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Phylogenetic studies of tospoviruses (Family: *Bunyaviridae*) based on intergenic region sequences of small and medium genomic RNAs

Brief Report

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Summary. Analysis of the intergenic region (IGR) of S and M RNAs of tospoviruses (Family *Bunyaviridae*) indicated their heterogeneity both in length and sequence. In general, IGRs of M RNA were shorter in length compared to the IGRs of their respective S RNA species. Percent identity among the S RNA IGR sequences of distinct tospovirus species varied from 42 to 57%, whereas it was 79 to 99% among isolates of the same species. Similarly, when IGRs of M RNAs were compared, there was higher sequence identity among isolates of the same tospovirus species (84 to 98%) than among distinct tospovirus species (46 to 59%). Percent nucleotide identities and maximum likelihood trees of IGR sequences of S and M RNAs indicated that their sequence divergence is similar to that of nucleocapsid gene at inter and intra-species levels. This is the first detailed sequence analysis of IGRs of S and M RNAs of known tospoviruses.

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The genus *Tospovirus* of the family *Bunyaviridae* contains plant viruses that are transmitted in a circulative, propagative manner by thrips (reviewed in [11, 18, 19, 33]). Tospoviruses are quasi-spherical in shape (80–110 nm in diameter) and possess a characteristic lipid envelope. The genome consists of three RNA

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segments, small (S), medium (M) and large (L). While L RNA is in negative polarity with a single open reading frame (ORF), M and S genomic RNAs have two ORFs each, with ambisense coding strategies (reviewed in [11, 18, 19]). Both S and M RNAs possess an intergenic region (IGR) with a high A-U content capable of forming a stable hairpin structure. The sequence conservation found at the top of the hairpins of the IGRs of both S and M RNAs suggests a possible role for this sequence as a signal for transcription termination (reviewed in [11]). In addition, the IGR of S RNA of tomato spotted wilt virus (TSWV) was correlated with its competitiveness in reassortant isolates [24].

A set of descriptors has been established based on which a tospovirus can be identified and classified to the species level (reviewed in [18,19]). Sequence analysis of the N gene of various tospoviruses revealed a high degree of sequence divergence at the species level, which is being used as one of the important criteria to delineate individual tospoviruses to the species level ([7, 16], reviewed in [18, 19]). So far, twelve definitive tospovirus species have been described (summarized in [18]).

In our previous studies, analysis of the IGRs of M RNA of different TSWV isolates from several naturally infected host species from Georgia indicated a high degree of sequence homology [3]. In this report, we further extended this analysis of the IGRs of S and M RNAS of all known tospoviruses by using quartet puzzling tree search method, a heuristic tree search procedure for reconstructing maximum likelihood tree topologies [30, 31].

The S RNA IGR of a TSWV isolate from naturally infected tobacco in Georgia (TSWV-GA-TB, Table 1) was amplified through immunocapture reverse tran-

Table 1. Sources of small (S) RNA intergenic region (IGR) sequences of tospoviruses used in the study

Isolate	Host	Country	GenBank acc. no.	Reference
TSWV-GA-TB	Tobacco	USA	AF135036	This study
TSWV-D	Dahlia	Netherlands	AF020660	[24]
TSWV-10	Peanut	USA	AF020569	[25]
TSWV-L3	Tobacco	Bulgaria	D13926	[17]
TSWV-BR-01	Tomato	Brazil	D00645	[8]
TSWV-B	Tomato	Brazil	L12048	[22]
INSV	Impatiens	Netherlands	X66972	[9]
PBNV	Peanut	India	U27809	[26]
WSMV	Watermelon	Taiwan	U78734	[34]
Tospo-To	Tomato	Taiwan	Z46419	[12]
PYSV	Peanut	India	AF013994	[28]
IYSV	Iris	Netherlands	AF001387	[5]
PCFSV	Peanut	Taiwan	AF080526	[35]

TSWV Tomato spotted wilt virus; *INSV* impatiens necrotic spot virus; *PBNV* peanut bud necrosis virus; *WSMV* watermelon silver mottle virus; *PYSV* peanut yellow spot virus; *IYSV* iris yellow spot virus; *PCFSV* peanut chlorotic fanspot virus

Table 2. Sources of medium (M) RNA intergenic region (IGR) sequences of tospoviruses used in the study

Isolate	Host	Country	GenBank acc. no.	Reference
TSWV-GA-TB	Tobacco	USA	AF135033	[3]
TSWV-FL-PT	Peanut	USA	AF135035	[3]
TSWV-BR-01	Tomato	Brazil	S48091	[14]
TSWV-JP	Pepper	Japan	AB01096	[20]
INSV	Impatiens	USA	M74904	[15]
PBNV	Peanut	India	U42555	[27]
WSMV	Watermelon	Taiwan	U75379	[4]

TSWV Tomato spotted wilt virus; *INSV* impatiens necrotic spot virus; *PBNV* peanut bud necrosis virus; *WSMV* watermelon silver mottle virus

scription polymerase chain reaction (IC-RT-PCR) as described previously [13]. Primers used for amplification were designed from published sequence [8]. The genome sense primer (5′TGCTTCAGGATCAAAATAA 3′) was derived from the coding region of the NSs gene sequence and the genome anti-sense primer (5′AATGAATGAAGATCAGCTGA 3′) was derived from the coding region of the NP gene. Amplification was performed in an automated thermal cycler (Perkin Elmer, GeneAmp 2400) programmed for one cycle of 42 C for 45 min for cDNA synthesis, and 40 cycles of amplification with the following parameters: 60 sec of denaturation at 90 °C, 2 min of annealing at 42 °C, and 1 min of extension at 72 °C followed by one cycle of final extension for 60 min at 72 °C.

Following PCR, reaction products were analyzed by agarose gel electrophoresis. The band corresponding to S RNA IGR was excised, purified, and ligated to pGEM-T vector (Promega Corporation, Madison, WI). Competent *Escherichia coli* (strain DH5$\propto$) was transformed and recombinant clones identified by restriction endonuclease digestion by following standard procedures [25]. A selected recombinant clone was sequenced at the Molecular Genetics Instrumentation Facility, University of Georgia, Athens, GA. Other S and M RNA IGR sequences used in the study were obtained from GenBank [2] (Table 1, 2). Percent identity between IGR sequences was determined using the GAP program in the University of Wisconsin Genetics Computer Group (UWGCG) package [10]. The BLAST program [1] was used to identify related sequences available from the GenBank database [2]. CLUSTALW [32] was used to generate alignments of multiple sequences. The sequences were manually verified and edited to ensure the optimality of alignments.

The suitability of the data set for reconstructing phylogenetic trees was verified by performing likelihood mapping analysis (LMA) [30] using the PUZZLE program. LMA offers a method to investigate the support for the internal branches of a tree without first having to construct an overall tree and to graphically visualize the phylogenetic content of a sequence alignment. The method is based on an analysis of maximum likelihoods for the three fully resolved tree topologies

that can be computed for each group of four sequences (a quartet). The resulting distribution of points for all quartets inside an equilateral triangle show whether the data set is evolving in a star-like or tree-like fashion.

Maximum likelihood trees were then constructed using the PUZZLE program [29] included within the PAUP 4.0 phylogenetic package [31]. The trees were constructed using 1000 puzzling steps. The program implements a fast tree search algorithm that automatically assigns estimations of support to each internal branch. In the first step, all possible quartet maximum likelihood trees are reconstructed, which are then combined to an overall tree in the puzzling step. Finally, a majority rule consensus of all intermediate trees is computed resulting in the quartet puzzling tree [29]. The unrooted trees were generated using Treeview [21].

Analysis of the IGR sequences of S and M RNAs of tospoviruses indicated their heterogeneity both in length and sequence. In general, IGRs of M RNA were shorter in length compared to the IGRs of their respective S RNA species (Tables 3, 4). Among different species of tospoviruses, IGR sequences of TSWV had the least number of nucleotides while the maximum number was found in the case of Tospo-To and watermelon silver mottle virus (WSMV) (Tables 3, 4). Variation in the length of IGRs was also observed among different isolates of TSWV.

Pairwise comparisons of S RNA IGR sequences of tospoviruses indicated a high degree of identity among different isolates of a species (Table 3). Percent identity among the S RNA IGR sequences of various isolates of TSWV ranged from 79 to 91. However, the IGR of TSWV-B showed only 49 to 57% identity with

Table 3. Percent nucleotide sequence identity between small (S) RNA intergenic region (IGR) of tospoviruses

Isolate	TSWV-GA-TB	TSWV-D	TSWV-10	TSWV-L3	TSWV-BR-01	TSWV-B	INSV	PBNV	WSMV	Tospo-To	PYSV	IYSV	PCFSV
TSWV-GA-TB (589)	100	88	98	87	91	53	53	50	46	47	44	48	45
TSWV-D (535)		100	79	96	89	52	55	49	51	50	45	55	49
TSWV-10 (597)			100	87	90	49	53	52	46	46	46	54	45
TSWV-L3 (585)				100	89	52	53	48	47	49	47	49	52
TSWV-BR-01 (503)					100	57	57	51	50	50	44	50	46
TSWV-B (629)						100	49	46	49	47	44	45	50
INSV (642)							100	47	52	50	46	49	49
PBNV (773)								100	51	52	45	47	48
WSMV (1261)									100	99	43	44	46
Tospo-To (1262)										100	42	47	46
PYSV (653)											100	49	49
IYSV (810)												100	46
PCFSV (455)													100

Number of nucleotides in the respective isolate is given in parenthesis

Table 4. Percent nucleotide sequence identity between medium (M) RNA intergenic region (IGR) of tospoviruses

Isolate	TSWV-BR-01	TSWV-GA-TB	TSWV-FL-PT	TSWV-JP	INSV	PBNV	WSMV
TSWV-BR-01 (319)	100	84	85	89	59	51	50
TSWV-GA-TB (272)		100	98	93	53	47	50
TSWV-FL-PT (271)			100	94	59	48	51
TSWV-JP (253)				100	52	46	52
INSV (478)					100	57	52
PBNV (408)						100	57
WSMV (477)							100

Number of nucleotides in the respective isolate is given in parenthesis

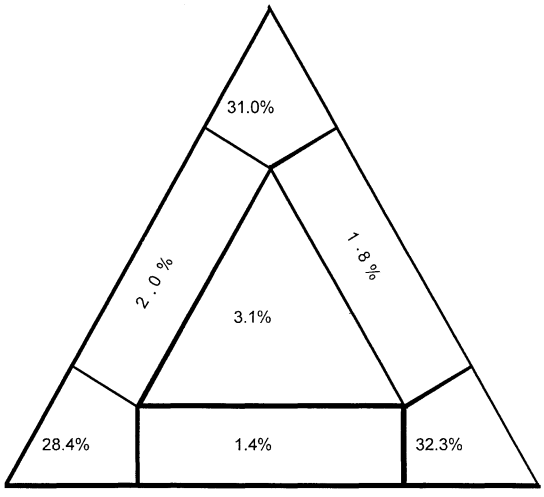

Fig. 1. Likelihood mapping analysis of intergenic region sequences of small RNA of known tospoviruses. The three regions located at the three corners of the triangle represent well-resolved phylogeny. The central region represents star-like evolution while the three sides of the triangle represent the three regions where it was difficult to distinguish between two of the three topologies that have equal likelihood with the third topology having a probability of zero [30]

different TSWV isolates. A 44 to 57% sequence identity was observed between IGR sequences of TSWV isolates and other tospovirus species (Table 3). There was 99% identity between WSMV and Tospo-To (Table 3), which are considered strains of the same species [34]. In general, identity between the S RNA IGR sequences of distinct tospovirus species varied from 42 to 57%, whereas it was 79 to 99% among strains of the same species (Table 3).

LMA revealed that 91.7% of the quartets belonged within the three regions (located at the three corners of the equi-lateral triangle) that represent well resolved phylogeny (Fig. 1). Only 3.1% of the quartets exhibited star-like evolution while 5.2% of the quartets were found to be unresolved (Fig. 1). The LMA results verified the suitability of the data set for tree reconstruction.

Quartet puzzling tree based on the S RNA IGR sequences of known tospoviruses revealed close clustering of all known TSWV isolates (except for TSWV-B) and showed them to be distinct from other topovirus species (Fig. 2). Further, TSWV isolates from a particular geographical area were closer to each other than to the isolates from other geographical areas, irrespective of the host from

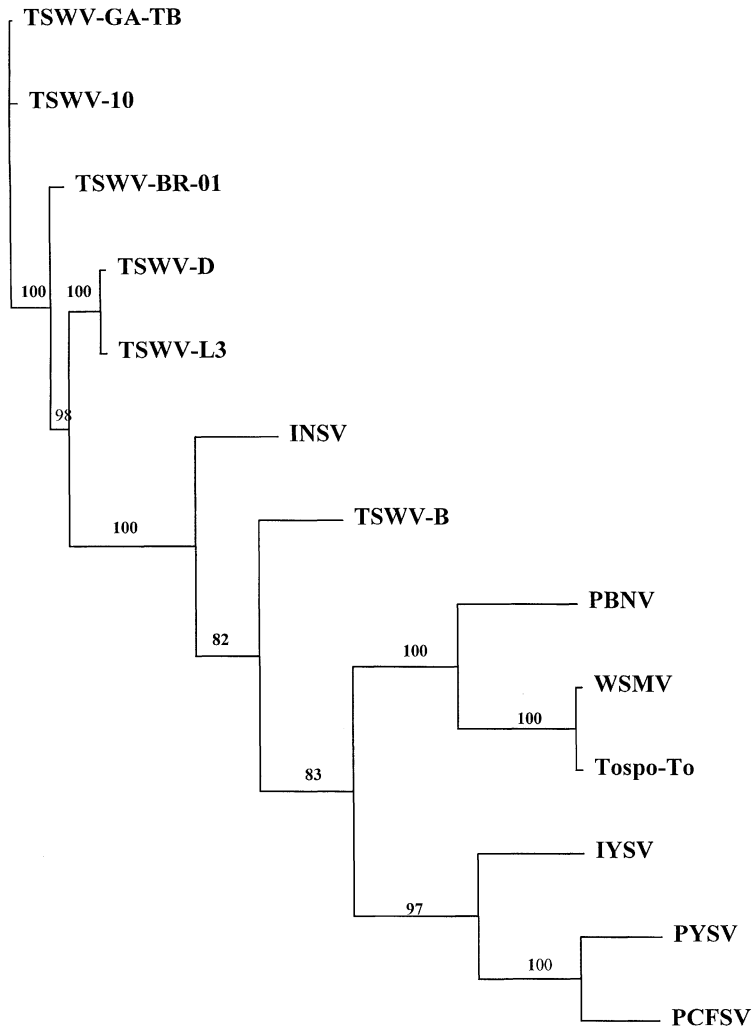

Fig. 2. Phylogenetic tree derived from the S RNA intergenic region of tospoviruses. Source of each sequence is listed in Table 1. The PUZZLE program [29] available in PAUP 4.0 [31] was used to construct the phylogenetic tree (based on 1000 puzzling steps). The phylogram was generated as an unrooted tree using TreeView [21]. The percent reliability value given above each branch indicates how often the corresponding cluster was found among the 1000 intermediate trees. Vertical length of the branches was arbitrary

which they were isolated (Table 1, Fig. 2). A similar pattern was observed when N gene sequences of several TSWV isolates from Georgia were compared [23]. Not surprisingly, WSMV and Tospo-To formed another close cluster (Fig. 2), while TSWV-B [22] and other tospoviruses such as impatiens necrotic spot virus (INSV) [16], peanut yellow spot virus (PYSV) [28], iris yellow spot virus (IYSV) [5] and peanut chlorotic fan spot virus (PCFSV) [35] reflected their distinctiveness as species in the dendrogram (Fig. 2).

Comparison of the M RNA IGR sequences also revealed a high degree of identity (84–98%) among various TSWV isolates (Tables 2, 4). In our previous study

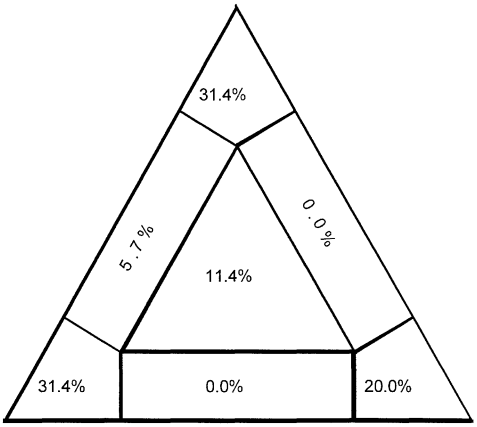

Fig. 3. Likelihood mapping analysis of intergenic region sequences of medium RNA of known tospoviruses. The three regions located at the three corners of the triangle represent well-resolved phylogeny. The central region represents star-like evolution while the three sides of the triangle represent the three regions where it was difficult to distinguish between two of the three topologies that have equal likelihood with the third topology having a probability of zero [30]

involving six isolates of TSWV from different naturally infected plant species from Georgia and a peanut isolate of TSWV from Florida, there was 83–100% sequence identity in their M RNA IGR region [3]. In contrast, TSWV isolates shared only 46 to 59% identity with other distinct tospoviruses in this region. As observed with S RNA IGRs, M RNA IGR sequences were more conserved among TSWV isolates within a geographical area than when compared to TSWV isolates from other geographical areas (Table 4). The M RNA IGRs of PBNV and WSMV, two distinct species shared a 57% identity supporting their earlier classification as distinct species [4, 27].

LMA of M IGR sequence alignments revealed that 82.9% of all quartets were found to be within the three regions representing a well resolved phylogeny. While 5.7% of them were unresolved, 11.4% represented star-like evolution (Fig. 3). Even though the percentage of quartets exhibiting star like pattern was high (11.4), the high percentage (82.9) of the well resolved quartets supported the suitability of the data set for phylogenetic tree reconstruction.

Quartet puzzling tree based on the M RNA IGR sequences revealed close clustering of TSWV isolates from US and showed them to be divergent from TSWV isolates from Brazil and Japan (Fig. 4). Other tospoviruses used in the study were discernible from each other indicating their distinctive species nature (Fig. 4).

The NP sequence identity, vector specificity and host range are currently being used as criteria for identification and differentiation of tospoviruses to the species level [6, 18, 19]. Our analysis of the IGR sequences of all available S and M RNAs of tospoviruses displayed similar differential sequence conservation among inter and intra species of tospoviruses. Greater variation in the length of the IGR was noticed between distinct species than among the strains of the same species (Tables 3, 4). Except for TSWV-B (Table 1), all other TSWV isolates showed sequence identity of 79 to 91% and 84 to 98% in their S and M RNA IGRs, respectively. TSWV-B shared 78% and 63% identity in the NP sequence with that of TSWV and INSV respectively [22], which is below the threshold level of 90% [18]. Thus TSWV-B is a distinct species based on both NP and IGR sequences. Although

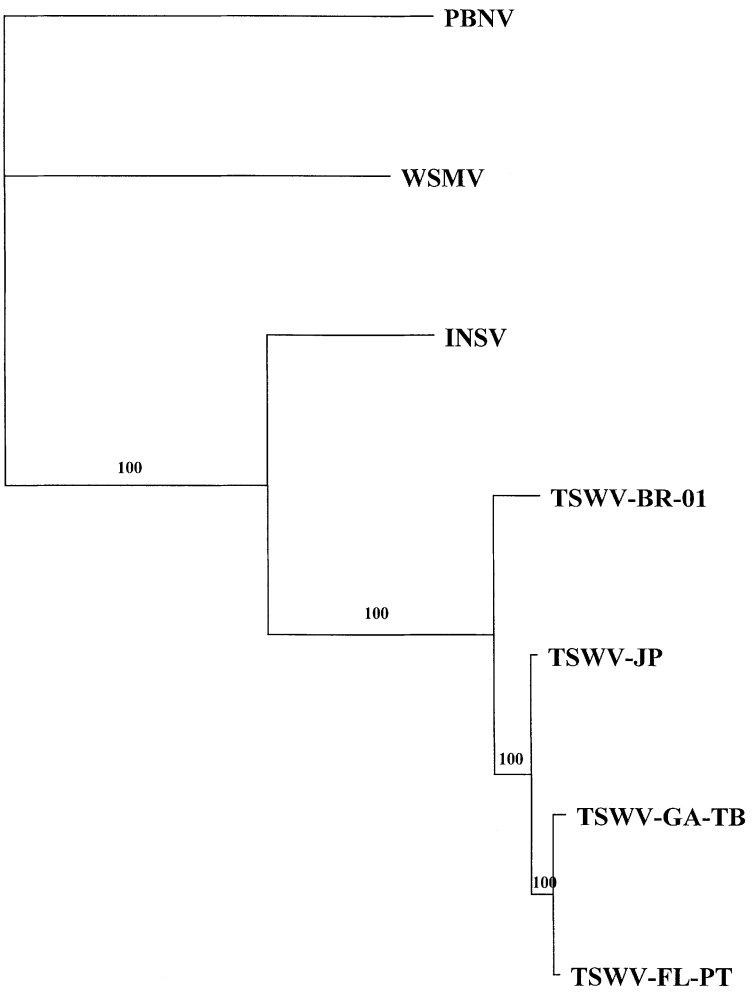

Fig. 4. Phylogenetic tree derived from the M RNA intergenic region of tospoviruses. Source of each sequence is listed in Table 2. PUZZLE program [29] available in PAUP 4.0 [31] was used to construct the phylogenetic tree (based on 1000 puzzling steps). The phylogram was generated as an unrooted tree using TreeView [21]. The percent reliability value given above each branch indicates how often the corresponding cluster was found among the 1000 intermediate trees. Vertical length of the branches was arbitrary

PBNV, WSMV and Tospo-To are grouped in the same serogroup [18], based on the host range and NP sequence similarities these viruses are considered as strains of the same virus, while PBNV is identified as a distinct species [12, 26, 34]. Our analysis based on the S RNA IGR similarities also placed PBNV as a distinct species from that of WSMV and Tospo-To. In addition, the low percent similarities in the IGRs of the S RNAs of recently described tospoviruses such as PYSV [28], IYSV [5] and PCFSV [35] support their identification as distinct tospovirus species. The potential biological significance of IGRs of tospoviruses is just beginning to be understood [24], our study identified the sequence divergence of IGRs of M and S RNAs along the lines of individual tospovirus species.

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