Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences

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The nucleotide sequences of the nucleoprotein (N) genes of seven tospovirus isolates representing three serogroups were determined and used to establish phylogenetic parameters to delineate species within the *Tospovirus* genus of the Bunyaviridae. A high sequence divergence (55·9 % identity at the nucleotide level) was observed between isolates of serogroup I (tomato spotted wilt virus) and isolates of serogroup III (*Impatiens* necrotic spot virus). The serogroup II isolates take an intermediate position. Their N genes have 75 % identity with those of serogroup I isolates and 57 % with those of serogroup III isolates. Whereas the isolates within serogroups I or III have almost identical sequences, the two isolates BR-03 and SA-05 of serogroup II diverged significantly from each other (82·1 % sequence identity). The results obtained support the conclusion that, in addition to the species TSWV and INSV, the serogroup II isolates BR-03 and SA-05 have to be considered as distinct species within the genus *Tospovirus* for which the names tomato chlorotic spot virus and groundnut ringspot virus, respectively, are proposed.

Introduction

Virus isolates described as tomato spotted wilt virus (TSWV) cause serious diseases world-wide in many crops and infect a considerable number of different plant species (Peters *et al.*, 1991). Whereas in the past TSWV infections were mainly found in (sub)tropical regions, devastating outbreaks have also occurred in non-solanaceous crops in the northern hemisphere in the last decade. This expansion has been caused by the spread of the thrips *Frankliniella occidentalis* (Perg.), an efficient vector of TSWV, over the U.S.A. and Canada, and its subsequent invasion in Europe (Cho, 1986; Marchoux, 1990; Vaira *et al.*, 1992).

Virus particles of TSWV isolates are spherical (70 to 110 nm in diameter) with a lipid membrane covered with surface projections formed by glycoproteins. The viral genome consists of three linear ssRNA segments, denoted L, M and S, complexed with nucleocapsid protein (N) and presumably with a viral transcriptase. The complete nucleotide sequences have become available for the genome of a Brazilian isolate (BR-01). The L RNA (8·9 kb) is of negative polarity and encodes a putative RNA polymerase of 331K (de Haan *et al.*, 1991). The two other genomic RNAs use ambisense coding strategies. The M RNA (4·8 kb) codes for a precursor to the two envelope proteins G1 (78K) and G2 (58K) and a non-structural protein denoted NSm (Kormelink *et al.*, 1992*a*, *b*). The S RNA (2·9 kb) encodes the N protein (28·8K) and another non-structural protein (NSs, 52·4K) (de Haan *et al.*, 1990; Kormelink *et al.*, 1991).

Based on the detailed knowledge of the BR-01 isolate, TSWV has been classified as the sole member of the newly created genus *Tospovirus* within the family Bunyaviridae (Francki *et al.*, 1991). In view of the world-wide distribution of tospovirus isolates able to infect a high number of plant species (more than 500 have been reported), one may question whether these virus isolates show enough variation to consider them as belonging to more than one single virus species. De Ávila *et al.* (1990, 1992*a*, *b*) showed that a selection of 21 isolates originating from different geographical areas and crops can be divided into three distinct serogroups by using polyclonal antibodies directed against their N protein. Most isolates studied belong to serogroup I, including type isolate BR-01. The serogroup I isolates reacted only weakly with antibodies to serogroup II viruses, and not at all with antibodies raised against serogroup III viruses. This serogroup consists of almost completely identical isolates from *Impatiens* plants in the U.S.A. (TSWV-I; Law & Moyer, 1990, 1991) and in The Netherlands (NL-07; de Ávila *et al.*, 1992*a*; de Haan *et al.*, 1992), differing entirely from the serogroup I and II viruses serologically (Law & Moyer, 1990; Law *et al.*, 1991; de Ávila *et al.*, 1992*a*, *b*). Therefore, serogroup III isolates may be considered as belonging to a different species, denoted *Impatiens* necrotic spot virus (INSV), whereas serogroup

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I may represent a species for which the name TSWV should be reserved. Furthermore, subgroup II splits into two distinct serotypes (de Ávila *et al.*, 1990), each of which possibly represents additional species. Other distinct TSWV-like isolates have been found in groundnut (Chanekar *et al.*, 1979; Reddy *et al.*, 1991, 1992) and one in watermelon (Kameya-Iwaki *et al.*, 1988), but their relationships to serogroups I, II and III have not yet been fully characterized.

To establish criteria for defining the various tospoviruses as species, the nucleotide sequences of the N genes and the amino acid composition of their products of seven isolates, preliminarily classified into three serogroups, were determined and compared.

Methods

Virus isolates. Isolate BR-03 was collected from tomato in Brazil and SA-05 was kindly supplied to Dr G. Adam (Braunschweig, Germany) by G. Pietersen after isolation from groundnut in South Africa. Both isolates, classified as serogroup II members (de Ávila *et al.*, 1990, 1992*b*) were multiplied in *Nicotiana rustica* L. var. America. RNA was extracted from purified nucleocapsids as described previously (de Ávila *et al.*, 1990; de Haan *et al.*, 1991).

DNA clones and nucleotide sequence analysis. cDNA was synthesized according to Gubler & Hoffman (1983). The RNA was primed by a synthetic oligonucleotide (5′ CCCGGATCCTGCAGAGCAATTG-TGTCA 3′), containing a *Bam*HI site (underlined), complementary to the first 15 nucleotides at the 3′ end of S RNA which is conserved between isolates BR-01 and NL-07 (serogroup III; de Haan *et al.*, 1992). ds cDNA was made blunt-ended with T4 DNA polymerase and subsequently digested with *Bam*HI, resulting in *Bam*HI/blunt-end cDNA fragments. These fragments were cloned in pUC19 plasmid vectors which had been digested with *Bam*HI and *Sma*I (Yanisch-Perron *et al.*, 1985). The specificity of clones was confirmed by Northern blot hybridization (data not shown). Clones covering the N gene of BR-03 and SA-05 were selected and their nucleotide sequences determined with alkaline-denatured plasmid DNA as templates, using either the standard M13 forward and reverse sequencing primers (Zhang *et al.*, 1988) or synthetic oligonucleotides complementary to previously determined sequences. Sequence alignments were performed using the GCG Wisconsin software package (Devereux *et al.*, 1984).

Results

Molecular cloning and sequence analysis of the N genes of isolates BR-03 and SA-05

The sequence of the N gene of various serogroup I and III isolates has been reported previously (de Haan *et al.*, 1990, 1992; Maiss *et al.*, 1991; Law & Moyer, 1991). To obtain sequence information of two distinct serogroup II isolates (BR-03 from Brazil and SA-05 from South Africa) their N genes were cloned. Using a specific primer complementary to the 3′ termini of the genomic S RNA of BR-01 or NL-07, several cDNA clones of BR-03 and SA-05 isolates, approximately 1 kb long and containing the complete coding regions of the respective N proteins were obtained. Sequence determination of these clones revealed that for both isolates the N gene ranged from nucleotides 153 to 942 (numbered from the 5′ end of the virus-complementary strand) (Fig. 1), corresponding to an N protein of 258 amino acid residues with an M_r of 28677 (BR-03) or 28836 (SA-05). Both these figures and the homology of the predicted gene products to the N protein of isolate of BR-01 (see below) confirm that these cloned sequences represented the N genes of the respective isolates. An alignment of the N gene sequences of serogroup II isolates BR-03 and SA-05 with those of serogroup III isolates BR-01 and NL-07 is presented in Fig. 1.

Divergence among S RNA sequences of different tospovirus isolates

The determined nucleotide sequences of the S RNA of BR-03 and SA-05 were compared with those of BR-01 (TSWV) and NL-07 (INSV). The alignment shown in Fig. 1 reveals that only the first 15 nucleotides at the 3′ termini are fully conserved, the remaining part of the 3′ non-coding sequence showing a remarkably lower degree of similarity. In this region (until the start of the N gene) the serogroup II isolates show 65·8 (BR-03) to 70·9% identity (SA-05) with serogroup I and only 46 to 47% with serogroup III. The similarity in the 3′ non-translated region of the S RNA of serogroup I and III isolates was estimated to be 50·7% (de Haan *et al.*, 1992). In contrast, the sequence of this region is highly conserved (99%) among isolates within serogroups I or III, but is less conserved between the two serogroup II isolates (88·1%, Fig. 1 and Table 1).

The same conclusions that were derived from the non-coding region can be drawn from the translated region within the various determined S RNA sequences, i.e. the N genes show similar levels of divergence and conservation between and within serogroups, respectively (Fig. 1, Table 1).

Divergence among N protein sequences of different tospovirus isolates

Since the serogrouping of tospoviruses (de Ávila *et al.*, 1990, 1992*a*, *b*) has been based on analyses using poly- and monoclonal antibodies directed to the N protein, it is worthwhile to determine whether this grouping agrees with the rates of divergence in the N protein sequences. Moreover, this sequence divergence could be used as a molecular parameter, and thus a true phylogenetic criterion for the classification of tospoviruses into species and strains. To this end an alignment was made of the newly determined serogroup II N protein sequences with those of previously published sequence data from

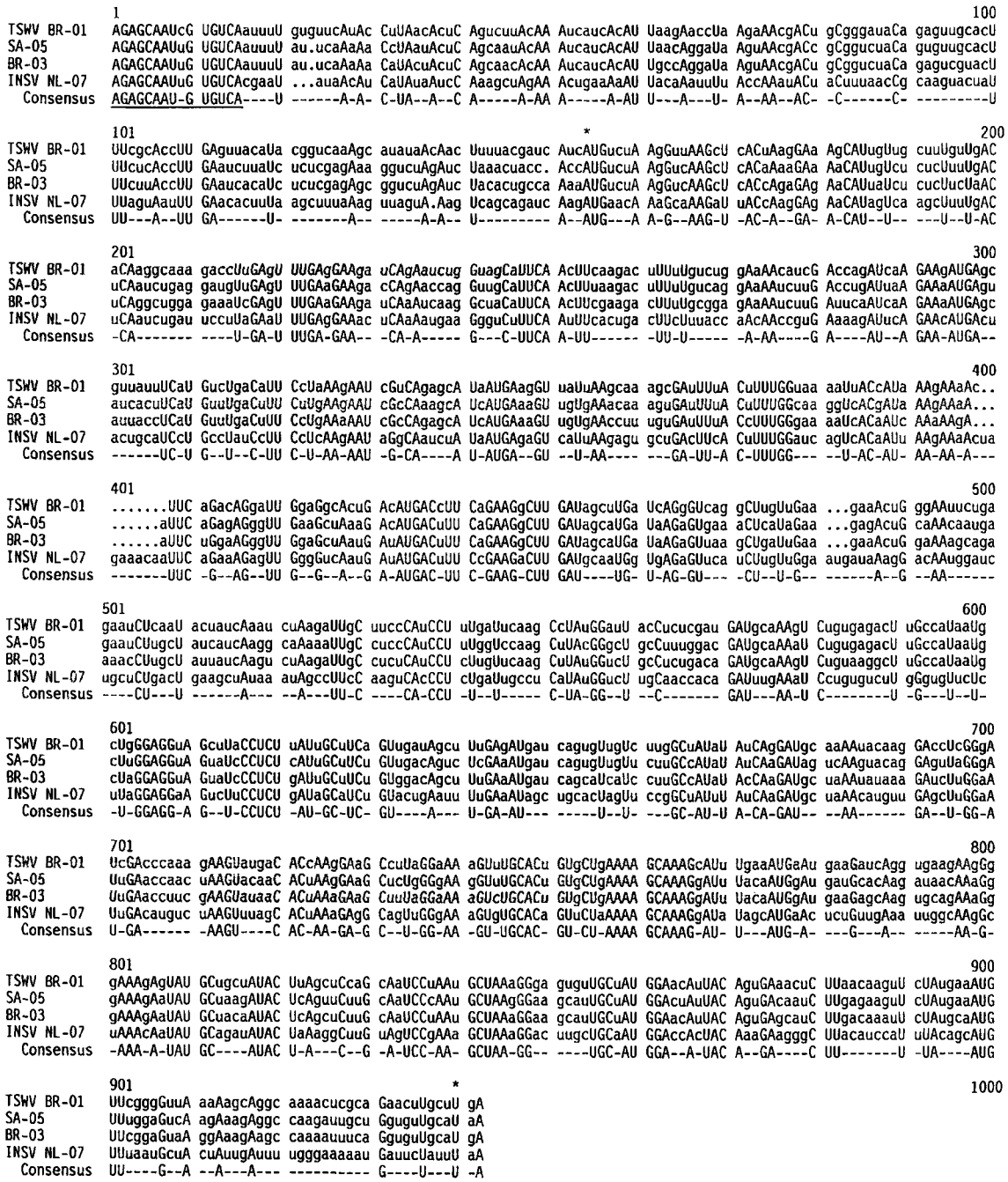

Fig. 1. Alignment of the 3′ part of the S RNA molecules carrying the N gene of four tospovirus isolates representing three serogroups. Nucleotides are numbered from the 5′ end of the virus-complementary strand. The nucleotide sequences of the isolates TSWV-L3, TSWV-Haw (serogroup I) and TSWV-I (serogroup III) are not shown since they are almost identical to that of BR-01 (serogroup I) and NL-07 (serogroup III), respectively. Asterisks indicate translational start and termination codons. Dots represent gaps introduced to reach an optimal alignment. The 3′-terminal consensus sequence is underlined.

serogroup I isolates BR-01, HAW (from Hawaii), L3 from Bulgaria (de Haan *et al.*, 1990; Maiss *et al.*, 1991; T. L. German, personal communication) and NL-07 (de Haan *et al.*, 1992; Law *et al.*, 1991). The alignment is shown in Fig. 2 and the calculated percentages of sequence identity in Table 1. The N protein sequences of the two serogroup II isolates shared 76·4% (BR-03) to 78·2% (SA-05) identity with all serogroup I isolates, the latter showing $\geqslant 99\%$ identity to each other. The similarities in the protein sequences are considerably lower (56 to 54%) between serogroup II and serogroup III isolates; this is similar to the relationship between serogroup I and III N protein sequences (Table 1). As found for the whole sequenced S RNA region, the N

Table 1. *Nucleotide and amino acid sequence identities (%) of the 3′ part of the S RNA molecules of four tospovirus isolates encoding the N protein*

	BR-03	SA-05	NL-07
BR-01	65·8*	70·9	50·7
	74·2†	75·9	55·9
	76·4‡	78·2	55·4
BR-03		88·1	46·0
		82·1	57·6
		81·0	56·0
SA-05			47·0
			57·3
			54·0

* Nucleotide sequence identity of 3′ non-coding region of the S RNA.
† Nucleotide sequence identity of the N gene.
‡ Amino acid sequence identity of the N protein.

protein sequences of both serogroup II isolates also diverged significantly, showing only 81% identity. Despite this clear distinction, both serogroup II isolates are serologically more closely related to each other than to the isolates of the other serogroups (de Avila *et al.*, 1990, 1992*b*).

Finally, examination of the alignment of the N protein sequences (Fig. 2) of the three serogroups reveals that four main domains in the N proteins are highly conserved particularly between residues 51 and 204. Analysis of the N proteins showed two hydrophilic domains located between residues 30 and 40, and 160 and 190. A highly conserved hydrophobic domain was also identified between residues 125 and 160. It is noteworthy that the positions of the methionine residues in the N proteins of all isolates studied are conserved and that mainly alpha helix-forming amino acids are present.

Discussion

Traditionally, the classification of Bunyaviridae has been based mainly on serology, which resulted in a categorization of these viruses into serogroups and serotypes (Elliott, 1990). Following this classification, and using the antigenic properties of the N protein, three serogroups (I to III) have thus far been recognized within *Tospovirus*, a genus of the Bunyaviridae (Francki *et al.*, 1991). Since the International Committee on Taxonomy of Viruses has recently introduced the species concept in virus taxonomy, it is important to define criteria to delineate species and to avoid the use of less discriminative concepts such as serogroups and -types. In this paper the nucleotide sequences of the N protein genes of two serogroup II isolates, BR-03 and SA-05, have been determined and compared with those of serogroup I and III isolates to establish discriminative and phylogenetic criteria to define *Tospovirus* species. The crucial role played by this protein in processes such as regulating the switch from transcription to replication, functioning in the replication complex and encapsidating genomic and anti-genomic RNA (Beaton & Krug, 1984, 1986) supports its selection for defining taxonomic criteria for tospoviruses.

The phenotypic and molecular characteristics of the serogroup I and III isolates are basically very different

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          1                                                                  100
TSWV BR-01 MsKvKlTkEs IvaLLTQgkd lEFEEdQnlv aFNFktFcle NidqIkkMsv iSCLtFLKNR QSIMkVikqs DFTFGkiTIK Kt...SdRig gTDMTFRRLD
TSWV L3    MsKvKlTkEs IvaLLTQgkd lEFEEdQnlv aFNFktFcle NldqIkkMsv iSCLtFLKNR QSIMkVikqs DFTFGkiTIK Kt...SdRig aTDMTFRRLD
TSWV Haw   MsKvKlTkEs IvaLLTQgkd lEFEEdQnlv aFNFktFcle NldqIkkMsi iSCLtFLKNR QSIMkVikqs DFTFGkiTIK Kt...SdRig aTDMTFRRLD
SA-05      MsKvKlTkEn IvsLLTQsed vEFEEdQnqv aFNFktFcqe NldlIkkMsi tSCLtFLKNR QSIMkVvkqs DFTFGkvTIK Kn...SeRve akDMTFRRLD
BR-03      MsKvKlTrEn IisLLTQage iEFEEdQika tFNFedFcge NldsIkkMsi tSCLtFLKNR QSIMkVvnlc DFTFGkiTIK Kn...SgRvg anDMTFRRLD
INSV NL-07 MnKaKiTkEn IvkLLTQsds lEFEEtQneg sFNFtdFftn NrekIqnMtt aSCLsFLKNR QSIMrViksa DFTFGsvTIK KtrnnSeRvg vnDMTFRRLD
INSV I     MnKaKiTkEn IvkLLTQsds lEFEEtQneg sFNFtdFftn NrekIqnMtt aSCLsFLKNR QSIMrViksa DFTFGsvTIK KtrnnSeRvg vnDMTFRRLD
Consensus  M-K-K-T-E- I--LLTQ--- -EFEE-Q--- -FNF--F--- N---I--M-- -SCL-FLKNR QSIM-V---- DFTFG--TIK K----S-R-- --DMTFRRLD

          101                                                                200
TSWV BR-01 sliRVrLve. etgnsenLnt ikskIaSHPL iqaYGLpldD aKSvrLaimL GGSlPLIASV dsfEmisvVL AIYQDakykd LGIdpkKyOT kEAlGKVCTV
TSWV L3    sliRVrLve. etgnsenLnt ikskIaSHPL iqaYGLpldD aKSvrLaimL GGSlPLIASV dsfEmisvVL AIYQDakykd LGIdpkKyOT kEAlGKVCTV
TSWV Haw   sliRVrLve. etgnsenLnt ikskIaSHPL iqaYGLpldD aKSvrLaimL GGSlPLIASV dsfEmisvVL AIYQDakykd LGIdpkKyOT rEAlGKVCTV
SA-05      smiRVkLie. etannenLai ikakIaSHPL vqaYGLpldD aKSvrLaimL GGSiPLIASV dslEmisvVL AIYQDsqvqe LGIeptKynT kEAlGKVCTV
BR-03      smiRVkLie. etgkaenLai ikskIaSHPL vqaYGLpltD aKSvrLaimL GGSiPLIASV dsfEmisiiL AIYQDakykd LGIepsKynT kEAlGKVCTV
INSV NL-07 amvRVhLvgm ikdngsaLte ainslpSHPL iasYGLattD lKScvLgvlL GGSlPLIASV lnfEiaalpL AIYQDakhve LGIdmsKfsT kEAvGKVCTV
INSV I     amvRVhLvgm ikdngsaLte ainslpSHPL iasYGLattD lKScvLgvlL GGSlPLIASV lnfEiaalvL AIYQDakhve LGIdmsKfsT kEAvGKVCTV
Consensus  ---RV-L--- -------L-- ----I-SHPL ---YGL---D -KS--L---L GGS-PLIASV ---E-----L AIYQD----- LGI---K--T -EA-GKVCTV

          201                                                  262
TSWV BR-01 LKSKafeMne dqvkKgKeYA aILsssnPnA KGsvAMehYs EtLnkfYeMF gvkkqaklae la
TSWV L3    LKSKafeMne dqvKKgKeYA aILsssnPnA KGsiAMehYs EtLnkfYeMF gvkkqaklte la
TSWV Haw   LKSKafeMne dqvkKgKeYA aILsssnPnA KGsiAMehYs EtLnkfYeMF gvkkqaklte la
SA-05      LKSKgftMdd aqdnKgKeYA kILsssnPnA KGsiAMdyYs DnLekfYeMF gvkkeakiag va
BR-03      LKSKgftMde eqvqKgKeYA tILsscnPnA KGsiAMehYs EhLdkfYaMF gvrkeakisg va
INSV NL-07 LKSKgysMns veigKaKqYA dILkacsPkA KGlaAMdhYk EgLtsiYsMF natidfgknd si
INSV I     LKSKgysMns veigKaKqYA dILkacsPkA KGlaAMdhYk EgLtsiYsMF natidfgknd si
Consensus  LKSK---M-- ----K-K-YA -IL----P-A KG--AM--Y- E-L---Y-MF ----------
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Fig. 2. Alignment of the N protein sequences of seven tospovirus isolates representing three serogroups. Dots represent gaps introduced to reach optimal alignment.

Table 2. *Proposed species within the genus* Tospovirus (*Bunyaviridae*)

Isolate	Origin		Sero-group	Sero-type	Species	Reference
	Country	Host				
BR-01	Brazil	Tomato	I		TSWV	de Haan *et al.* (1990) de Ávila *et al.* (1990)
BR-03	Brazil	Tomato	II	I	TCSV	de Ávila *et al.* (1990, 1992*b*)
SA-05	South Africa	Groundnut	II	II	GRSV	de Ávila *et al.* (1990, 1992*b*)
TSWV-I	U.S.A.	*Impatiens*	III		INSV	Law *et al.* (1991, 1992)
NL-07	Netherlands	*Impatiens*				de Ávila *et al.* (1992*a*, *b*) de Haan *et al.* (1992)

(Table 1; Law & Moyer, 1990; Law *et al.*, 1991; de Haan *et al.*, 1992; de Ávila *et al.*, 1992*a*) and their N protein sequences have diverged to such an extent (55·4% identical) that these two groups can certainly be defined as two different species. Serogroup I contains the original TSWV isolates, including the type isolate BR-01 (de Ávila *et al.*, 1990), and hence we propose that the name TSWV be employed for isolates of serogroup I (Table 2). The isolates of serogroup III represent a second species, recently proposed as INSV (Law & Moyer, 1990; Law *et al.*, 1991, 1992; de Ávila *et al.*, 1992*a*; de Haan *et al.*, 1992).

With respect to serogroup II isolates it was evident from serological studies that they differ notifiably from TSWV and INSV (de Ávila *et al.*, 1992*b*). Their taxonomic status could not unequivocally be clarified owing to overlapping phenotypic characteristics such as host range, symptom expression and cytopathology. Serology using monoclonal antibodies against the N protein showed that these isolates could be divided into two serotypes. Analysis of the nucleotide sequences of the N gene, the amino acid sequence of the N protein and the nucleotide sequences of the untranslated 3′ end regions of the S segment showed that these sequences are 82·1, 81·0 and 88·1% identical, respectively, between BR-03 (serotype I) and SA-05 (serotype II) (Table 1). Although the rate of divergence between these two isolates is less than that between TSWV and INSV isolates, we propose that they should be considered to be two distinct species. This proposal is supported by the observation that the N genes within the TSWV and INSV species, with isolates originating either from Brazil, Hawaii and Bulgaria, or from the U.S.A. and The Netherlands, have almost 100% identical nucleotide sequences. The two novel species may be named tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV) for the serotype I (BR-03) and II (SA-05) viruses (Table 2). The relationship between these *Tospovirus* species, based on nucleotide and amino acid sequence homology, is represented diagrammatically in Fig. 3.

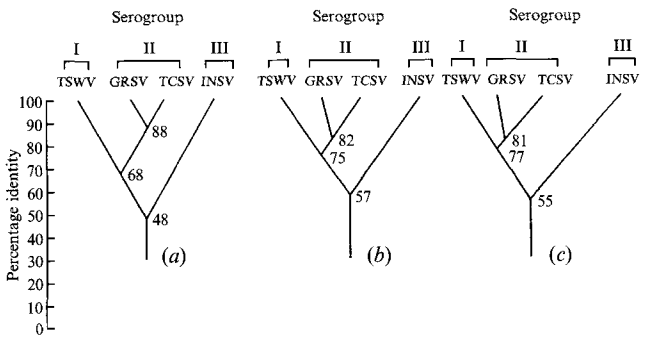

Fig. 3. Putative phylogenetic trees of tospoviruses based on the percentage identity of the nucleotide sequences of the non-coding (*a*) and coding (*b*) regions of the N gene encoded by the S RNA segment. (*c*) Amino acid sequence identity in the nucleocapsid protein.

Other tospoviruses, not yet fully characterized, are currently reported as possible new species (Kameya-Iwaki *et al.*, 1988; Chanekar *et al.*, 1979; Reddy *et al.*, 1991, 1992) but clarification of their taxonomic position awaits further studies.

It is noteworthy that the similarity in N proteins among the three serogroups (now species) of the *Tospovirus* genus is higher than among serogroups of the *Bunyavirus* and *Phlebovirus* genera. The N proteins of six viruses representing three serogroups in the genus *Bunyavirus* show an overall sequence similarity of 40%, whereas $\geqslant$ 80% similarity is observed within a serogroup (Elliott, 1990). The *Phlebovirus* genus N proteins show a degree of similarity which varies from 54 to 30% among serogroups and a higher relatedness within single serogroups (Simons *et al.*, 1990; Giordi *et al.*, 1991). However, the amino acid sequence identity of the *Hantavirus* genus N proteins varies from 61 to 83% (Arikawa *et al.*, 1990; Stohwasser *et al.*, 1990). These values resemble those now found for the *Tospovirus* genus. The similar degrees of relatedness found in both genera might be explained by stronger constraints on the evolution of the N protein genes. The limited number of vectors used by tospoviruses (Sakimura, 1962) and the non-biological transmission of the hantaviruses between

rodents and humans (Gonzales-Scarano & Nathanson, 1990) may be two of these constraints. The viruses of the other two genera, *Bunyavirus* and *Phlebovirus*, displaying higher divergence in the N protein, are transmitted by vector species belonging to different families or orders such as mosquitoes, *Culicoides*, phlebotomines and ticks (Gonzales-Scarano & Nathanson, 1990; Peters, 1991).

The Bunyaviridae are classified into five genera based on their mode of transmission, genome coding strategy and composition of the 3′- and 5′-terminal sequences (Francki *et al.*, 1991). The viruses within the four genera consisting of viruses infecting animals are basically classified into antigenic groups using haemagglutinin and neutralizing antigenic determinants present on virus glycoproteins and complement fixation associated with the N protein (Gonzales-Scarano & Nathanson, 1990). These serological and molecular characteristics of some viruses support the existence of several virus species within each genus of this family (Gonzales-Scarano & Nathanson, 1990; Elliott, 1990). However, the formulation of parameters or criteria by which species could be distinguished taxonomically within these genera, as now proposed for tospoviruses is awaited.

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