analysed, wherein each band with a different electrophoretic mobility was assigned a position number and based on the presence or absence of the band it was named with binary digits as 1 or 0. Only polymorphic bands were considered for analysis. The binary data corresponding to each enzyme was entered in to MS Excel spread sheet (Microsoft office, USA) and saved for further analysis.

Cluster analysis: The binary data obtained was fed to NTSYSpc 2.02i software (Rohlf, 1998) to get similarity coefficient and based on this, a dendrogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering algorithm.

Determination of polymorphism: The percentage of polymorphism of the isolates was assessed by using the formula given by Smith *et al*. (1997).

$$\text{Polymorphism}\,(\%) = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Results and Discussion

The 24 *E. turcicum* isolates collected from eleven different surveyed mandals of Bapatla, Guntur and Prakasam districts were identified using ITS 1 and ITS 4 primers, previously designed by White *et al*. (1990) to amplify the internal transcribed spacer (ITS) regions located between 18 S rRNA and 28 S rRNA genes. DNA (20 ng) extracted from 24 *E. turcicum* isolates were amplified and resulted in an amplicon of 600 bp (Plate 2) and no size variation in amplified ITS regions confirmed their identity as *E. turcicum*. A total of five restriction endonucleases, *i.e.,* three hexa cutters (*ECOR* I, *BamH* I and *HIND* III), and two tetra cutters (*Hae* III and *Taq* I) were used to notice the polymorphism in restriction sites of ITS region. Analysis was performed using the banding pattern obtained for all the restriction enzymes used.

ECOR I based banding pattern revealed the existence of a single restriction site in all the 24 *E. turcicum* isolates which resulted in two digested products (300 bp and 600 bp) (Plate 3) with no polymorphism. With *BamH* I, a total of four bands were obtained where in three were polymorphic indicating 75% polymorphism and the band size ranged from 200-600 bp (Plate 4). *HIND* III restriction enzyme resulted in a total of five bands that ranged from 200-600 bp with four polymorphic bands with 80.00% polymorphism among isolates (Table 1 and Plate 5). Of the tetra cutters, *Taq* I showed no polymorphism in restriction sites of ITS region and produced only amplified product (600bp) without any digestion while *Hae* III was found with 80.00 per cent polymorphism with four polymorphic bands that ranged from 300 to 800 bp (Table 1 and Plates 6 and 7). A total of 16 bands were produced by all the enzymes, out of which, 10 polymorphic bands were produced with five different restriction endonucleases and resulted in an average polymorphism of 47.00% with band size ranged from 200 bp to 800 bp with PIC value of 0.26. BamHI and *HIND* III generated a PIC value of 0.45 and 0.46 respectively while *Hae* III had a PIC value of 0.39 (Table 1). Similarity matrix was calculated using binary data.

The genetic similarity coefficients of all the enzymes were taken as distance and the clustering was done using UPGMA (Unweighted Pair Group Method with Arthmetic mean). The similarity coefficients between the isolates varied between 0.56 and 1.00. The data revealed that the isolate PNR 2 did not vary with DVT, BVM, MRK (1.0), while APK 2 was found to have 94% similarity with CBL1, LAM1, EDM (0.94) and also between PNR 1 and PNR 2, BPT, IRP, MRK (0.94) (Table 2).

Table 1 : Polymorphism and PIC values for different tetra and hexa restriction enzymes

S.No.	Name of the Restriction enzyme	Total no. of bands	No. of polymorphic bands	Polymorphism (%)	Band size (bp)	PIC value
1.	*ECOR* I	2	0	0.00	300-600	0.00
2.	*BamH* I	4	3	75.00	200-600	0.45
3.	*HIND* III	5	4	80.00	200-600	0.46
4.	*Hae* III	5	4	80.00	300-800	0.39
5.	Taq I	1	0	0.00	600	0.00
	Total	16	10			
	Average			47.00	200-800	0.26

Dendrogram using NTSYSpc.2.02i software (Rohlf, 1998) revealed the distribution of 24 isolates into two major clusters irrespective of their geographical distribution, where 15 isolates were in cluster A and nine isolates were aligned in another cluster (cluster B). Four isolates (PNR 2, DVT, BVM and MRK) from the cluster A showed 100 per cent similarity. The isolates in cluster A, APK1 and SMG; PNR1 and PNR2, DVT, BVM, MRK; BPT1 and BPT; LAM 2 and KTP were closely related (94 per cent similarity) and in cluster B, APK 2 and CBL1; IRP and KMR were closely related (Fig. 1).

The difference in the banding patterns obtained with hexa and tetra cutters revealed polymorphism within the isolates which may be due to variations in single nucleotide resulting in variation in restriction sites. According to Latha and Mukharjee (2002), analysis of ITS1-5.8S-ITS2 region of the cDNA produced a band of ~ 600 bp with size variation. Restriction analysis of this region showed inter and intra-specific polymorphisms. Rita *et al.* (2002) reported that the PCR fragments generated using the pair of primers ITS1-ITS4 as 580 to 620 base pairs in length.

Plate 1 : Genomic DNA of all 24 *E. turcicum* isolates.

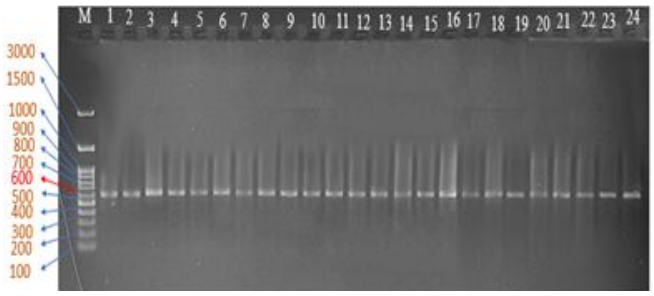

Plate 2 : Amplicons obtained using universal Internal Transcribed Spacer (ITS) primers in all 24 *E. turcicum*

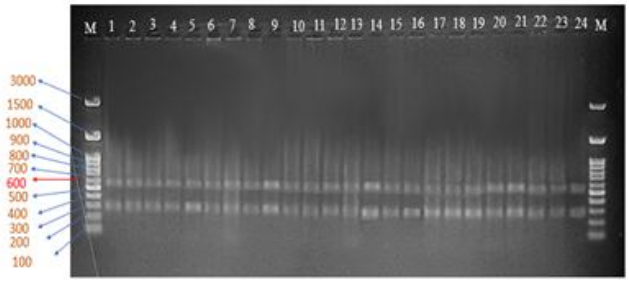

Plate 3 : *EcoR* I Restriction products of amplicons obtained using ITS primers.

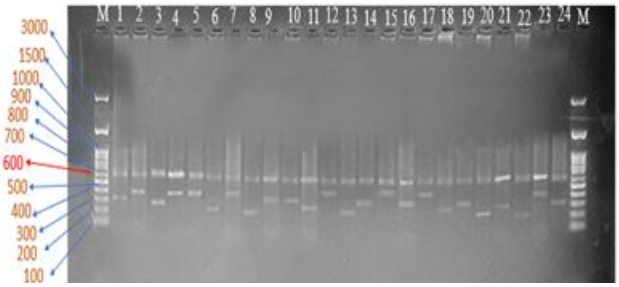

Plate 4 : *BamH* I Restriction products of amplicons obtained using ITS primers.

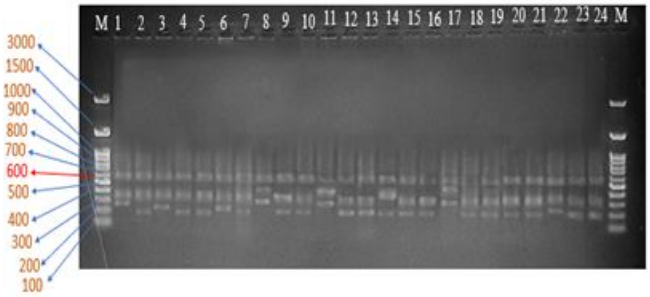

Plate 5 : *Hind* III Restriction products of amplicons obtained using ITS primers.

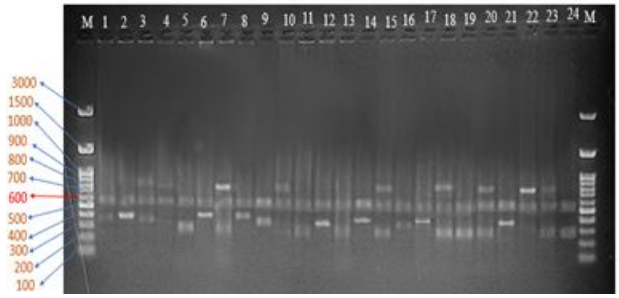

Plate 6 : *Hae* III Restriction products of amplicons obtained using ITS primers.

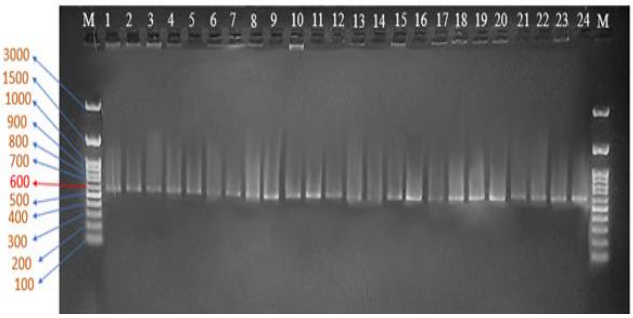

Plate 7 : *Taq* I Restriction products of amplicons obtained using ITS primers.

Table 2 : Similarity matrix of *E. turcicum* isolates from maize and sorghum.

	APK1	APK2	BPT1	BPT2	CBL1	CBL2	LAM1	LAM2	PNR1	PNR2	TNL1	TNL2	BPT	DVT	IRP	BVM	RJP	RDG	SMG	ELC	MRK	KTP	KMR	EDM
AP K 1	1																							
APK 2	0.75	1																						
BPT 1	0.88	0.75	1																					
BPT 2	0.63	0.88	0.63	1																				
CBL 1	0.69	0.94	0.69	0.94	1																			
CBL 2	0.69	0.81	0.81	0.81	0.75	1																		
LAM 1	0.69	0.94	0.69	0.81	0.88	0.75	1																	
LAM 2	0.75	0.63	0.88	0.75	0.69	0.81	0.56	1																
PNR 1	0.81	0.69	0.69	0.69	0.63	0.75	0.75	0.69	1															
PNR 2	0.88	0.75	0.75	0.75	0.69	0.81	0.69	0.75	0.94	1														
TNL 1	0.81	0.69	0.81	0.69	0.75	0.63	0.63	0.81	0.63	0.69	1													
TNL 2	0.75	0.75	0.75	0.75	0.69	0.81	0.69	0.75	0.81	0.88	0.69	1												
BPT	0.81	0.81	0.94	0.69	0.75	0.88	0.75	0.81	0.75	0.81	0.75	0.81	1											
DVT	0.88	0.75	0.75	0.75	0.69	0.81	0.69	0.75	0.94	1.00	0.69	0.88	0.81	1										
IRP	0.69	0.69	0.69	0.69	0.75	0.63	0.75	0.69	0.75	0.69	0.75	0.81	0.75	0.69	1									
BVM	0.88	0.75	0.75	0.75	0.69	0.81	0.69	0.75	0.94	1.00	0.69	0.88	0.81	1.00	0.69	1								
RJP	0.75	0.63	0.75	0.63	0.56	0.69	0.56	0.75	0.69	0.75	0.81	0.88	0.69	0.75	0.69	0.75	1							
RDG	0.69	0.69	0.69	0.69	0.75	0.63	0.75	0.69	0.75	0.69	0.75	0.69	0.75	0.69	0.88	0.69	0.56	1						
SMG	0.94	0.81	0.81	0.69	0.75	0.75	0.75	0.69	0.88	0.94	0.75	0.81	0.88	0.94	0.75	0.94	0.69	0.75	1					
ELC	0.75	0.75	0.88	0.75	0.81	0.81	0.69	0.88	0.69	0.75	0.81	0.75	0.94	0.75	0.81	0.75	0.63	0.81	0.81	1				
MRK	0.88	0.75	0.75	0.75	0.69	0.81	0.69	0.75	0.94	1.00	0.69	0.88	0.81	1.00	0.69	0.56	0.81	0.69	0.94	0.75	1			
KTP	0.69	0.56	0.81	0.69	0.63	0.75	0.63	0.94	0.75	0.69	0.75	0.69	0.75	0.69	0.75	0.69	0.69	0.75	0.63	0.81	0.69	1		
KMR	0.75	0.75	0.75	0.75	0.81	0.69	0.69	0.75	0.69	0.75	0.81	0.88	0.81	0.75	0.94	0.75	0.75	0.81	0.81	0.88	0.75	0.69	1	
EDM	0.81	0.94	0.81	0.81	0.88	0.75	0.88	0.69	0.75	0.81	0.75	0.81	0.88	0.81	0.75	0.81	0.69	0.75	0.88	0.81	0.81	0.63	0.81	1

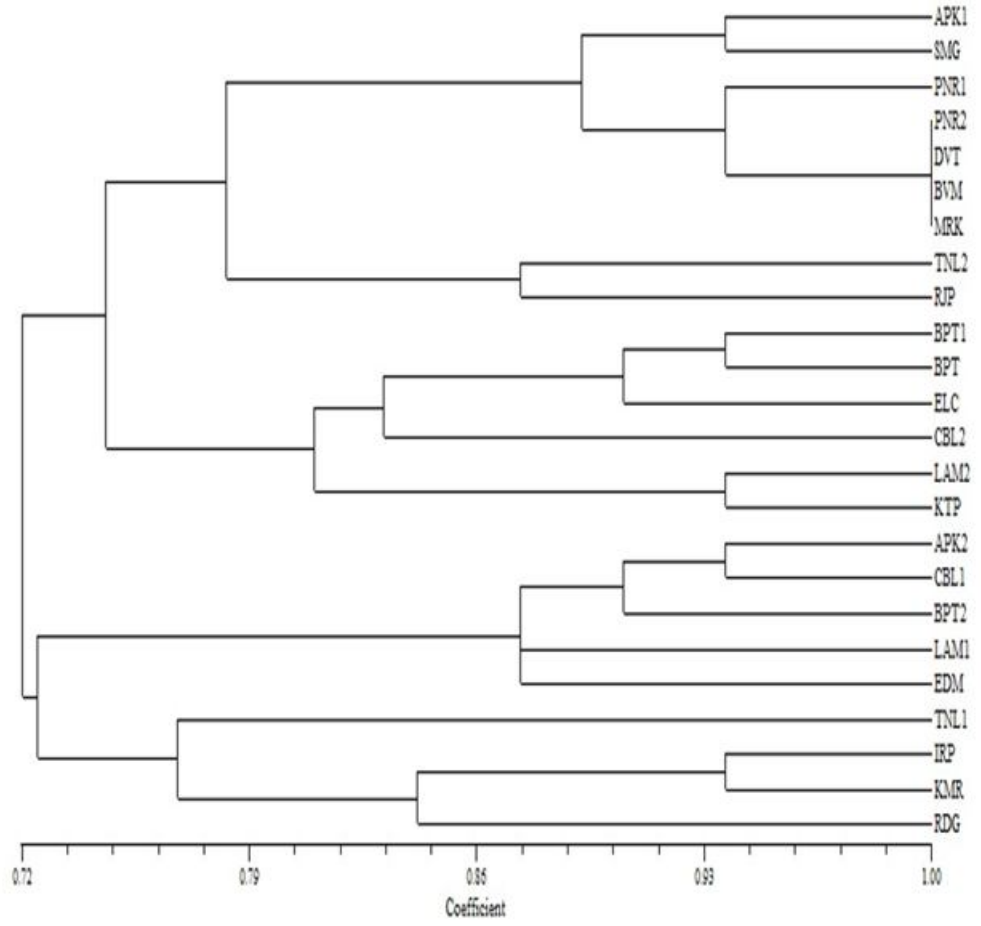

Fig. 1 : Dendrogram depicting clustering of *E. turcicum* isolates collected from maize and sorghum

The isolates were reported to show length polymorphism in this region of the genome and observed that *B. oryzae* isolates from rice revealed two digestion fragments when tested with the enzymes *HaeIII, EcoRI, AluI* and *RsaI*. Bakonyi *et al.* (1995) reported that the restriction profiles of the maize *S. turcica* isolates have two fragments ranged from 200-600 bp when digested with the enzymes *HaeIII, MspI,*

EcoRI, AluI and *HpaII* and revealed polymorphism among the isolates. The restriction profiles of the isolates from wheat *Bipolaris sorokiniana* obtained after digestion with the enzymes *HaeIII, MspI, RsaI, EcoRI, AluI,* and *HpaII* were reported to exhibit two fragments (300 – 600 bp) and four fragments (200-800 bp) with the enzymes *HinfI, BamHI* and *HindIII* (Matsumura *et al.*, 1988). A total of 1.458 alleles were observed among the isolates of three districts *i.e.*, Bapatla, Guntur and Prakasam. The highest mean values of per cent polymorphism, Na, Ne, I and h were observed among the isolates obtained from Guntur (68.75%, 1.688, 1.376, 0.335 and 0.233 respectively), while the lowest were obtained with the isolates from Bapatla district (43.75%, 1.250, 1.338, 0.271 and 0.188 respectively). These results indicated more genetic variability among the genomes of the isolates collected from Guntur district, while, least genetic variability among the genomes of Bapatla district (Table 3).

Principle Coordinate Analysis (PCoA): The differentiation occurred in any population at different geographical locations was represented by different axis and these axes together represent total divergence. Certain proportion of total variability was created at each axis and to understand this differentiation, variances (Eigen values), variance (%), cumulative variance (%) were presented in Table 4 and Fig 2.

Table 3 : Genetic diversity using GenAlex 6.5 software for twenty-four isolates of *E. turcicum* collected from Guntur, Bapatla and Prakasam districts.

Population	N		%P	Na	Ne	I	h	uh
Bapatla	4	Mean	43.75%	1.250	1.338	0.271	0.188	0.250
		SE		0.194	0.104	0.080	0.056	0.075
Guntur	14	Mean	68.75%	1.688	1.376	0.335	0.233	0.251
		SE		0.120	0.081	0.067	0.046	0.049
Prakasam	6	Mean	50.00%	1.438	1.309	0.275	0.184	0.221
		SE		0.157	0.091	0.073	0.050	0.060
TOTAL	24	**Mean**	**54.17%**	**1.458**	**1.341**	**0.300**	**0.202**	**0.241**
		SE	**7.51%**	**0.094**	**0.053**	**0.042**	**0.029**	**0.035**

N: No. of isolates within the population; %P: percentage of polymorphic loci; Na: No. of observed alleles within population; Ne: No. of effective alleles; I: Shannon's Information Index; h: gene diversity, uh: unbiased diversity

Table 4 : Eigen values, variance (%), cumulative variance (%) and component loading of *E. turcicum* isolates

	PC1	PC2	PC3
Eigen Value	2.217	1.882	1.200
Per cent Variance	24.42	20.73	13.22
Cum. Per cent Variance	24.42	45.14	58.36

To understand the genetic diversity among the genomes of *E. turcicum* isolates with respect to the data obtained from different hexa and tetra cutter restriction endonucleases, PCoA was performed through GenAlex 6.5 software. A total of 45.14% of overall variance was attributed with first two principle coordinates. Principal Component 1 (PC 1) represented 24.42% of the variation, while PC 2 accounted for 20.73% of the total variation. The isolates were plotted on 2-dimensional plot (Fig. 4.12) and can be classified into single group, included all the 24 isolates (APK-1, APK-2, CBL-1, CBL-2, BPT-1, BPT-2, LAM-1, LAM-2, PNR-1, PNR-2, TNL-1, TNL-2, BPT, DVT, IRP, BVM, RJP, RDG, SMG, ELC, MRK, KTP, KMR and EDM). It was also observed that isolates from different geographical regions were found in same group, indicating that there was no correlation between the geographical distribution and genomic diversity.

The results were in accordance with Petkar *et al.* (2019) who analysed 99 *F. o.* f. sp. *niveum* isolates through PCoA from four different regions of southeastern United States and named them as GA1, GA2, GA 3 and FL and reported that all GA isolates belonging to three different regions could cluster together due to least diversity, whereas, Florida population (FL) clustered separately being more diverse. Similar results were reported by Nourollahi and Aliaran (2017), who did PCoA for *F. o.* f. sp. *ciceri* isolates from six different countries in western Iran. Sivaramakrishnan *et al.* (2002) did PCoA for RAPD and AFLP marker data on 43 Fusarium wilt pathogen isolates of chickpea, the results indicated that AFLP was more effective in bringing out the polymorphism among the pathogen isolates and placing these in different race-specific clusters in contrast to RAPDS.

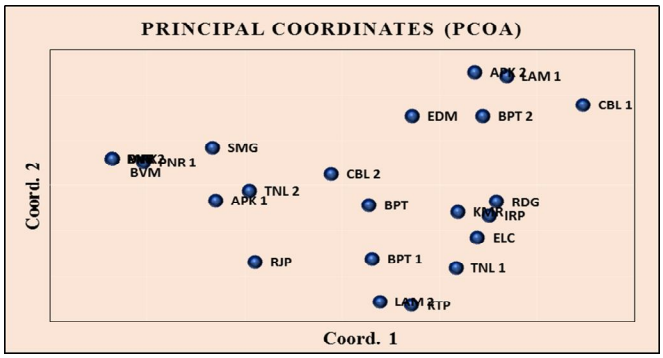

Fig. 2 : Principal coordinate analysis based on the data of restriction enzymes

Conclusion

Molecular diversity analysis of *E. turcicum* isolates using ITS primers revealed two clusters with 72% similarity. Polymorphism was observed by digesting the amplicon with different restriction enzymes. *HIND III* and *Bam HI* restriction enzymes showed 80 per cent of polymorphism among the isolates. Principle coordinate analysis revealed that, more genetic diversity existed between the isolates collected from the Guntur district.

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Conflict of Interests

The authors declare that there is no conflict of interest related to this article.

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