

## Molecular analysis of whitefly-transmitted tomato geminiviruses from Southeast and East Asia

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### ABSTRACT

Tomato cultures are under the constant threat of geminiviruses transmitted by the whitefly *Bemisia tabaci*. In the affected regions, yield losses often reach 100%. In Southeast and East Asia, as well as in many countries of the Old World, these viruses have been termed tomato yellow leaf curl virus (TYLCV) or tomato leaf curl virus (ToLCV). These viral diseases are becoming more widespread, usually following appearance or increases in whitefly populations. Although leaf curl-related diseases of tomato have been reported throughout Southeast and East Asia, the molecular data available on these viruses is still scarce. The genome of TYLCV and ToLCV isolates has been cloned from infected tomato plants from Bangladesh, China, India, Malaysia, Sri Lanka, Taiwan, The Philippines and Thailand, and sequenced partially or entirely. DNA and protein sequence comparisons indicate that many TYLCV and ToLCV isolates analyzed so far constitute distinct virus species and are not strains of the same species. Analysis of their coat proteins indicate that these viruses group into two branches that include the viruses from China, Malaysia, Taiwan and Thailand (together with the virus from Australia) and those from Bangladesh, India and Sri Lanka. The whitefly-transmitted tomato geminiviruses from Southeast and East Asia constitute a cluster of geminiviruses distinct from those of the Middle East, Southeast Europe and of the Americas.

**Key words:** geminivirus, tomato, tomato leaf curl virus, tomato yellow leaf curl virus, virus, whitefly

### INTRODUCTION

Tomatoes cultivated in regions within a large tropical and subtropical belt around the globe are under the constant threat of whitefly-transmitted geminiviruses. Losses due to these viruses often reach 100% of the crop (Green and Kalloo 1994). Disease symptoms include inhibition of growth, yellowing and curling of leaves and stunting. In the Old World (Middle East, Southwest Europe, Tropical Africa, East and Southeast Asia, and Australia) these diseases are caused by a group of viruses denominated tomato yellow leaf curl virus (TYLCV) or tomato leaf curl virus (ToLCV). The

geminiviruses infecting tomato from the New World are genetically different from the TYLCV/ToLCV complex (Rybicki 1994, Padidam *et al.* 1995a). The most prominent were termed as tomato mottle virus in Florida (TMoV), chino del tomate virus in Mexico (CdTV), tomato golden mosaic virus in central and south America (TGMV), tomato yellow mosaic virus in south America (TYMV) (Polston and Anderson 1997).

Geminiviruses infecting tomatoes are rapidly spreading to regions where they were unknown before, threatening tomato production. In Asia, a TYLCV has recently conquered new areas in Iran

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**Abbreviations:** aa- amino acid, PCR- polymerase chain reaction, ToLCV- tomato leaf curl virus, TYLCV - tomato yellow leaf curl virus., IR- intergenic region, CP- capsid protein, Rep- replication- associated protein

(Hajimorad *et al.* 1996), in Asian Republics of the former USSR - Azerbaidjan, Turkmenistan and Uzbekistan (Anonymous 1993), and in China (Liu *et al.* 1997). The spread of TYLCV/ToLCV-like diseases and of many other whitefly-transmitted geminiviruses has often paralleled the worldwide expansion of the B biotype of *B. tabaci* (also known as the silverleaf whitefly *B. argentifolii*, Perrring *et al.* 1993, Bedford *et al.* 1994a, Brown 1995). In Asia (India, Pakistan), non-B whitefly biotypes (H, K) have been detected by esterase banding patterns; these biotypes were able to transmit TYLCV/ToLCV (Bedford *et al.* 1992).

A great deal of information has accumulated during the last decade on the molecular properties of geminiviruses in general and on that of TYLCV/ToLCV in particular (Rybicki 1994, Padidam *et al.* 1995, Czosnek and Laterrot 1997). Until the end of the eighties, it was considered that the genome of the whitefly-transmitted geminiviruses was composed of two circular single-stranded DNA genomic molecules of about 2.8 kb each (denominated DNA A and DNA B) (Lazarowitz 1992). Today, both the ToLCVs and the TYLCVs include members that have one (monopartite) (Navot *et al.* 1991, Kheyr-Pour *et al.* 1991, Dry *et al.* 1993, Noris *et al.* 1994, Chatchawankanphanich *et al.* 1995, Crespi *et al.* 1995) and two (bipartite) genomic components (Rochester *et al.* 1994, Padidam *et al.* 1995b). The monopartite TYLCV/ToLCV genome strand (or DNA-A of bipartite viruses) encodes two genes (V1 and V2), including the capsid protein (V1) (Kallender *et al.* 1988). The virus genome complementary DNA encodes four genes (C1 to C4), including the replication-associate protein (C1 or Rep) necessary for virus replication (Elmer *et al.* 1988, Laufs *et al.* 1995). The DNA-B genome encodes two genes, BR1 and BL1, involved in virus translocation (Ingham *et al.* 1995, Noueiry *et al.* 1994). A 150 to 300 nucleotide-long intergenic region (IR) (almost identical in DNA-A and B) contains a conserved stem-loop structure of 30 nucleotides bearing the origin of replication (Heyraud-Nitschke *et al.* 1995).

Many tomato diseases in Asia have been attributed to TYLCV or ToLCV (Green and Kalloo 1994). However, with the exception of a few isolates, the viruses causing these diseases have been poorly characterized, if at all. In this paper, we describe the status of the TYLCV/ToLCV diseases in Southeast and East Asia and summarize the molecular data available at the end of 1998 in relation with the other known geminiviruses affecting tomatoes.

## MATERIALS AND METHODS

### Comparison of sequences of TYLCV/ToLCV genes and proteins

The nucleotide sequences of the virus genomes and the amino acid sequences derived from the genes encoding the capsid (CP) and replication-associated (Rep) proteins were retrieved from the gene banks. The sequences of the geminivirus isolates infecting tomato used in this study are indicated in Table 2. Sequences were aligned using the DNAMAN software (Lynnon BioSoft, Quebec, Canada). Homology trees were set up with the distance matrix using the UPGMA method (Sneath and Sokal 1973). Phylogenetic trees were setup with the distance matrix using the Neighbor-Joining method (Saitou and Nei 1987). Bootstrap tests (5,000 replicates) were carried out to assess the confidence value of the phylogenetic trees. TYLCV/ToLCV isolates have been named according to Padidam *et al.* (1997). The sequence of the leafhopper-transmitted wheat dwarf geminivirus (WDV, MacDowell *et al.* 1985) was used as outgroup.

## RESULTS AND DISCUSSION

### Distribution of TYLCV/ToLCV diseases in Southeast and East Asia

Table 1 shows the spread of the TYLCV/ToLCV-related diseases as compiled from the literature. These diseases have been described in almost all countries of Southeast and East Asia, some as early as 1948 (Vasudeva and Samraj 1948), while others are recent. Everywhere, these diseases are associated with serious losses of yield, making TYLCV/ToLCV an important factor in local agricultural economies. Generally TYLCV/ToLCV has been identified by the appearance of the diseased plant associated with large whitefly populations. Geminiviral particles have been observed using the electron microscope only in a few cases (viruses from India, Taiwan, Thailand and Pakistan). In some cases, antibodies raised against heterologous geminiviruses have been used to identify or to confirm the tomato pathogen as a geminivirus (China, India, Indonesia, Malaysia, Pakistan, The Philippines, Taiwan and Thailand). Heterologous cloned geminiviral DNA have been used as probes to screen for the presence of infected tomato plants in hybridization assays (China, India, Nepal, Pakistan, Taiwan and Thailand). The genome of TYLCV/ToLCV isolates has been cloned and sequenced (entirely or partially) from China, India,

Table 1. Spread and characterization of TYLCV/ToLCV in Southeast and East Asia.

Country	First report	Serology	Particles	Hybridization (H), PCR (P)	DNA Sequence
India	1948	+	+	H,P	complete A, A+B
The Philippines	1971	+	-	P	A partial
Bangladesh	1974	-	-	P	A partial
Thailand	1980	+	+	H	A+B complete
Pakistan	1982	+	+	H,P	-
Taiwan	1984	+	+	H,P	A complete
Indonesia	1985	+	-	-	-
Malaysia	1986	-	-	P	A partial
Cambodia	1989	-	-	-	-
China	1992	+	-	H	A complete
Nepal	1990	-	-	H,P	-
Sri Lanka	1995	-	-	P	A partial

<sup>1</sup>Based on symptoms and on the presence of large whitefly populations. See references in text.

and Thailand. Recently the polymerase chain reaction (PCR) has been applied to the diagnosis of TYLCV/ToLCV (Bangladesh, China, India, The Philippines, Sri Lanka, Taiwan and Thailand). Genomic DNA fragments of ToLCV from Bangladesh, Malaysia, Sri Lanka, Taiwan and The Philippines have been PCR-amplified using degenerate primers allowing amplification of whitefly-transmitted geminivirus DNA (Rojas *et al.* 1993), cloned and sequenced.

A whitefly-transmitted disease causing yellowing and curling of tomato leaves was reported in India as early as 1948 and termed tomato leaf curl virus disease (Vasudeva and Samraj 1948). This was the first report of the presence of ToLCV in Asia. The disease is serious in all tomato growing areas throughout India and yield losses may reach 100% (Datar 1984). Extensive investigations over the years have shown that these diseases were caused by a complex of whitefly-transmitted geminiviruses (Muniyappa *et al.* 1991, Reddy *et al.* 1981). Several ToLCV isolates from different regions have been cloned and sequenced: from New Delhi, Lucknow and Bangalore. ToLCV isolates from New Delhi have a bipartite genome (Padidam *et al.* 1995), while those from Bangalore seem to possess a single genomic component (Chatchawankanphanich *et al.* 1993, 1995; Hong and Harrison 1995).

In Bangladesh, ToLCV was first reported in 1974 (Talukdar 1974, Akanda 1991) and presently occurs in almost all the districts of the country. It is the most destructive disease of tomato in that country and geminivirus-related diseases may cause the loss of 100% of the crop. Many strains seem to exist but none has been identified and characterized yet. We have recently amplified by PCR a 1.4 kbp DNA fragment from ToLCV-infected tomato using the PALv1978 and PARc715 degenerate primer pairs that have been used to amplify and clone several whitefly-transmitted geminiviruses (Rojas *et al.* 1993). This fragment has been sequenced; it includes the 233 N terminal amino acids (a.a.) of the Rep protein, the IR and the 118 N-terminal a.a. of the coat protein of a DNA-A-like genomic component (Green

Table 2. Origin of TYLCV/ToLCV isolates used for sequence comparison.

Country (region)	Abbreviation	GenBank accession <sup>1</sup>	Reference
China	TYLCV-CN	(C) D88773	Liu <i>et al.</i> 1997
Cuba	TYLCV-CU	(C) U65089	Martinez Zubiaur <i>et al.</i> 96
Dominican Republic	TYLCV-DO	(C) AF024715	Nakhla <i>et al.</i> 1994
Egypt	TYLCV-EG	(C) L12219	Nakhla <i>et al.</i> 1993
Israel, virulent	TYLCV-IL	(C) X15656	Navot <i>et al.</i> 1991
Israel, mild	TYLCV-IL/Mild	(C) X76319	Antignus & Cohen, 1994
Italy (Sardinia)	TYLCV-IT/Sar	(C) X61153	Kheyr-Pour <i>et al.</i> 1991
Italy (Sicily)	TYLCV-IT/Sic	(C) Z28390	Crespi <i>et al.</i> 1995
Senegal	TYLCV-SN	(C) D88800	Liu <i>et al.</i> 1996
Saudi Arabia (North)	TYLCV-NSA	(P)	Hong and Harrison 1995
Saudi Arabia (South)	TYLCV-SSA	(P)	Hong and Harrison 1995
Spain	TYLCV-ES	(C) Z25751	Noris <i>et al.</i> 1994
Thailand	TYLCV-TH	(C) X63016	Rochester <i>et al.</i> 1994
Yemen	TYLCV-YE	(P) X79429	Bedford <i>et al.</i> 1994b
Bangladesh	ToLCV-BD1	(P)	Shih <i>et al.</i> , 1998
Bangladesh	ToLCV-BD2	(P)	Shih <i>et al.</i> , 1998
India (New Delhi) mild	ToLCV-Nde/Mld	(C) U15016	Padidam <i>et al.</i> 1995b
India (New Delhi) severe	ToLCV-Nde/Svr	(C) U15015, U15017	Padidam <i>et al.</i> 1995b
India (Lucknow)	ToLCV-Luc	(P) X78956, X89653	Shrivastava <i>et al.</i> 1995
India (Bangalore)	ToLCV-Ban1	(P) L12738, L11746, L12739	Chatchawankanphanich <i>et al.</i> 1993
India (Bangalore)	ToLCV-Ban2	(C) Z48182	Hong and Harrison 1995
India (Bangalore)	ToLCV-Ban3	(C) U38239	Chatchawankanphanich <i>et al.</i> 1995
India (Bangalore)	ToLCV-Ban4	(C)	Muniyappa <i>et al.</i> , unpublished
Malaysia	ToLCV-MY	(C)	Shi <i>et al.</i> , 1998b
Sri Lanka	ToLCV-SL	(P)	Green <i>et al.</i> , unpublished
The Philippines	ToLCV-PH	(P)	Shih <i>et al.</i> , 1997
Taiwan	ToLCV-TW	(C) U88692	Chiang <i>et al.</i> 1997
Australia	ToLCV-AUS	(C) S53251	Dry <i>et al.</i> 1993
Brazil	TGMV	(C) K02029	Hamilton <i>et al.</i> 1984
USA (Florida)	ToMoV	(C) L14460	Abouzzid <i>et al.</i> 1992
Czechoslovakia	WDV	(C) X02869	MacDowell <i>et al.</i> 1985

<sup>1</sup>Viruses have been sequenced completely (C) or partially (P).

*et al.* unpublished).

In Pakistan tomato plants showing ToLCV-like symptoms were identified in the early 1980s. Now symptoms are present in all tomato-growing regions in Sind and Punjab. Extracts of diseased plants reacted with an antiserum against TYLCV (Solangi *et al.* 1983, Rana *et al.* 1992). An isolate of ToLCV was obtained in 1993 and maintained in graft-inoculated tomato (Harrison *et al.*, 1997). No molecular data are available on this isolate. Universal DNA primers for whitefly-transmitted geminiviruses were used to amplify a full-length DNA A-like geminiviral genome from ToLCV-diseased tomato plants. Similarly, degenerate primers pBLv2040 and PCRc1, which amplify DNA B, were used to amplify a 650 bp fragment. The amplified DNA A and DNA B were used as probes in Southern blot hybridization to detect ToLCV in diseased plants from Punjab. Therefore a bipartite geminivirus is associated with tomato leaf curl disease in Pakistan (Mansoor *et al.* 1997).

In Thailand whitefly-transmitted diseases of tomato were known from the late 1970s and the causal agent was named tomato yellow leaf curl virus (TYLCV-TH) (Giatgong 1980, Thanapas *et al.* 1983). TYLCV has become a serious problem in the mid-1980s. The virus was isolated from infected tomato samples (Attathom *et al.* 1990) and its genome has been completely sequenced (Rochester

*et al.* 1994). Contrary to the TYLCV isolates from the Middle East and South-Western Europe, TYLCV-TH has a genome composed of two molecules, DNA A- and DNA-B. Agroinoculation of cloned viral genomes has indicated that DNA-A is sufficient to induce disease symptoms, while DNA-B enhances them (Rochester *et al.* 1990).

In Taiwan, ToLCV-related disease symptoms in tomato were first observed in 1981 (AVRDC 1984, Green *et al.* 1987). The disease that was sporadic then became suddenly critical in 1993 following a dramatic increase in the whitefly populations in southern and central Taiwan. Electron microscopic examination revealed geminate virus particles. DNA-A clones of the Taiwan tomato leaf curl virus (TLCV-TW) have been sequenced (Chiang *et al.* 1997). It is not known whether the cloned viral genome is infective and whether TLCV-TW has a DNA-B genomic component.

In the Philippines, a ToLCV-like disease has been described in the early 1980s (AVRDC 1985). We have recently amplified by PCR a 1.4 kbp TLCV DNA fragment from infected tomatoes using the PALv1978 and PARc715 primer pairs, and sequenced it (Shi *et al.*, 1997). It comprises the 272 N terminal a.a. of the Rep protein, the IR and the 82 N-terminal a.a. of the coat protein.

In Nepal, tomato with leaf curl symptoms associated with the presence of whiteflies was first reported in the late 1980s in the western part of the country. ToLCV was identified in 1990 (Dahal *et al.* 1993, Ghimire and Pradhanang 1996). The disease started to be of economical importance after 1994 and has been increasing every year. The identity of ToLCV was confirmed by hybridization using DNA probes from ToLCV-Ban1-3 and TYLCV-TH (Green *et al.* unpublished).

In China the occurrence of yellow leaf curl disease in tomato was first discovered in GuangXi Province in 1992 where losses reached 20% (Y. Liu and P. Tien, personal communication). The genome of a TYLCV-CN DNA-A like component has been cloned and sequenced (Liu *et al.* 1997). It is not known whether TYLCV-CN has a monopartite or a bipartite genome.

Diseases with TYLCV/ToLCV-related symptoms have also been described on tomato from Cambodia (Rowell *et al.* 1989, Marom 1993), Indonesia (AVRDC 1985) and Malaysia (Abu Kassim and Abu Bakar 1986). These diseases have not been characterized and molecular data is lacking. Recently we have used the PALv1978 and PARc715 primer pairs to PCR-amplify 1.4 kbp viral DNA fragment from infected tomatoes from Malaysia (Shi

*et al.* 1998b) and Sri Lanka (Green *et al.*, unpublished). The sequence of the ToLCV-SL and ToLCV-MY fragments comprise the N terminal moiety of the Rep protein, the IR and the N-terminal moiety of the coat protein.

In Japan a disease called tomato yellow dwarf, has been reported from tomato (Osaki and Inouye 1978). The disease now occurs mainly in southwestern Japan as a result of a surge of whitefly populations but it is not considered of economical importance. The disease agent has been referred to tobacco leaf curl geminivirus (Kobatake *et al.* 1981).

Although formally not part of Southeast Asia, Australia is sufficiently close to interrelate with Asia. A monopartite whitefly-transmitted geminivirus, denominated ToLCV-AU, causes severe crop damage to tomato in the northern part of Australia. An infectious clone has been sequenced (Dry *et al.* 1993).

#### Relationship between TYLCV/ToLCV isolates based on comparisons of the intergenic region (IR), capsid (CP) and replication-associated (Rep) proteins

Up to date the complete nucleotide sequences of eleven isolates of TYLCV and of eight isolates of ToLCV have been published or are available in databases. In addition, sequences of genes and gene fragments from many other isolates have been published in the literature and in databases. We have compared the nucleotide and protein sequences of TYLCV/ToLCV isolates from Eastern and Southeastern Asia among them and with isolates from other regions of the world. The comparison was based on three genetic elements of the virus: (1). The IR, the least conserved region of the geminiviruses (Rybicki 1994, Padidam *et al.* 1995), except for the 30 nucleotide stretch forming a stem-loop structure and containing the origin of replication (Laufs *et al.* 1995); (2). the CP, assembled to form the capsid and necessary for transmission by the whitefly vector (Azzam *et al.* 1994, Briddon *et al.* 1990); (3). the Rep, necessary for replication in the infected plant (Elmer *et al.* 1988, Laufs *et al.* 1995).

Figure 1 shows homology trees obtained by aligning nucleotide sequences of the IR and the amino acid sequences of the CP and Rep of TYLCV/ToLCV isolates from Asia and of tomato geminiviruses from other regions of the world. All the TYLCV/ToLCV isolates from East and Southeast Asia analyzed so far constitute different species, not strains from the same virus as are, for example, TYLCV from Israel and from Egypt (Nakhla *et al.*

1993). Even the four ToLCV isolates from Bangalore, India, cannot be considered as strains of the same virus, and are different from the ToLCVs from New Delhi and from Lucknow. For example, CP comparisons indicated that the Indian ToLCVs share between 84 to 94 % homology; the four isolates from Bangalore share 87 to 90% homology. The Indian ToLCVs share 69 to 77 % homology with the Thai TYLCV. Similarly TYLCV from China is different from TYLCV/ToLCV from neighboring Thailand and Taiwan, sharing 78 to 83 % homology with these viruses, about the same level of homology it shares with the Mediterranean TYLCV isolates. ToLCV from Australia is different from those of Southeast and East Asia. Comparisons of IR and Rep provide identical conclusions.

Figure 2 shows phylogenetic trees based on nucleotide sequences of the IR and amino acid sequences of the CP and Rep of TYLCV/ToLCV isolates from Asia and from other regions of the world. As observed before (Kheyr-Pour *et al.* 1991, Nakhla *et al.* 1993, Rybicki 1994, Hong and Harrison 1995, Padidam *et al.* 1995) the relationships based on the CP reflects the geographical origin of the various isolates. The tree presents three main branches representing isolates from the Eastern Mediterranean

basin (Italy, Spain), the Middle East (Israel, Egypt, North Saudi Arabia) and the Far East. The latter divides into two subgroups: the first comprises isolates from Australia, China, Malaysia, Taiwan and Thailand; the second isolates from Bangladesh, India and Sri Lanka. ToLCV from the Philippines behaves as an outgroup because only the sequence of a small stretch of the CP is available. Tomato geminiviruses from the New World constitute a clear distinct group. The Caribbean TYLCV groups with the Middle Eastern isolates, indicating that they are recent imports. The geographical grouping of the whitefly-transmitted tomato geminiviruses according to similarities of their capsid protein may reflect the co-evolution of local virus-whitefly complexes (McGrath and Harrison 1995).

The tree based on the Rep protein reveals a geminivirus grouping similar to that obtained with the CP, although less stringent. The Rep protein of geminiviruses from the same geographical group shows a much lesser degree of homology than that exhibited by the CP of the same viruses. Nonetheless, it is clear that the viruses from Asia and Southeast Asia constitute a homogenous group, separated from those of Southeast Europe and the Middle East. Variability of the CP is constrained to assure virion

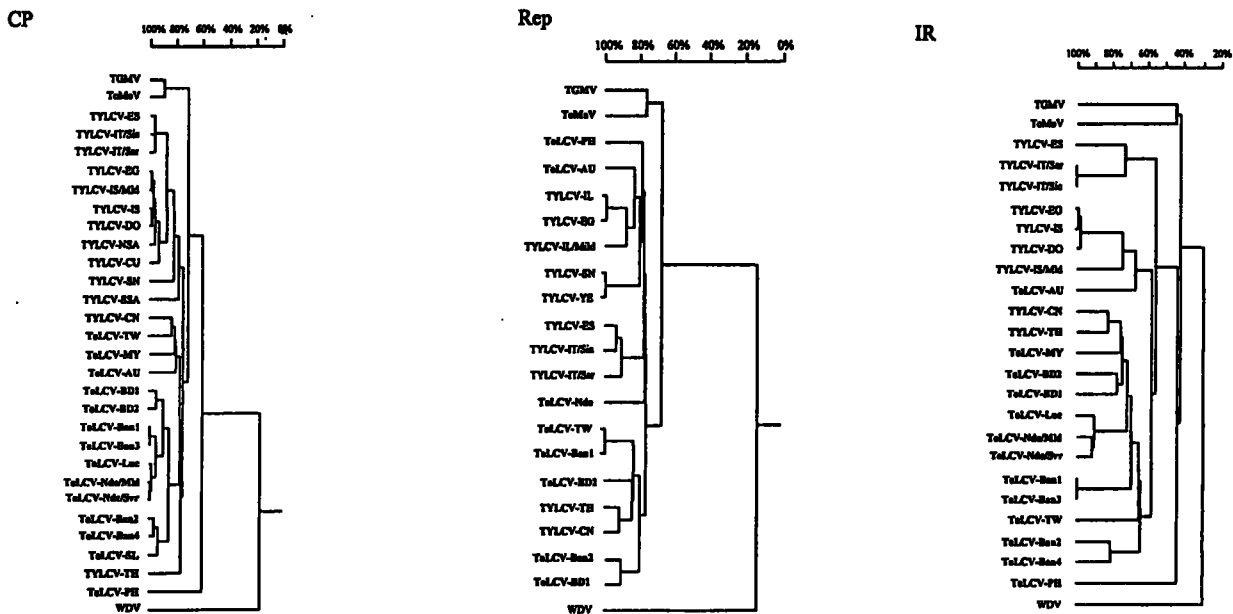


Figure 1. Dendrograms showing homologies among the capsid proteins (CP), the replication-associated protein (Rep) and the intergenic region (IR) of TYLCV/ToLCV isolates from Southeast and East Asia (in bold capitals) with other whitefly-transmitted tomato geminiviruses. Scale represents percent homology. The sequences of the CP, Rep and IR of WDV are included as outgroups. Only the 118, 108, 82 and 114 N-terminal amino acids from ToLCV-BD, TYLCV-CU, ToLCV-PH and ToLCV-SL CP are available, respectively. Only 233, 229 and 132 N-terminal amino acids of ToLCV-BD, ToLCV-PH and TYLCV-YE Rep proteins are available, respectively. The sequences of the CP, Rep and IR of WDV are included as out group. See Table 2 for virus nomenclature.

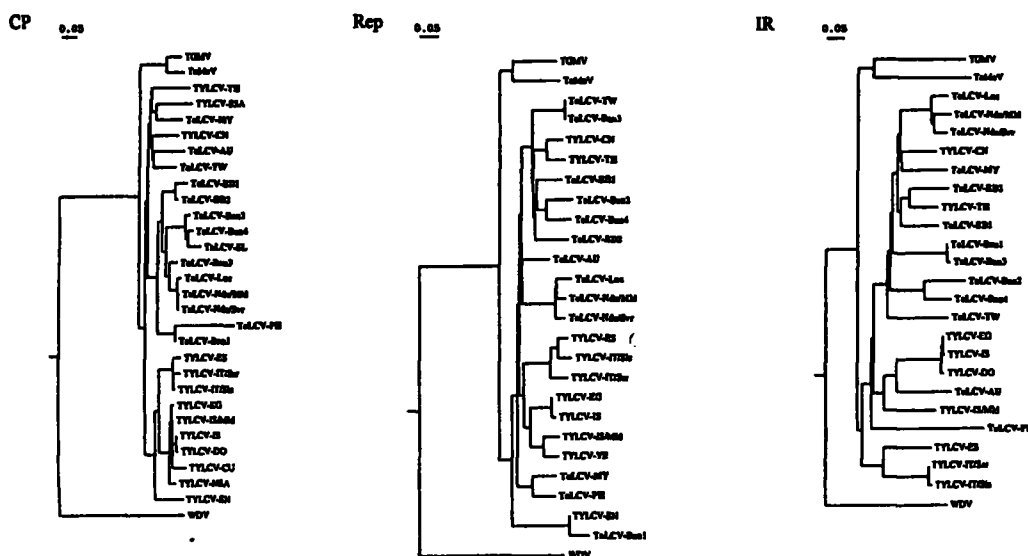


Figure 2. Phylogenetic trees showing relationships among the capsid proteins (CP), the replication-associated protein (Rep) and the intergenic region (IR) of TYLCV/ToLCV isolates from Southeast and East Asia (in bold capitals) with other whitefly-transmitted tomato geminiviruses. The sequences of the CP, Rep and IR of WDV are included as outgroup. The trees are rooted. The scale refers to branch lengths (percent of substitutions). See Table 2 for virus nomenclature.

assembly and recognition by the insect vector, hence most of the diversity of this protein is confined to its N-terminus (Padidam *et al.* 1995). In contrast the Rep protein seems to allow a higher degree of change, even though the domains ensuring binding to the virus origin of replication and replication are conserved (Jupin *et al.* 1995).

The tree based on the IR present the same general patterns as those obtained with the CP and Rep, although clustering is less pronounced probably because of a greater diversity of the IR, except for the conserved stem-loop sequence, some promoter elements and Rep binding sites (Fontes *et al.* 1994).

The great variability of the TYLCV/ToLCV complex worldwide should be considered when breeding programs for virus resistance are established. A tomato line tolerant/resistant to a particular TYLCV/ToLCV isolate may not be as effective against another distantly related virus isolate.

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