Nucleotide analysis of pome fruit virus isolates detected in apple and pear samples from Italy and India

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Abstract

In the framework of a joint research project between Italy and India field surveys were done in different pear and apple growing areas of North of India and Central and Southern Italy. Samples were collected from plants belonging to common and local varieties and molecularly analyzed for the detection of the main pome fruit viruses (*Apple stem pitting virus*, *Apple stem groving virus*, *Apple chlorotic leaf spot virus*, *Apple mosaic virus*) by using harmonized diagnostic protocols.

The sequence homology was evaluated and a phylogenetic tree was built, on the basis of which, the Indian isolate of ASGV showed maximum sequence identity at a nucleotide level to Italian isolates when analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similarly, a maximum identity, ranging from 90-93%, was found for the Italian isolates of ASPV and pear and apple isolates from Poland, while a sequence homology ranging from 83 to86% was observed within the Indian isolates of ASPV. Multiple alignment of the Indian pome ACLSV-isolates indicate maximum variability in the middle portion while the first 140 nucleotides are maximally conserved and shared a percent identity at nucleotide level of 86-100% with the Italian isolates.

The ApMV Indian isolates showed maximum (92-99%) sequence homology to the Korean isolate (AY125977) from apple. However, a comparison with other isolates from different host plant species revealed a clustering of Indian isolates with a Czech isolate from pear and a sequence homology of 84 to 98%. Phylogenetic analysis showed that sequence variability was independent to the geographical origin or the host for all the investigated viruses.

Keywords: ACLSV, ASPV, ASGV, ApMV, sequences analysis, Italy, India.

Introduction

Among the temperate fruit crops, apple and pear are widespread species of relevant economic importance in many countries all over the world. In India apple is one of the major cash crops especially in the North Western Himalayan region (states of Himachal Pradesh, Jammu & Kashmir and Uttarakhand), where the local economy is largely dependant on apple cultivation. In Europe, apple and pear crops are particularly important and Italy is the main producing country.

Pome fruit are affected by a number of diseases, including those of viral etiology. Particularly, *Apple chlorotic leaf spot virus* (ACLSV, *Trichovirus*), *Apple stem pitting virus* (ASPV, *Foveavirus*), *Apple mosaic virus* (ApMV, *Ilarvirus*) and *Apple stem grooving* virus (ASGV, *Capillivirus*) are common pathogens in pome fruit trees. These viruses frequently occur in mixed infections and can significantly reduce the fruit yield and quality (Posnette et al., 1963; Desvignes, 1999).

In the framework of a joint research project between Italy and India, a molecular investigation on pome fruit viruses from these two countries was carried out in order to evaluate possible genomic diversity and phylogenetic relationships among isolates of different geographical origin. In this work we report the results of the nucleotide sequence analysis performed on the gene encoding for the capsid protein (CP) of ACLSV, ASPV and ASGV isolates from Italy and India and the comparison with other isolates retrieved in GenBanik[J1].

(http://www.ncbi.nlm.nih.gov/Genbank/).

A nucleotide analysis of ApMV isolates from India is also reported.

Material and methods

Source of isolates: A total of 12 Italian isolates of ACLSV (4), ASPV (5) and ASGV (3) identified in apple and pear trees from the regions of Latium and Sicily (Central and Southern Italy) were molecularly analyzed and compared with 19 Indian isolates of ACLSV (16), ASPV (2) and ASGV (1) identified in apple, pear and quince trees from the pome

fruit growing belts of Himachal Pradesh (North Western India). Seven ApMV isolates from India was also included in the analysis. No ApMV isolates were found in the samples collected from theItalian regions. Analyzed isolates are listed in table 1.

RNA extraction and cDNA synthesis and amplification: Total RNA was extracted from phloem tissue by use of the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacture's instructions with some modifications. For cDNA synthesis and amplification, specific primer pairs amplifying fragments of the complete or partial coat protein region of each virus were used: ACLSV, ASPV and ASGV primers as described by Menzel et al. (2002) were used in Italian and Indian (ASPV) labs; degenerate primers amplifying the complete coat protein and part of 3'UTR of ACLSV (Rana et al., 2008a) and the ASGV primer pairs 6396R/5641F (MacKenzie et al., 1997) were used in the Indian lab; finally, the primer pair PAPMCP3/PAPMCP5 (Choi et al., 2003) was used in the Indian lab for cDNA synthesis and amplification of ApMV. Synthesis of cDNA and amplification were performed as detailed in Rana et al., (2008b) at standardized temperatures of annealing for each virus specific primers.

Cloning of cDNA products, sequencing and sequence analysis: Amplified cDNA fragments of the analyzed isolates were cloned into pGEM T-Easy vector as directed by the supplier (Promega, USA) and then sequenced. A BLAST search was performed to verify the correspondence of the cloned amplicons with the investigated viruses. One recombinant plasmid containing cDNA was selected for each isolate for sequence comparisons. For each virus, a nucleotide sequence analysis was then carried out by generating a multiple alignment of both Indian and Italian isolate sequences using Clustal[J2] (Larkin et al., 2007). Finally, in order to investigate the phylogenetic relationships among isolates of different geographical origin, nucleotide sequences of Indian and Italian isolates were compared to corresponding regions of ACLSV, ApMV, ASPV, ASGV isolates worldwide identified in pome fruits downloaded from the NCBI database[J3] (http://www.ncbi.nlm.nih.gov/sites/genome). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura et al., 2007).

Tab. 1 List of the Indian and Italian virus isolates used in the nucleotide sequence analyses.

Virus	Isolate	Country	Host	Accession no.
ACLSV	India1	India	Apple	AM494505
	India2	India	Apple	AM494506
	India3	India	Apple	AM494507
	India4	India	Apple	AM494508
	India5	India	Apple	AM494509
	India6	India	Apple	AM494510
	India7	India	Apple	AM494511
	India8	India	Apple	AM494512
	India9	India	Apple	AM494513
	India10	India	Apple	AM494514
	India11	India	Apple	AM408891
	India12	India	Apple	AM409322
	India13	India	Apple	AM709776
	India14	India	Apple	AM709777
	India17	India	Pear	AM882704
	India21	India	Quince	AM498049
	ACLSV_MLO13P	Italy	Apple	
	ACLSV_PRO37P	Italy	Pear	
	ACLSV_RM22	Italy	Pear	
	ACLSV_VT2	Italy	Pear	
ASPV	Solan-SD3	India	Apple	FM863704
	Solan-SD4	India	Apple	FM863705
	ASPV_10M	Italy	Apple	
	ASPV_RM3	Italy	Apple	
	ASPV_VT36	Italy	Apple	
	ASPV_RM22	Italy	Pear	
	ASPV_VT2	Italy	Pear	
ASGV	India1	India	Apple	FM204881
	ASGV_VT36	Italy	Apple	
	ASGV_VT31	Italy	Apple	
	ASGV_10M	Italy	Apple	
ApMV	India1	India	Apple	FM178274
	India2	India	Apple	FJ429311
	India3	India	Apple	FJ429309
	India4	India	Apple	FN435317
	India5	India	Apple	FN435316
	India6	India	Apple	FN435315
	India7	India	Apple	FN43514

Results

Apple stem grooving virus: A high degree of identity (99%) at the nucleotide level was shown for the Italian isolates. Comparison with the Indian isolate showed an identity of 98% (Tab. 2). Moreover, the Indian isolate had the most identical sequence when Italian isolates were analyzed by BLAST. A clear clustering of Indian and Italian isolates into a closely related group was revealed by the phylogenetic analysis (Fig. 1). The ASGV-apple isolate from India shared a sequence identity of 87% with another ASGV Indian strain (FM393044) from pear (data not shown).

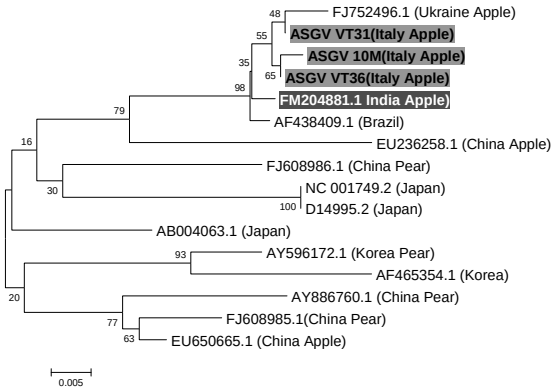

Fig. 1 Phylogenetic relationships among ASGV isolates from pear and apple worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red, Indian isolates; marked in gree, Italian isolates.

Tab. 2 Nucleotide sequence identity (%) among Italian and Indian ASGV isolates.

	ASGV 10M	ASGV VT36	ASGV VT31	FM204881
ASGV 10M	-	99	99	98
ASGV VT36		-	99	98
ASGV VT31			-	98
FM204881				-

Apple stem pitting virus: An identity percentage ranging from 91 to94 was shared among the Italian isolates, while a sequence homology ranging from 83 to 86% was observed in comparison with the Indian ones (Tab. 3). Maximum identity ranging from 90 to93% was shown for the Italian isolates with pear and apple isolates from Poland (data not shown) when blasted with the ASPV-CP sequences from database. Phylogenetic analysis performed on all the apple and pear ASPV-CP isolates identified worldwide showed the presence of two main groups of isolates, reported as I and II: the Italian and Indian isolates clustered into the same group I, but with a high level of divergence clustering in two possible different sub-clusters (Fig. 2).

Tab. 3 Nucleotide sequence identity (%) among Italian and Indian ASPV isolates.

	ASPV 10M	ASPV RM3	ASPV VT36	ASPV RM22	ASPV VT2	FM863704	FM863705
ASPV 10M	-	91	93	92	93	83	83
ASPV RM3		-	94	91	92	84	84
ASPV VT36			-	92	92	86	86
ASPV RM22				-	94	84	84
ASPV VT2					-	86	86
FM863704						-	100
FM863705							-

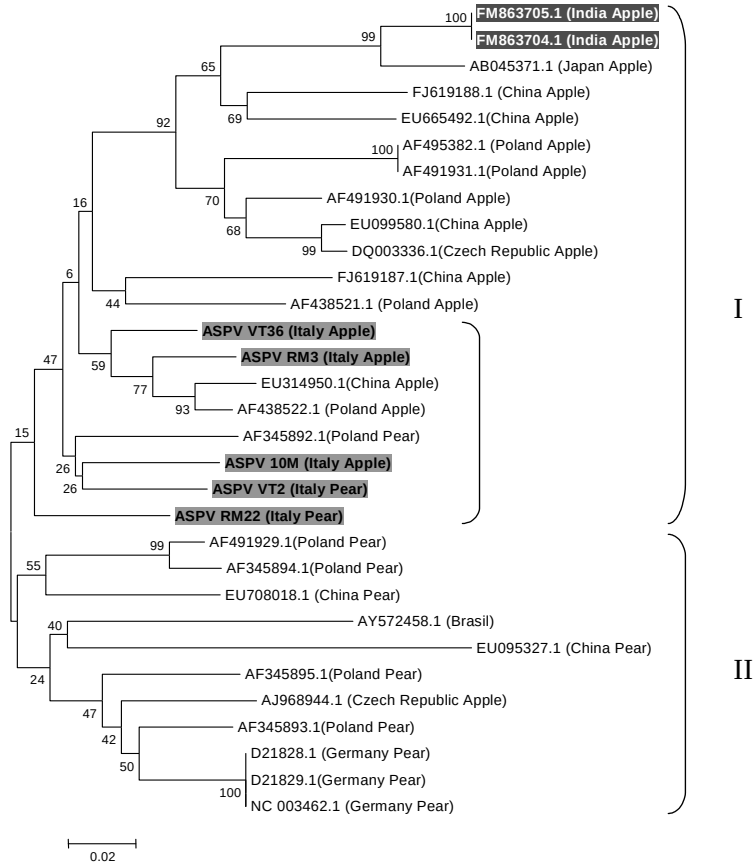

Fig. 2 Phylogenetic relationships among ASPV isolates from pear and apple worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red, Indian isolates; marked in gree, Italian isolates.

Apple chlorotic leaf spot virus: The 20 ACLSV-apple isolates (16 Indian and 4 Italian) shared a percent identity at nucleotide level ranged from 86 to100. When compared among themselves the Italian isolates showed a sequence identity of 88-96%, while Indian isolates shared 82-93% (data not shown). An Indian ACLSV-quince isolate had a nucleotide sequence identity of 84% with a partial ACLSV-CP from Greece. A multiple alignment of the Indian pome ACLSV-isolates indicates maximum variability in the middle portion, while the first 140 nucleotides are maximally conserved. According with the classification proposed by Yaegashi et al. (2007), based on changes of the five highly conserved amino acids at positions 40, 59, 75, 130 and 184, India 2, 5, 6, 8, 9, 10,13, 17 and Italian MLO13 P, RM22 isolates resulted as 'B6 type', while India 1, 3, 4, 7, 11, 12, 14, 21 and Italian PRO37P, VT2 isolates as 'P205 type'. In a phylogenetic analysis no clustering on basis of location or host was evident (Fig. 3).

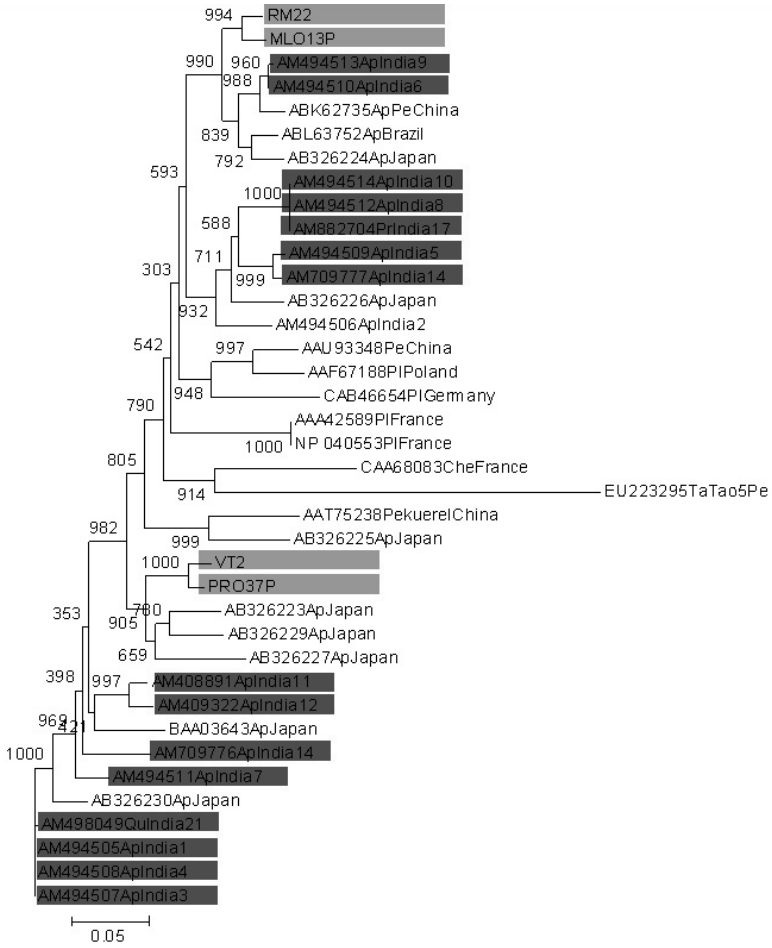

Fig. 3 Phylogenetic relationships among ACLSV isolates from pear and apple worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red, Indian isolates; marked in gree, Italian isolates.

Apple mosaic virus: All the Indian sequences shared 92-99% sequence identity at a nucleotide level when compared among themselves and 59-60% with the PNRSV-apple isolate from India, used as an outgroup. The ApMV apple isolates India1 (FM178274), India2 (FJ429311), India3 (FJ429309), India4 (FN435317), India5 (FN435316), India6 (FN435315), India7 (FN43514) showed maximum (92-99%) sequence identity to the Korean isolate (AY125977) from apple. However, comparison with other isolates, characterized from different host plant species, revealed clustering of Indian isolates with a Czech isolate from pear and a sequence identity ranging from 84 to 98% was observed (data not shown). No clear clustering of ApMV-CP isolates from apple and other hosts was revealed by phylogenetic analysis (Fig. 4).

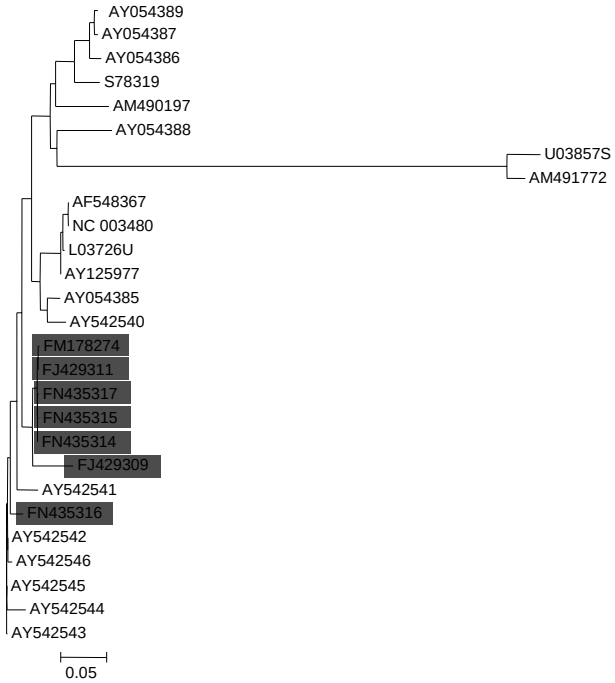

Fig. 4 Phylogenetic relationships among ApMV isolates from different hosts worldwide identified based on the nucleotide sequences of the coat protein. The tree was produced using the N-J Tree option of MEGA4. Marked in red the Indian isolates.

Discussion

On the basis of the obtained results new and improved knowledge on the genomic and phylogenetic relationship of pome fruit viruses has been obtained. In particular, this study provided further knowledge about the genetic diversity of ASPV and ASGV, especially in Italy, from where little information was available.

The nucleotide analysis performed on the investigated viruses confirmed the high level of molecular variability among different isolates of ACLSV (Candresse et al., 1995; Pasquini et al., 1998; Krizbai et al., 2001; Al Rwahnih et al., 2004) and ASPV (Nemchinov et al., 1998) both in Italian and Indian isolates. In contrast, a very high degree of similarity among Indian and Italian isolates was observed for ASGV, confirming the low coat protein gene variability among isolates from distinct regions, previously ascertained by other authors (Nickel et al., 2001). The phylogenetic analysis generated using the nucleotide sequences of virus isolates identified worldwide showed that the sequence variability was generally independent of the geographical origin of the isolates. This is probably due to the exchange of propagative plant material that currently occurs between different countries. Only for ASPV a possible correlation with the geographical origin resulted evident between Italian and Indian isolates, that clustered in two distinct sub-clusters. Nevertheless, more ASPV isolates from these countries should be investigated to confirm this evidence. No correlation based on host plant was shown by the phylogenetic analyses for all the investigated viruses. In particular, no clear clustering of ApMV-CP isolates of apple and other hosts was found, as previously observed by Lee et al. (2002), so classifying into subgroups need further characterization and analysis. The nucleotide sequences of two ASPV-CP (FM863705, FM863704) and three ApMV-CP (FJ429311, FJ429309, FM178274) Indian isolates were submitted to the GenBank database.

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Literature

Al-Rwahnih, M.; Turturo, C.; Minafra, A.; Saldarelli, P.; Myrta, A.; Pallás, V.; Savino, V.; 2004: Molecular variability of *Apple chlorotic leaf spot virus* in different hosts and geographical regions. Journal of Plant Pathology, **86** (2), 117-122.

Candresse, T.; Lanneau, M.; Revers, F.; Macquaire, G.; German, S.; Dunez, J.; Grasseau, N.; Malinowski, T.; Barba, M.; Hadidi, A.; 1995: An immunocapture PCR assay adapted to the detection and the analysis of the molecular variability of *Apple chlorotic leaf spot virus.* Acta Horticulturae **386**, 136-147.

Choi, S. H. and Ryu, K.H.; 2003: Rapid screening of *Apple mosaic virus* in cultivated apples by RT-PCR. Plant Pathology Journal **19** (3), 159-161.

Desvignes, J.C.; 1999: Virus diseases of fruit trees. EPPO Bull. 29, 239 - 252**.**

Krizbai, L.; Ember, I.; Németh, M.; Kölber, M.; Pasquini, G.; Faggioli, F.; Clark, M.F.; 2001: Characterization of Hungarian isolates of *Apple chlorotic leaf spot virus*. Acta Horticulturae **550** (1), 291-295.

Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; Thompson, J.D.; Gibson, T.J. and Higgins, D.G.; 2007: ClustalW and ClustalX version 2. Bioinformatics, **23** (21), 2947-2948.

Lee, G. P.; Ryu K.H.; Kim, H.R.; Kim, C.S.; Lee, D.W.; Kim, J. S.; Park, M.H.; Noh, Y.M.; Choi, S.H.; Han, D.H.; Lee, C.H.; 2002: Cloning and phylogenetic characterization of coat protein genes of two isolates of *Apple mosaic virus* from 'Fuji' apple. The Plant Pathology Journal **18**(5), 259-265.

Mackenzie, D.J.; Maclean, M.A.; Mukerji, S.; Green, M.; 1997: An improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Disease **81** (2)**,** 222-226.

Menzel, W.; Jelkmann, W.; Maiss, E.; 2002: Detection of four apple viruses by multiplex RT – PCR assays with coamplification of plant mRNA as internal control. Journal of Virological Methods, **99**, 81-92.

Nemchinov, L.; Hadidi, A.; Faggioli, F.; 1998: PCR-detection of *Apple stem pitting virus* from pome fruit hosts and sequence variability among viral isolates. Acta Horticulturae, 472, 67-73.

Nickel, O.; Fajardo, T. V. M.; Jelkmann, W.; Kuhn, G. B.; 2001: Sequence analysis of the capsid protein gene of an isolate of *Apple stem grooving virus*, and its survey in Southern Brazil. Fitopatologia Brasileira **26** (3) 655-659.

Pasquini, G.; Faggioli, F.; Pilotti, M.; Lumia, V.; Barba, M.; Hadidi, A; 1998: Characterization of *Apple chlorotic leaf spot virus* isolates from Italy. Acta Horticulturae **472**, 195-202.

Posnette, A.F.; 1963: Vein yellows and red mottle of pear. In: Virus diseases of apples and pears. Tech. Commun. Bur. Hort. E. Mailing **30**, 93-96.

Rana,T.; Chandel,V.; Hallan,V.; Zaidi, A.A.; 2008a: *Cydonia oblonga* as reservoir of *Apple cholorotic leaf spot virus* in India. Plant Pathology **57** (2), 393.

Rana,T.; Chandel,V.; Hallan,V.; Zaidi, A.A.; 2008b: Characterisation of Apple chlorotic leaf spot virus infecting almonds in India. Australasian Plant Disease Notes **3**, 65-67.

Tamura, K.; Dudley, J.; Nei, M.; Kumar, S.; 2007: *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution **24**, 1596-1599.

Yaegashi, H.; Isogai, M.; Tajima, H.; Sano, T.; Yoshikawa, N.; 2007: Combinations of two amino acids (Ala40 and Phe75 or Ser40 and Tyr75) in the coat protein of *Apple chlorotic leaf spot virus* are crucial for infectivity. Journal of General Virology **88** (9), 2611-2618.