

Molecular Characterization of *Tospovirus* Transmitting Thrips Populations from India

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Abstract: The presence of *Tospovirus* in thrips vector, *Scirtothrips dorsalis* has been detected for the first time in India using RT-PCR analysis. Similarly RT-PCR analysis with GBNV infected tomato leaves also resulted in the amplification of cDNA corresponding to the N gene (approximately 830 bp) of Coimbatore isolate. Dendrogram constructed on the basis of RAPD similarity matrix revealed that *S. dorsalis* population from tomato, groundnut and chillies had at least 75% similarity while; only 50% similarity existed between the *Frankliniella schultzei* populations from cowpea and sunnhemp. *Thrips tabaci* from cotton was distantly related with *S. dorsalis* and *Frankliniella schultzei* with lowest similarity indices (less than 0.464). This was also confirmed by the ISSR analysis with the same six thrips populations. It is evident from the present study that RAPD markers were more informative than ISSR in differentiating host-associated populations of thrips.

Key words: *Tospovirus*, *Scirtothrips dorsalis*, *Thrips tabaci*, *Frankliniella schultzei*, ISSR, RAPD, RT-PCR

INTRODUCTION

Tospoviruses belonging to the family Bunyaviridae^[1] are one among the ten most devastating plant viruses^[2] with a host range of over 1050 species^[3]. *Tospoviruses* represent the only plant virus group that is biologically transmitted by thrips^[4]. Indeed, *Tospoviruses* are not known to exist in crops or in nature in the absence of thrips^[5]. Only ten species of thrips (Thysanoptera: Thripidae) were reported as vectors of tospoviruses out of more than 5000 species recorded worldwide^[6]. Thus less than 0.2 per cent of the Thysanoptera species were known to be associated with *Tospoviruses*, and there was no evidence that many more thrips species are likely to be implicated^[7]. Only five of the 160 described species of *Frankliniella*, four of the 280 species of the genus *Thrips* and just one of the 90 species of *Scirtothrips* were reported as vectors of *Tospoviruses*^[6, 7]. Although the dependency of *Tospoviruses* on thrips is so strong, it is surprising that so little attention has been paid to characterize the *Tospovirus* transmitting thrips populations in India. It has already been suggested that molecular tools need to be exploited to discriminate populations differing in important traits such as virus transmission^[8]. PCR based techniques have already been applied in the field of intra- and inter-specific polymorphism in Thysanoptera species^[9-11] and suitable DNA polymorphism was detected to distinguish populations originating from geographically different locations^[11]. However, the use of molecular markers in the field of thrips vectors is scanty. Hence molecular markers *viz.*, RAPD (Random Amplified Polymorphic DNA) and

ISSR (Inter Simple Sequence Repeat) were employed to characterize the *Tospovirus* transmitting thrips populations collected from the state of Tamil Nadu, India.

MATERIALS AND METHODS

Sampling sites: Three different species of thrips namely, *Frankliniella schultzei* (Trybom), *Scirtothrips dorsalis* Hood and *Thrips tabaci* Lindeman found associated with the *Tospovirus* transmission in six different host plants *viz.*, groundnut, tomato, chillies, cowpea, sunnhemp, and cotton were collected in and around Coimbatore, Tamil Nadu, India.

DNA extraction: The DNA was extracted from 100 individuals representing each population by CTAB (hexadecyl trimethyl ammonium bromide) procedure^[12] as modified by Mendel *et al.*^[13]. The quantity and quality of the isolated DNA were determined using DyNA Quant 200 - Fluorimeter and 0.8% agarose gel stained with ethidium bromide.

PCR amplification

RAPD analysis: The RAPD analysis was performed with ten decamer primers supplied by Operon Technologies Inc., California, USA (Table 1). The PCR amplification was carried out in 10µl reaction mixture containing 25ng genomic DNA, 8mM dNTPs, 10X assay buffer, 0.2µM primer and 0.4 U *Taq* DNA polymerase. Amplification was performed in PTC-100TM thermocycler (MJ Research Inc., USA) programmed as one cycle of initial denaturation at 94°C

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for 2 min; 40 cycles each of 94°C for 1 min., 47 – 55°C for 1 min., 72°C for 1 min.; and final extension at 72°C for 7 min. The PCR products were separated in 1.5 % agarose gel electrophoresis.

ISSR analysis: ISSR analysis was performed with five ISSR primers supplied by Sigma Genosys Ltd., Bangalore (Table 1). PCR amplification was carried out in 15µl reaction volume containing 20 -30 ng of genomic DNA, 0.80 µM of primer, 0.66 mM each of dATP, dGTP, dCTP and dTTP, 1.5 mM assay buffer and 0.03 units of *Taq* DNA polymerase. Amplification was done using a PTC-100™ thermocycler (MJ Research Inc.) programmed for initial denaturation at 94°C for 5 min, 40 cycles of 1 min denaturation at 94°C, 1 min annealing at respective temperatures and 2 min extension at 72 °C and final extension at 72 °C for 5 min. The PCR products were electrophoresed in a 4 per cent denaturing poly acrylamide gel electrophoresis^[14] and resolved by silver staining procedure^[15].

Analysis of PCR amplification profiles: Clearly resolved, unambiguous polymorphic bands were scored visually for their presence or absence in each RAPD as well as ISSR primer. The scores were obtained in the form of a matrix with '1' and '0', which indicate the presence and absence of bands respectively. The genetic diversity among the thrips populations were evaluated by calculating the Jaccard's Similarity Coefficient^[16] using the NTSYS-PC software, version 2.0^[17] and dendrogram was constructed by the Unweighted Pair-Group method (UPGMA).

ISOLATION OF RNA AND DETECTION OF TOSPOVIRUS

Isolation of total RNA: RNA from *S. dorsalis* and GBNV (Groundnut Bud Necrosis Virus) infected tomato leaves was extracted using TRI Reagent (Sigma–Aldrich, USA) as per the protocol described by Chomczynski and Sacchi^[18] with necessary modifications. Isolated total RNA was used as a template for amplification in the reverse transcription and polymerase chain reaction (RT -PCR)

PCR amplification: RT-PCR^[19] was performed with the degenerate primers (5' TTACAATTCCAGCGAAGGACC 3' and 5' ATGTCTAACGTT/CAAGCAG/ACTC 3'- NCBI Accession # 515817 and PB 27809) to amplify GBNV N gene from first strand cDNA as described by Sambrook *et al.*^[14] with a few modifications. PCR was carried out in 50 µl reaction mixture containing 2.0 µl of first strand cDNA, 5.0 µl of 10 X PCR buffer (10m M Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin), 20 mM each of dNTPs, 100ng each of forward and reverse primers and 1.5 U of *Taq*

Table 1: List of RAPD and ISSR primers used for genetic diversity analysis

Primer	Sequence (5'-3')
RAPD Primers	
OPA 01	CAGGCCCTTC
OPA 07	GAAACGGGTG
OPA 08	GTGACGTAGG
OPA 13	CAGACCCAC
OPC 02	GTGAGGCGTC
OPC 08	TGGACCGGTG
OPC 15	GACGGATCAG
OPE 04	GTGACATGCC
OPE 08	TCACCACGGT
OPE 15	ACGCACAACC
ISSR Primers	
ISSR 01	ATATATATATATATATT
ISSR 02	AT ATATATATATATATG
ISSR 03	ATATATATATATATATC
ISSR 05	TATATATATATATATAC
ISSR 10	GAGAGAGAGAGAGAGAT

DNA polymerase (Bengaluru Genei Pvt. Ltd., Bangalore, India) in PTC-100™ thermal cycler (MJ Research, Inc., USA) with temperature profile of initial denaturation at 94°C for 2 min., 40 cycles of melting at 94°C for 30 sec, annealing at 58°C for 1 min, synthesis at 72°C for 1 min followed by final extension at 72°C for 10 min. The amplified product was electrophoresed on 1.0% agarose gel.

RESULTS AND DISCUSSION

Survey at the experimental fields of Tamil Nadu Agricultural University revealed that there were two different species of thrips *viz.*, *F. schultzei* and *S. dorsalis*, found associated with GBNV transmission in different host plants. *F. schultzei* was found to be the vector of GBNV in cowpea and sunnhemp whereas, *S. dorsalis* in groundnut, tomato and chillies.

RAPD analysis: DNA based molecular marker technique is a powerful method in genetic diversity analysis of insect pests^[20]. The major advantage of the PCR based marker systems is the generation of fragments from both single and multi loci. All the ten RAPD primers used in this study generated scorable PCR products by amplifying the template DNA with *Taq* polymerase. Of the ten primers tested, the number of RAPD products generated was higher for the primers OPA 08 (Fig. 1) and OPE 15 (17), while lowest number of markers (4) were produced by the primer, OPA 01 (Table 2). Such a wide variation in the number of markers produced by these arbitrary primers may be attributed to the differences in the binding sites throughout genome of the genotypes studied. Generation of higher number of markers or alleles in the present study indicated the higher amount of genetic diversity among different populations of *Tospovirus* transmitting thrips. Among the ten primers, nine primers showed 100 per cent polymorphism except OPE 08 (83.3 %). RAPD primers produced the highest

Table 2: Total number of markers and per cent polymorphism detected using RAPD primers

Primer	Max. number of markers generated	Polymorphic markers	Monomorphic markers	Polymorphism (%)
OPA 01	4	4	-	100
OPA 07	12	12	-	100
OPA 08	17	17	-	100
OPA 13	10	10	-	100
OPC 02	12	12	-	100
OPC 08	14	14	-	100
OPC 15	9	9	-	100
OPE 04	15	15	-	100
OPE 08	6	5	1	83.3
OPE 15	17	17	-	100

Table 3: Similarity index values among six thrips populations obtained through RAPD-PCR analysis

	<i>S. dorsalis</i> (tomato)	<i>T. tabaci</i> (cotton)	<i>F. schultzei</i> (cowpea)	<i>S. dorsalis</i> (chillies)	<i>S. dorsalis</i> (groundnut)	<i>F. schultzei</i> (sunhemp)
<i>S. dorsalis</i> (tomato)	1.000					
<i>T. tabaci</i> (cotton)	0.463	1.000				
<i>F. schultzei</i> (cowpea)	0.116	0.096	1.000			
<i>S. dorsalis</i> (chillies)	0.783	0.402	0.137	1.000		
<i>S. dorsalis</i> (groundnut)	0.803	0.423	0.115	0.759	1.000	
<i>F. schultzei</i> (sunhemp)	0.216	0.227	0.500	0.188	0.198	1.000

Table 4: Total number of markers and per cent polymorphism detected using ISSR primers

Primer	Max. number of markers generated	Polymorphic markers	Monomorphic markers	Polymorphism (%)
ISSR 02	12	11	1	91.7
ISSR 03	11	10	1	90.9
ISSR 05	16	16	-	100
ISSR 09	11	7	4	63.6
ISSR 10	16	7	9	43.8

Table 5: Similarity index values among six thrips populations obtained through ISSR-PCR analysis

	<i>S. dorsalis</i> (tomato)	<i>T. tabaci</i> (cotton)	<i>F. schultzei</i> (cowpea)	<i>S. dorsalis</i> (groundnut)	<i>F. schultzei</i> (sunhemp)	<i>S. dorsalis</i> (chillies)
<i>S. dorsalis</i> (tomato)	1.000					
<i>T. tabaci</i> (cotton)	0.466	1.000				
<i>F. schultzei</i> (cowpea)	0.500	0.444	1.000			
<i>S. dorsalis</i> (groundnut)	0.900	0.410	0.492	1.000		
<i>F. schultzei</i> (sunhemp)	0.596	0.464	0.826	0.559	1.000	
<i>S. dorsalis</i> (chillies)	0.979	0.458	0.492	0.920	0.586	1.000

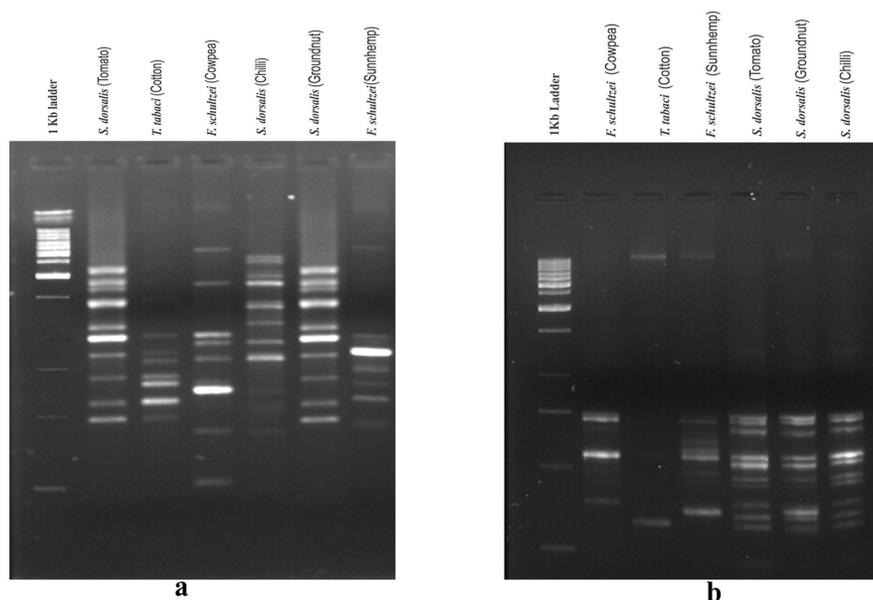


Fig. 1: Molecular polymorphism among six thrips populations after PCR amplification with a) OPA 08 b) OPC 08

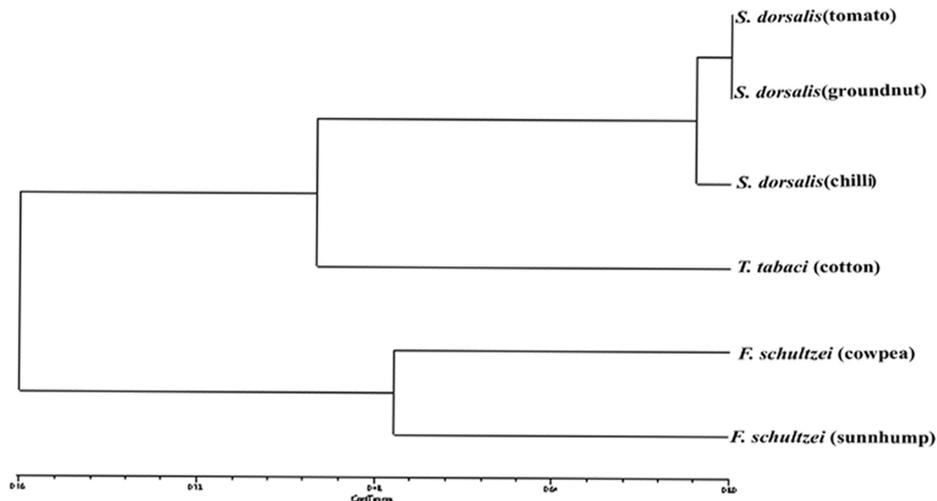


Fig. 2: Cluster dendrogram showing the relationship among six thrips populations using RAPD primers

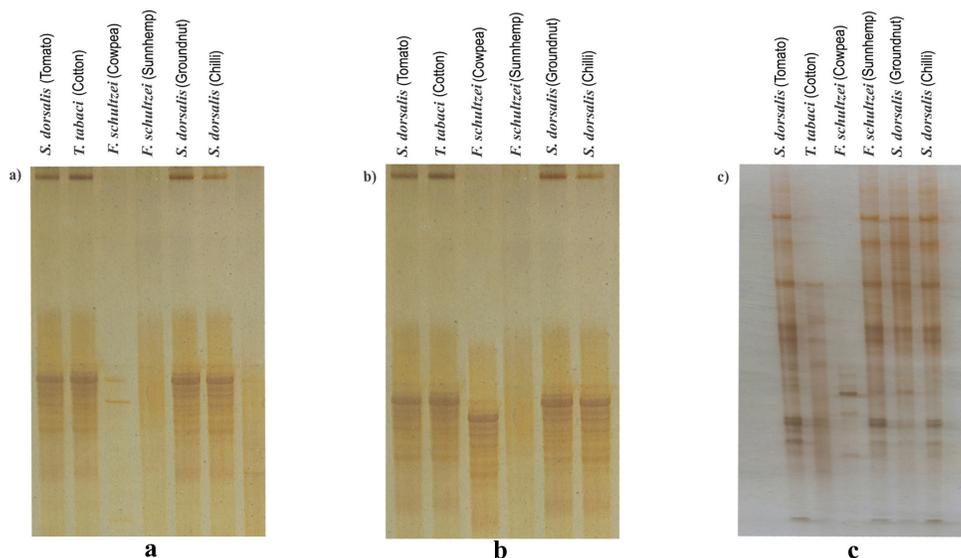


Fig. 3: Molecular polymorphism generated among six thrips populations using a) ISSR 02 b) ISSR 03 c) ISSR 10

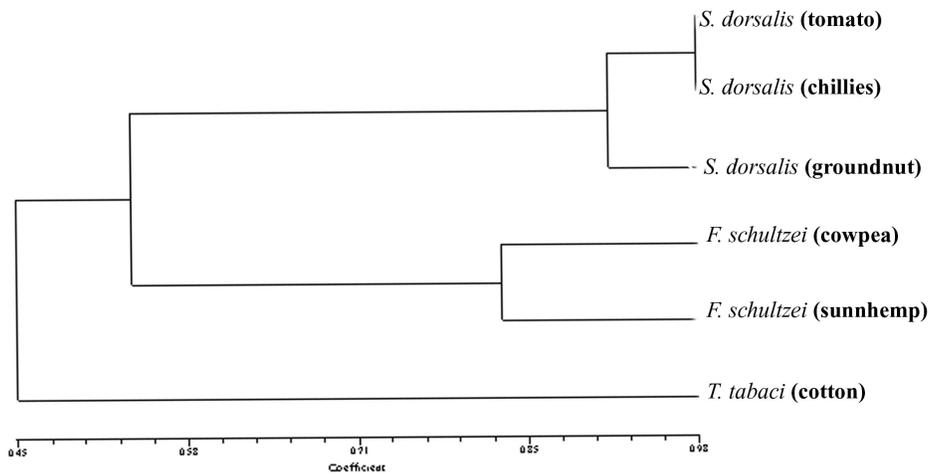


Fig. 4: Cluster dendrogram showing the relationship among six thrips populations using ISSR primers

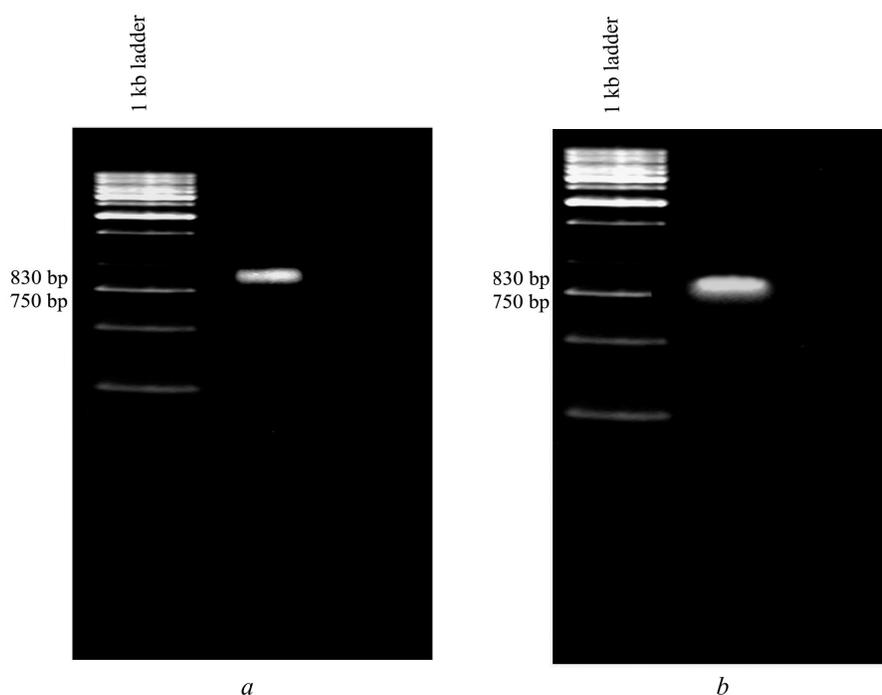


Fig. 5: Amplification of cDNA through RT-PCR from a) infected tomato plant b) *S. dorsalis*

number of markers, because these primers scan the whole genome at random. The similarity index values obtained for each pair wise comparison among the six thrips populations based on 116 RAPD markers ranged from 0.096 to 0.803 (Table 3). *S. dorsalis* from tomato and groundnut showed the highest similarity index (0.803) followed by *S. dorsalis* from tomato and chillies (0.783). *T. tabaci* population showed less similarity with other two thrips species (9.6 – 42.3%). The RAPD marker system distinguished the different genera into different clusters as expected since they were morphologically and genetically well differentiated. From dendrogram it could be deduced that *S. dorsalis* population from different hosts had at least 75 % similarity while, only 50 % similarity existed between the *F. schultzei* population from cowpea and sunnhemp (Fig. 2). This might be the host-induced genetic variation as observed in thrips and other polyphagous insect pests such as *Helicoverpa armigera* Hubner^[21, 22]. The establishment of this type of diversity has to be checked again by using other marker systems such as ISSR and SSR, because RAPD scans the whole genome, whereas SSR and ISSR search for specific sites in the genome. Since SSR markers are not available, the six thrips populations were again analysed using ISSR markers.

ISSR analysis: The ISSR primers generated a total of 66 markers and the maximum numbers of markers were generated by ISSR 05 and ISSR 10 (16) (Table 4). Among the five primers tested, the highest per cent polymorphism was observed in ISSR 05 (100 %) while the lowest was in ISSR 10 (43.8%) (Fig. 3). The

similarity index values obtained for each pair wise comparison among the six thrips populations ranged from 0.410 (between *T. tabaci* from cotton and *S. dorsalis* from groundnut) to 0.979 (between *S. dorsalis* populations from tomato and chillies) (Table 5). *S. dorsalis* populations from different hosts had the highest similarity index (0.900 - 0.979), while *T. tabaci*, from cotton showed the lowest similarity indices (less than 0.464) with other populations. Dendrogram constructed on the basis of ISSR similarity matrix clearly indicated that the *T. tabaci* from cotton was distantly related with the remaining populations (Fig. 4). The present study, as well as those of others^[9-11, 21] clearly established the utility of molecular markers such as RAPD and ISSR in assessing the genetic diversity among the host-associated populations. It is evident from the present study that RAPD markers were more informative than ISSR in differentiating host-associated populations of different genera.

Detection of *Tospovirus* in tomato and *S. dorsalis*: *Tospoviruses* replicate in their thrips vectors, thus the insects not only spread the virus but also serve as a virus host^[23]. RT-PCR assay for the detection of *Tospovirus* was previously employed by several workers in crop plants as well as in vectors^[24-26]. In the present investigation the presence of *Tospovirus* was detected in *S. dorsalis* for the first time from India using RT-PCR analysis. Similarly RT-PCR analysis with GBNV infected tomato leaves also resulted in the amplification of cDNA corresponding to the N gene (approximately 830 bp) of the Coimbatore isolate of GBNV (Fig. 5). This confirms that the virus present in

tomato and *S. dorsalis* is GBNV, the type species of the genus *Tospovirus* in India.

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