Genetic Variability of Croatian *Citrus tristeza virus* Isolates

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ABSTRACT. About 30 citrus field samples were collected from different orchards in the Croatian coastal region. The majority of trees was grafted on *Poncirus trifoliata* and displayed no symptoms of *Citrus tristeza virus* (CTV) infection. However, about 50% of tested trees were found to be CTV-infected. The genetic variability and population structure of CTV were studied by SSCP and sequence analysis of the cloned p25 (CP) and p23 genes. The results of phylogenetic analysis, corroborated by biological characterization, showed that both CP and p23 sequences clustered into phylogenetic groups that include sequences of severe quick decline, seedling yellows and stem pitting isolates. Evidence of mixed infections and recombination events was also found.

Development of the modern citrus industry in Croatia started about 50 yr ago when more then 100 types of citrus were imported from Corsica, California, ex-USSR Republic of Georgia, and Japan (4). Unfortunately, many of them came into the country without any record of CTV-status. At present, citrus is grown on approximately 1500 ha mostly in two regions, primarily in the Neretva Valley (near Dubrovnik) and much less in the Kaštela region near Split. Due to the cool climate, the main citrus cultivated is Satsuma mandarin (*Citrus unshiu*), grafted on trifoliate rootstock in over 90% of the cases. Most of the Satsuma scions were introduced from Japan. Although ELISA tests in the last 20 yr confirmed the presence of CTV, no further steps regarding its characterization were taken until recently.

About 30, mostly symptomless samples, used in this study were collected in commercial orchards from Kaštela and one, more isolated, location on the island of Vis. ELISA results confirmed the incidence of about 50% CTV infection, with the highest infection rate in Satsuma mandarins. Nearly 100% of Satsumas tested were CTV positive. The bark patches from 11 out of 15 positive samples (Table 1) were successfully grafted on Madam Vinous sweet orange and, 6 mo later, this infected tissue was used for molecular characterization. The tissue was analyzed by immunocapture RT-PCR (5) using primers targeting the whole p25 and p23 genes (2, 3). PCR products of the expected size were obtained for both genes. After purification, amplified products were subjected to SSCP analysis (1). All samples showed complex SSCP patterns, suggesting the presence of more then one CTV variant. In order to separate different variants existing in the virus population of each plant sample, we used a standard procedure of cloning and *E. coli* transformation. The PCR products obtained from the transformed cells were subjected to additional SSCP analysis in order to identify different variants. PCR products from at least 10 transformed colonies were analyzed. The clear predominance of one genomic variant was confirmed in each sample tested. For each sample, PCR products of genes p25 and p23, displaying different SSCP patterns were considered to indicate different variants and they were sequenced (Macrogen Inc.). Neighbor-joining phylogenetic analysis was performed using 28 p25 and 39 p23 sequences of Croatian isolates, as well as 16 reference sequences from GenBank of biologically well-characterized isolates. The trees generated were evaluated by bootstrap analysis based on 1000 repetitions.

TABLE 1
DETAILS OF CROATIAN CTV ISOLATES ANALYZED PHYLOGENETICALLY IN FIG. 1

CTV isolate	Origin	*Citrus* variety	Symptoms on Madam Vinous indicator plant[a]	Phylogenetic grouping of sequences[b]				
				2	**3a**	**4**	**5**	**M**
3	Croatia (the island of Vis)	*C. sinensis* 'Wash. navel'	SP		●			
4	Croatia (the island of Vis)	*C. unshiu* 'Kuno'	0					●
6	Croatia (the island of Vis)	*C.unshiu* 'Kuno'	0	●				
B6	Croatia (Kaštela region)	*C. unshiu* 'Zorica Rana'	0	●				
S1	Croatia (Kaštela region)	*C. unshiu* 'Chahara'	0				●	
S2	Croatia (Kaštela region)	*C .unshiu* 'Chahara'	0	●				
S3	Croatia (Kaštela region)	*C. unshiu* 'Ichimaru'	SP	●	●			
S4	Croatia (Kaštela region)	*C. unshiu* 'Ichimaru'	SP		●			
Fu	Croatia (Kaštela region)	*C. sinensis* 'Fukumoto'	SP	●		●		
Ib	Croatia (Kaštela region)	*C. wilsonii*	SP			●	●	
Iuz	Croatia (Kaštela region)	*C. wilsonii*	0				●	

[a] 0- no symptoms were observed, SP- stem pitting symptoms
[b] phylogenetic groups are defined according to classification proposed by Zemzami et al. (2)

The trees showed seven clusters (data are shown for the p25 gene, Fig. 1). Since the clustering pattern was the same as in Zemzami et al. (5), we adopted the same nomenclature (groups 1 to 5, plus group M). Phylogenetic analysis of both genes gave the same clustering pattern and confirmed that Croatian sequences cluster together with the reference sequences from five phylogenetic groups (2, 3a, 4, 5 and M). Three of these groups (3a, 4, and 5) include CP sequences of reference isolates that cause severe symptoms of stem pitting, quick decline, and seedling yellows. The sequences of 64% of samples clustered with reference isolates from 'severe' groups 3a, 4, and 5. In 27% of cases, we detected within one sample a mixture of variants belonging to different phylogenetic groups. In these samples, the occurrence of recombinants between genomic variants from different phylogenetic groups was also detected. When analyzing trees growing in the same orchard (close vicinity) for 20-40 yr, we found that their CTV population structure was very different, indicating that vector-mediated transmission did not occur.

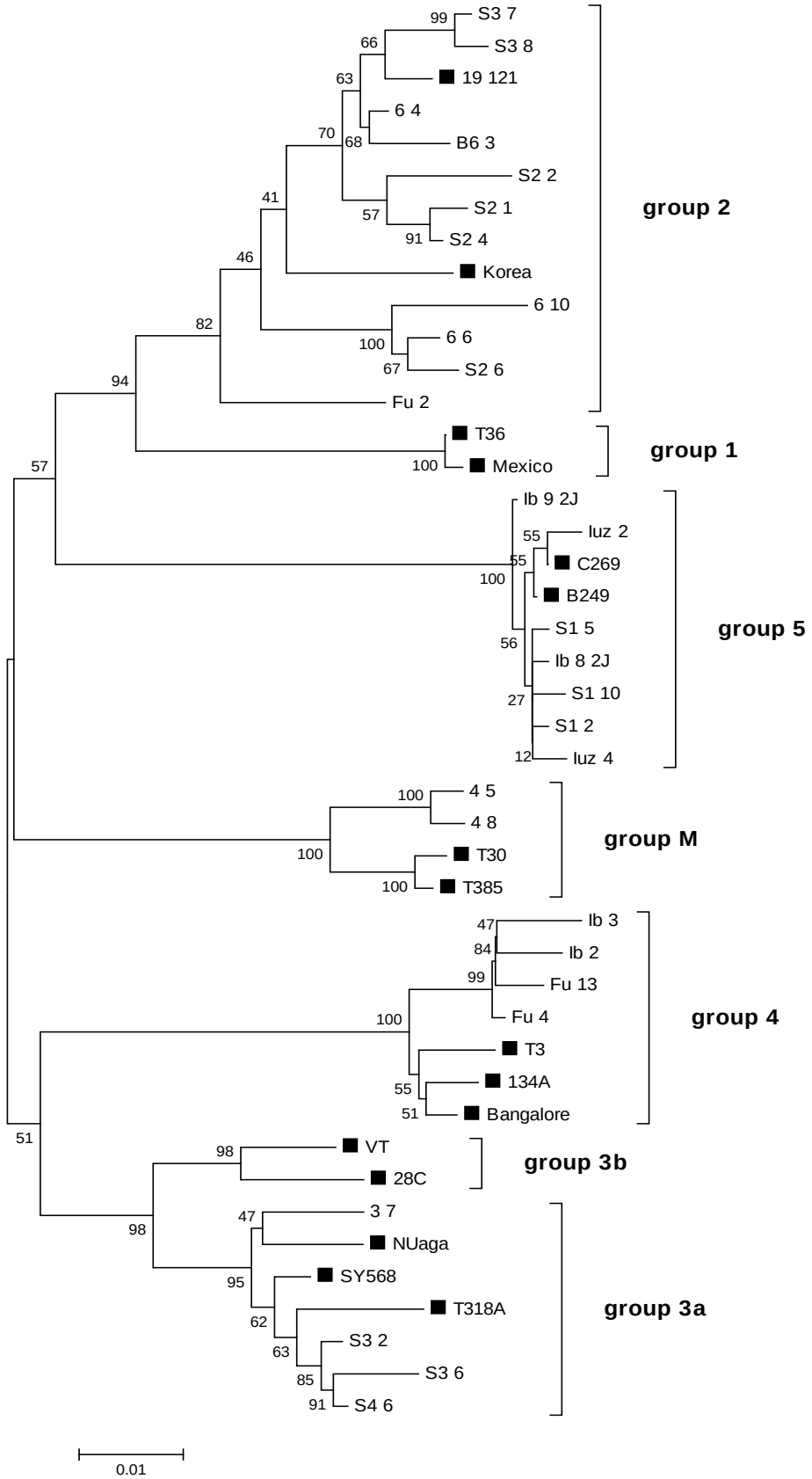

Fig. 1. Neighbor-joining phylogenetic tree obtained by the analysis of *Citrus tristeza virus* coat protein sequences of Croatian isolates. Reference sequences retrieved from GenBank are marked with squares.

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