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Molecular characterization of dasheen mosaic virus isolates infecting edible aroids in India

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Summary. – Dasheen mosaic virus (DsMV) infecting three major edible aroids namely *Amorphophallus paeoniifolius, Colocasia esculenta,* and *Xanthosoma sagittifolium* cultivated in India was characterized. Infected plants showing typical DsMV symptoms were subjected to reverse transcription-polymerase chain reaction, and an amplification of a 963 bp fragment which encoded the coat protein (CP) gene was obtained. BLAST analysis of the cloned DNA amplicon revealed the identity of the virus to be that of DsMV. Sequence identity matrix of the nucleotide sequences among the three isolates showed that the DsMV isolate infecting *A. paeoniifolius* and *C. esculenta* shared an identity as high as 93%, while the DsMV isolate from *X. sagittifolium* shared an identity of only 73% and 76% with the DsMV isolates from *A. paeoniifolius* and *C. esculenta,* respectively. Comparative analysis of the coat protein of the three DsMV isolates showed the presence of DVG motif (*A. paeoniifolius* and *C. esculenta*) and DTG motif in *X. sagittifolium* and several varying potential threonine and asparagine rich N-glycosylation motifs. Single amino acid substitution of the several conserved motifs occurs in all the three DsMV isolates. This is the first characterization of DsMV isolates infecting *A. paeoniifolius, C. esculenta,* and *X. sagittifolium* plants in India.

Keywords: Amorphophallus paeoniifolius**;** coat protein; Colocasia esculenta; potyvirus; RT-PCR; Xanthosoma sagittifolium

Introduction

Tuber crops form the means of sustenance for millions of people in the tropical and subtropical world. They have a higher biological efficiency as food producers and show the highest rate of dry matter production per day per unit area among all the crops. These crops are known to supply cheap source of energy especially for the weaker sections of the population. These attributes make tuber crops ideal for cultivation in the less developed countries of the world.

In India, among the tuber crops of economic importance, edible aroids of the family *Araceae*, including *Amorphophallus paeoniifolius* (Elephant foot yam), *Colocasia esculenta* (Taro) and *Xanthosoma sagittifolium* (Arrowleaf elephant ear, Cocoyam, Tannia) plays a very important role in the socio-economic development of the country. Elephant foot yam is widely used as vegetable in various cuisines and in preparation of indigenous ayurvedic medicines (Misra *et al.,* 2002). It has become a cash crop and is gaining popularity due to its high production potential (50–60 t/ha), nutritional and medicinal values, and high economic returns (Srinivas and Ramanathan, 2005). Taro is grown primarily as a vegetable food for its edible corms and forms an important staple in the diets of people throughout the tropics. In addition, taro is known to have medicinal properties, and has a very valuable place in the world agriculture scenario as this crop has an important role in the subsistence economies and in crop diversification. Another important edible tropical tuber crop, Cocoyam (Jennings, 1987; Onwueme and Charles, 1994), grown for its starchy corms forms an important source of nutrition for both humans as well as animals (Ndoumou *et al.,* 1995; Tambong *et al.,* 1997; Nyochembeng and Garton, 1998).

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Abbreviations: DsMV = dasheen mosaic virus; VMV = vanilla mosaic virus; RDP = Recombination detection programme; CP = capsid protein; Nib = gene for RNA-dependent RNA polymerase

All three important edible aroids cultivated in India are vegetatively propagated and are affected by viral disease. In spite of their importance in the diets of poor farmers, very little attention has been paid to study the diseases occurring on these crops in India. Among the field diseases of these edible aroids, infection caused by DsMV of the genus *Potyvirus* is widely spread. Repeated use of virus infected planting materials has increased the disease incidence in these edible aroids in the recent years. In the case of Elephant foot yam, occurrences of mosaic, puckering, leaf distortion, filiformy or shoestringing type symptoms are common. In India, Pandit *et al.* (2001), first reported the association of a DsMV with the mosaic disease of Elephant foot yam based on serological studies from the leaf samples collected from West Bengal. The infection caused by DsMV on the plant appeared to be severe resulting in reduced tuber yields. Nehalkhan *et al.* (2006), reported a 24–88% mosaic incidence with yield losses of 3.5–38% in Elephant foot yam growing fields, from the state of Uttar Pradesh. Disease incidence of 5–10 % was observed in other major Elephant foot yam growing states of India (Kerala, Andhra Pradesh, and Orissa) (Binoy *et al.,* unpublished data).

In case of taro, the leaves show a mosaic pattern along the veins, causing variation patterns in color, shapes, and sizes of the leaf, and sometimes produce whitish feathery symptoms, and reduced corm yield. Occurrence of mosaic disease on taro has been reported from Thiruvananthapuram, with typical symptoms resembling that of infection with DsMV (Malathi and Shanta, 1981). In case of Cocoyam, also whitish feathery mosaic symptoms are commonly observed. The other visible symptoms on the plants include leaf distortion, vein chlorosis, mosaic feathering along the veins (Zettler *et al.,* 1989), and in case of a severe attack, stunted plants result in reduced tuber yield. Our preliminary studies with immunosorbent electron microscopy and RT-PCR using potyvirus group specific primers have showed the association of DsMV with all the three infected edible aroids cultivated in India (Binoy *et al.,* 2010).

DsMV, an aphid-transmitted virus, was found to infect a wide variety of cultivated aroids and ornamental plants worldwide (Zettler *et al.,* 1978; Zettler and Hartman, 1986, 1987; Brunt *et al.,* 1996). Partial sequence characterization of DsMV infecting *Colocasia* (Pappu *et al.,*1994), *Xanthosoma (*Reyes *et al.,* 2009*)* and *Zantedeschia* (Chen *et al.,* 2001) and complete genome sequence from *Zantedeschia* have been reported (Chen *et al.,* 2001). However no sequence information of DsMV from *A. paeoniifolius, C. esculenta,* and *X. sagittifolium* in India has so far been reported. Therefore, the objective of the present study was to characterize the sequences of the CP gene of three DsMV isolates infecting these crops and to analyze the molecular and phylogenetic relationship among these isolates.

Materials and Methods

Virus source and RNA extraction. Diseased *A. paeoniifolius, C. esculenta*, and *X. sagittifolium* plants showing foliar mosaic symptoms were collected from Central Tuber Crops Research Institute (Trivandrum, Kerala) field, and total RNA was extracted from 100 mg of infected leaves using the Qiagen RNeasy Plant mini kit (Qiagen, USA), according to the manufacturer´s protocol.

RT-PCR. Total RNA was subjected to RT-PCR using a one step RT-PCR kit (Finnzymes) in a reaction mixture (20 µl) containing 5 µl RNA, 1 µl of 5 µmol/l oligo $(dT)_{16}$, 1 µl of 5 µmol/l each of DsMV(I)-F and DsMV(I)-R primers, 2.0 µl of 10x/5x reaction buffer, 0.5 µl of 10 mmol/l dNTPs, 0.5 µl of 50 mmol/l $MgCl_2$, 0.25 µl of AMV reverse transcriptase and 1.0 µl of Dynazyme DNA Polymerase in Eppendorf Mastercycler Gradient ES with the following profile: 48°C for 45 min, 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 1 min, 72°C for 1 min and one cycle of 72°C for 10 min. Degenerate forward primer DsMV (I)F and specific reverse primer DsMV (I)R were designed 21 nucleotides upstream of the 5' end of the CP gene comprising the partial NIb region (DsMV (I)F 5'-GATGCCTTTRMCTTTGARNTGWKSKGTG-3') and in the 3'untranslated region downstream of the CP gene (DsMV (I) R 5'-GCCAATAACTGTGGCCTGTT-3'), respectively, using the Primer Premier Software version 5, using the DsMV sequences available in the GenBank. The amplified product was analyzed on 1% agarose gel, stained with ethidium bromide and photographed with a UV-gel doc system (Alpha Imager HP, California).

Cloning and sequencing. The amplified PCR products were purified using the Gel extraction kit (Qiagen), cloned into the pGEM-T Easy vector (Promega) and transferred into *Escherichia coli* strain DH5α (Sambrook and Russel, 2001). Ten positive clones of each isolate carrying the insert were sequenced using T7 and SP6 primers and also with primers DsMV (I)F and DsMV (I)R.

Phylogenetic analysis. Sequences were edited using the BIOEDIT Software (Hall, 1999). Nucleotide and the deduced amino acid sequences were compared with those in the NCBI database using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/), with Blastn and Blastp, respectively. Deduced amino acid sequences were analyzed using the GCG program MOTIFS (Devereux *et al.,* 1984). Multiple sequence alignment of the virus isolates and the identity percentage between aligned sequences were calculated using Clustal W (Thompson *et al.,* 1994). Sequence identity matrix of the DsMV isolates under study was done using the BIOEDIT software. Phylogenetic analysis using nucleotide sequences was done between DsMV and other selected members of the genus *Potyvirus*. From the aligned sequences, a phylogenetic tree was constructed using the neighbor-joining method (Tajima and Nei, 1984) with 100 bootstrap replicates, and the tree was constructed using the TREECON software (Van de Peer and De Wachter, 1994).

Recombinant analysis. Recombination analysis of the three isolates was carried out in order to determine the genetic relation-

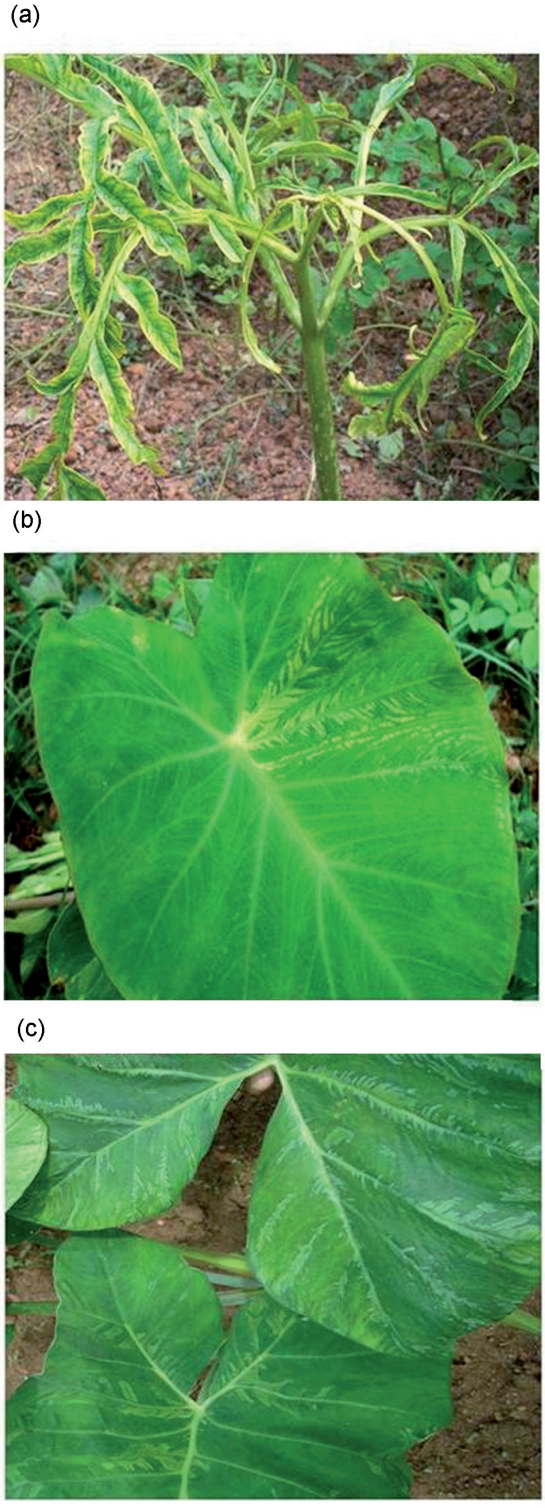

Fig. 1
Symptoms on leaves of edible aroids infected by DsMV
(a) *Amorphophallus paeoniifolius,* (b) *Colocasia esculenta,* (c) *Xanthosoma sagittifolium.*

ship, using different methods of the Recombination detection programme (RDP Version 3.0) (Martin and Rybicki, 2004).

Sequences. Nucleotides sequences have been submitted to NCBI database under the accession number: DsMV-Amp1 (FJ160764), DsMV-Ta1 (HQ207541) and DsMV-Xan1 (HQ207542). In case of DsMV-Amp1 (FJ160764), the sequence submitted was from an earlier clone (1223 bp) containing the NIb, CP, and the 3'UTR region, from which specific primers were designed for the amplification in the present study.

Results and Discussion

RT-PCR analysis of total RNA from infected leaves of all three aroid species (Fig. 1) with DsMV specific primers resulted in 963 bp amplicon (Fig. 2), which potentially encoded the complete CP gene of 313 amino acids. Sequence analysis of the three isolates using BLAST indicated that the virus is DsMV. All the three isolates shared 80–90% nt identity with the other DsMV sequences available in the GenBank. BLAST analysis of the CP nucleotide sequence indicated that the three DsMV isolates also showed a higher identity at the nucleotide and amino acid level respectively, with that of vanilla mosaic virus (VMV) (86–88% nt and 94–95% aa), and an moderately higher identity with that of Bean common mosaic virus (BCMV) (78–80% nt and 82–85% aa) (data not shown). The identities between these viruses and the dendrogram clustering of these viruses together (Fig. 3) clearly indicates the close relationship among these viruses, as was also reported earlier (Adams *et al.,* 2005; Reyes *et al.,* 2009; Farreyrol *et al.,* 2006). DsMV isolates from the three edible aroids under study differed from two other DsMV isolates (AY994104, AY994105) and with one VMV (AJ616719) from GenBank, in lacking 36 nucleotides at the 5'-end region of the CP and 54 nucleotides (140–194) compared to 9 DsMV isolates (AM910398, DMU00122, AF169832, EF199550, DQ925464, AM910407, AM910401, AM994104, and AY994105) and one VMV isolate (AJ616719) (data not shown).

Comparative analysis of the nucleotide sequence identity matrix of the three isolates indicated that DsMV-Amp1 and DsMV-Ta1 shared a higher identity ratio of 0.927, while DsMV-Xan1 showed an identity ratio of 0.756 and 0.728 with DsMV-Amp1 and DsMV-Ta1 respectively (Table 1). These data clearly show that the DsMV isolates obtained from *A. paeoniifolius* and *C. esculenta* could probably be the similar isolates, while the DsMV isolate from *Xanthosoma spp.* could be a different strain. Comparative to other DsMV isolates from the GenBank, the identity matrix shows that the DsMV-Amp1 and DsMV-Ta1 share the highest identity among themselves, while the DsMV-Xan1 showed the highest identity (0.8) with that of DsMV isolate from China (Acc.

No: EU420058), followed by the highest identity with that of DsMV-Amp1(0.76). The lower identity of the DsMV-Xan1 with other viral isolates clearly proves that the isolate is a completely new strain of DsMV. Moreover since the isolates were collected from the same geographical location, the data clearly prove the presence of varying DsMV strains/ isolates in the same location. Moreover in our other studies we have analyzed 12 different DsMV isolates (HQ207529 to HQ207540) from *A. paeoniifolius* collected from different geographical locations of Kerala, which exhibited high variability among the isolates (Binoy *et al.,* unpublished data). Analysis of our viral isolate DsMV-Amp1 with these viral isolates from the same host indicated a higher identity ranging between 74–96%, while the DsMV isolate from the host *C. esculenta*- DsMV-Ta1, indicated a lower identity with these isolates ranging from 64–71% (data not shown). The DsMV isolate from *Xanthosoma* – DsMV-Xan1 indicated an identity ranging between 72–86% with the 12 different DsMV isolates from *A. paeoniifolius.* These data prove that the DsMV isolates from the same host shared a higher identity, while from the different host appeared to be variable, except in the case of DsMV-Xan1 which showed an identity of 84–86% with certain DsMV isolates. This proves that the *Xanthosoma* isolate, percentage of identity is somewhere between the divergent line and that of the DsMV isolates from *A. paeoniifolius*. Moreover the DsMV isolate from the host plant *C. esculenta* (DsMV-Ta1) showed the highest identity with that of the DsMV-Amp1 isolate from a different host plant (from Trivandrum), and a lower identity with that of the other DsMV isolates from *A. paeoniifolius* from different locations. The constructed phylogenetic tree clearly shows that the two isolates DsMV-Amp1 and DsMV-Ta1 share a higher identity and are grouped in similar clade, while DsMV-Xan1 forms an entirely different clade (Fig. 3). The similar geographical location of the three DsMV isolates and the higher and lower sequence identity among these isolates could be attributed to the occurrence of recombination events in the evolution of these isolates. Recombination analysis of the three viral isolates using RDP showed the presence of single recombination event in the coat protein region of the viral isolates (Fig. 4). The recombination events were detected by GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq programmes of the RDP, with a recombination frequencies ranging from $1.281x10^{-02}$ to $7.181x10^{-01}$ (Bonferroni P-value 0.05). The recombination analysis indicated the possibility of DsMV-Amp1 to be a recombinant of DsMV-Xan1 (major parent) and DsMV-Ta1 (minor parent). However recombinant analysis of the other regions of the genome, which could possibly provide an insight in to the occurrence of these isolates with varying identities in the same geographical locations, needs to be carried out. The possibilities of recombination events are high in case of viral isolates found in similar geographical locations

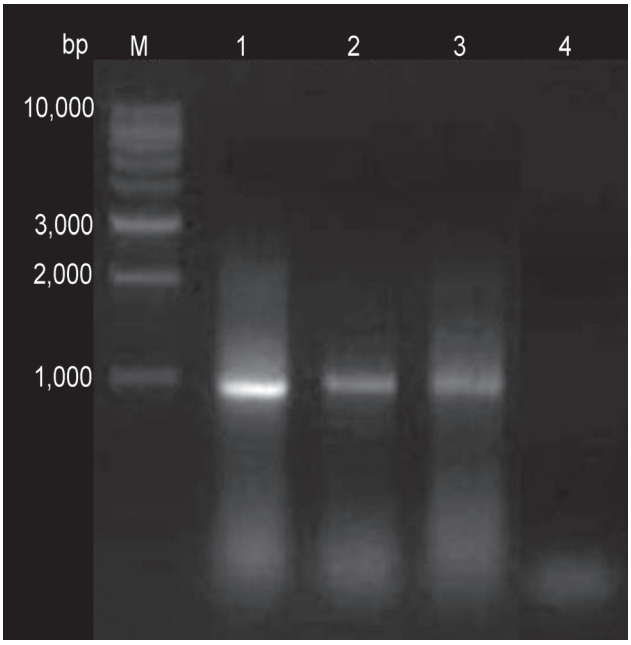

Fig. 2

RT-PCR analysis of DsMV infected edible aroids

Lane M: 1Kb marker; lane 1: infected *A. paeoniifolius* sample; lane 2: infected *C. esculenta* samples; lane 3: infected *X. sagittifolium* sample; lane 4: negative control.

(Bateson *et al.,* 2002; Garcıa-Arenal *et al.,* 2001), and could be adopted for the better adaptability to the environment and to the suitable host. Moreover, the other possible causes for the genetic variation such as genetic drift and selection (Garcıa-Arenal *et al.,* 2001) also need to be analyzed for a much better understanding.

Multiple sequence alignment of the CP of the three viral isolates under study showed that the N-terminal region of the CP varies considerably; however, two thirds of the C-terminal were highly conserved. Protease cleavage sites in potyvirus polyprotein sequences for the production of the CP are Q/A, Q/S or Q/G residues (Pappu *et al.,* 1994). DsMV-Amp1, DsMV-Ta1 and DsMV- Xan1 have the potential protease cleavage site at position 7 and 8, between Q/A to produce a CP of 313 amino acids (Fig. 5). The presence of the sequence DEVVL in the NIb region, upstream of the glutamine residue of the Q/A site at position 6 suggests that this is the protease digestion site in almost all the isolates. This region also correlates with the protease sites for other potyviruses (Yu *et al.,* 1989). The presence of the sequence ADDTV following the first putative cleavage site indicates that this site is likely active, as reported for Tobacco etch virus and Tobacco vein mottling virus (Pappu *et al.,* 1994; Yu *et al.,* 1989). In DsMV-Amp1 and DsMV-Xan1, the site ADDTV is conserved, while in DsMV-Ta1, a single amino acid substitution of V/G occurs. A DVG motif (residues 6 to 8) was observed in the DsMV-Amp1 and DsMV-Ta1, and

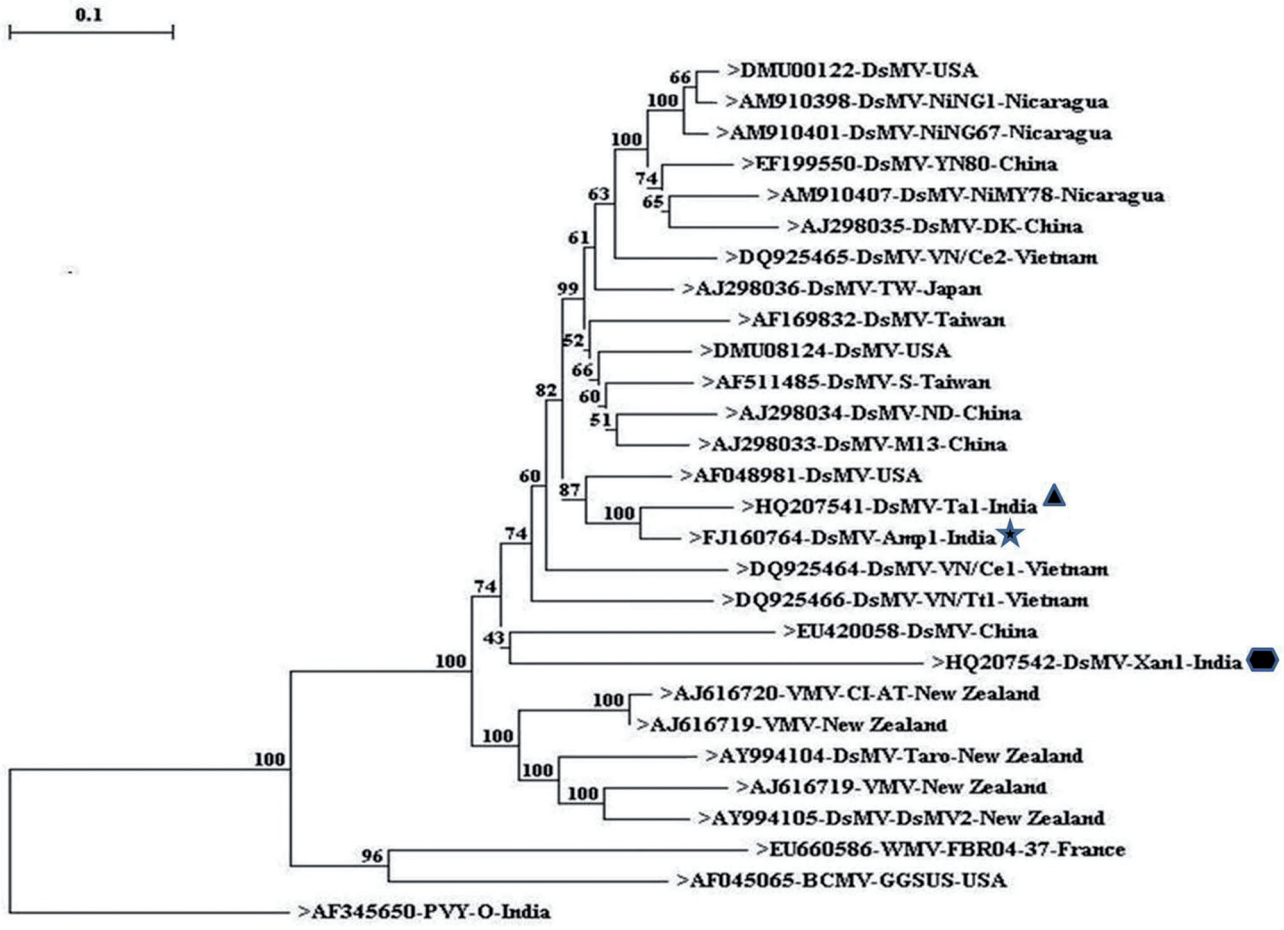

Fig. 3

Phylogenetic tree illustrating relationships of the three DsMV isolates DsMV-Amp1 ★, DsMV-Ta1 ▲ and DsMV-Xan1 ⬬ with different viral isolates using neighbor-joining method with 100 bootstrap replicates and with potato virus Y as outgroup

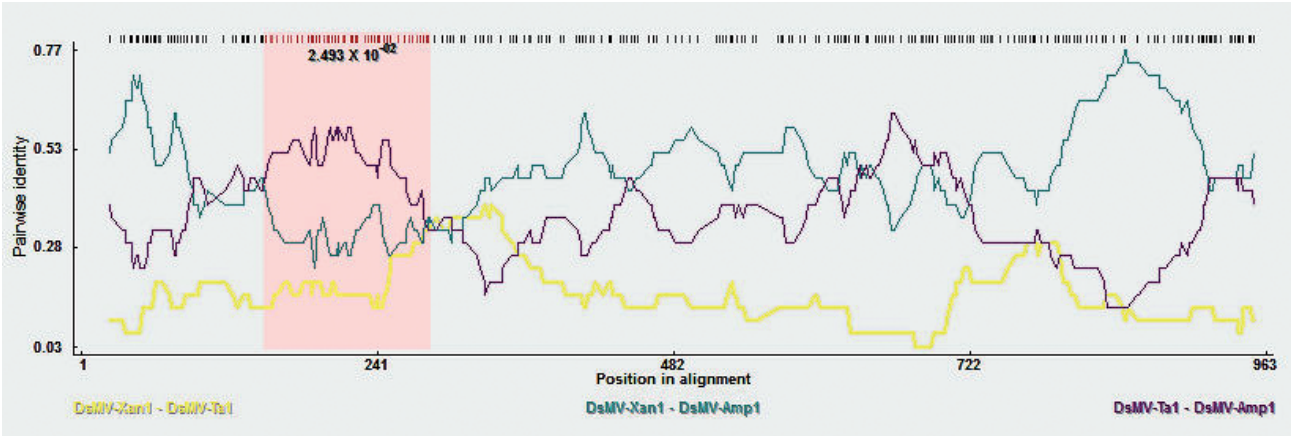

Fig. 4

Recombination rate plot of the three DsMV isolates using Recombination detection programme

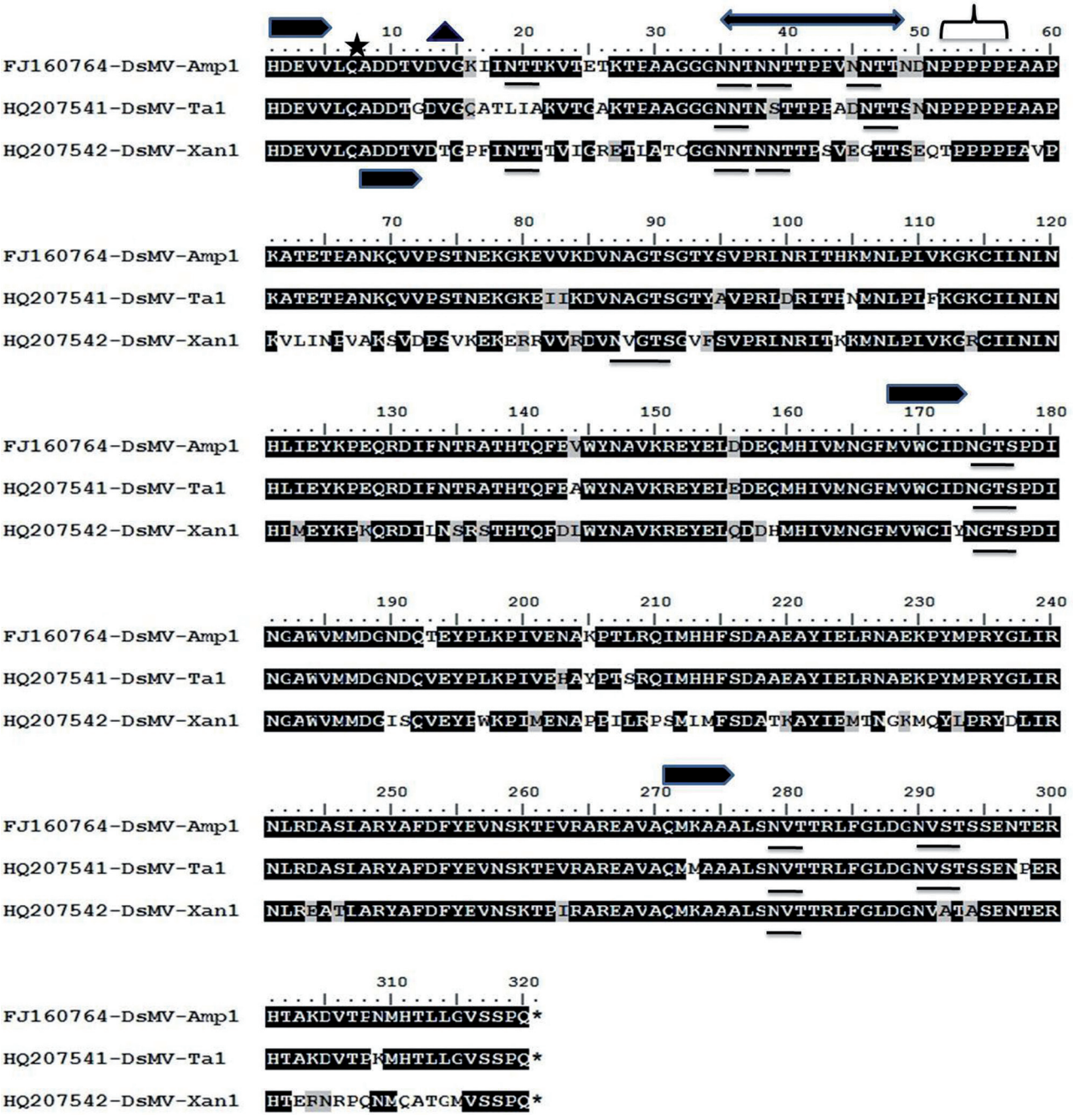

Fig. 5

Deduced amino acid sequence of the coat protein of DsMV of *A. paeoniifolius, C. esculenta,* and *X. sagittifolium*

indicates the putative protease cleavage site Q/A. The DAG motif is indicated by ▲ and the repetitive amino acid is indicated by ⟷. The proline residues are indicated by ⊓. The potential glycosylation motifs are underlined with bold lines —— and the conserved motifs (ADDTV, DEVVL, MVWCIDN, QMKAA) are indicated by ➡.

Table 1. Sequence identity matrix showing the relative nucleotide identity of the three DsMV isolate under study with other DsMV isolates

Seq->	DsMV-A. paeoniifolius	DsMV-Taro	DsMV-Xanthosoma	AF048981-DsMV	AJ298036-DsMV-TW	DQ925465-DsMV-VN/Ce2	AJ298033-DsMV-M13	AM910398-DsMV-NiNG1	DMU00122-DsMV	DMU00122-DsMV	AJ298034-DsMV-ND	AF511485-DsMV-S	AF169832-DsMV	EF199550-DsMV-YN80	AJ298035-DsMV-DK	DQ925464-DsMV-VN/Ce1	DQ925466-DsMV-VN/Tt1	AM910401-DsMV-NiNG67	AM910407-DsMV-NiNG78	AY994104-DsMV-Taro	EU420058-DsMV	AJ616720-VaMV-CI-AT
DsMV-A. paeoniifolius	ID																					
DsMV-Taro	0.927	ID																				
DsMV-Xanthosoma	0.75	0.728	ID																			
AF048981-DsMV	0.906	0.877	0.739	ID																		
AJ298036-DsMV-TW	0.877	0.854	0.725	0.871	ID																	
DQ925465-DsMV-VN/Ce2	0.862	0.836	0.72	0.865	0.904	ID																
AJ298033-DsMV-M13	0.869	0.841	0.72	0.881	0.891	0.88	ID															
AM910398-DsMV-NiNG1	0.796	0.77	0.67	0.798	0.818	0.822	0.81	ID														
DMU00122-DsMV	0.809	0.786	0.675	0.813	0.83	0.832	0.827	0.962	ID													
DMU08124-DsMV	0.862	0.839	0.712	0.878	0.899	0.861	0.894	0.809	0.82	ID												
AJ298034-DsMV-ND	0.867	0.834	0.715	0.869	0.884	0.861	0.907	0.819	0.823	0.891	ID											
AF511485-DsMV-S	0.872	0.842	0.721	0.872	0.883	0.871	0.914	0.808	0.827	0.901	0.886	ID										
AF169832-DsMV	0.819	0.791	0.678	0.821	0.844	0.816	0.834	0.818	0.836	0.84	0.819	0.841	ID									
EF199550-DsMV-YN80	0.792	0.774	0.662	0.793	0.818	0.828	0.809	0.913	0.925	0.811	0.804	0.808	0.826	ID								
AJ298035-DsMV-DK	0.819	0.794	0.693	0.812	0.825	0.833	0.82	0.83	0.839	0.816	0.813	0.811	0.814	0.861	ID							
DQ925464-DsMV-VN/Ce1	0.785	0.765	0.678	0.79	0.793	0.77	0.785	0.779	0.792	0.787	0.775	0.787	0.797	0.8	0.763	ID						
DQ925466-DsMV-VN/Tt1	0.737	0.73	0.627	0.734	0.732	0.72	0.738	0.715	0.697	0.726	0.719	0.721	0.696	0.704	0.724	0.672	ID					
AM910401-DsMV-NiNG67	0.794	0.774	0.673	0.801	0.817	0.828	0.813	0.973	0.944	0.808	0.808	0.808	0.819	0.917	0.839	0.792	0.716	ID				
AM910407-DsMV-NiNG78	0.77	0.75	0.65	0.78	0.78	0.79	0.78	0.9	0.88	0.79	0.77	0.79	0.81	0.9	0.85	0.8	0.71	0.91	ID			
AY994104-DsMV-Taro	0.701	0.688	0.623	0.692	0.697	0.697	0.689	0.717	0.713	0.694	0.691	0.693	0.715	0.725	0.72	0.71	0.646	0.718	0.72	ID		
EU420058-DsMV	0.683	0.669	0.8	0.693	0.686	0.682	0.676	0.675	0.664	0.681	0.681	0.677	0.645	0.662	0.68	0.645	0.774	0.671	0.67	0.616	ID	
AJ616720-VaMV-CI-AT	0.67	0.66	0.6	0.67	0.69	0.68	0.68	0.69	0.68	0.67	0.67	0.68	0.68	0.7	0.71	0.67	0.75	0.7	0.7	0.71	0.72	ID

DTG sequence in DsMV-Xan1 instead of the aphid transmission motif DAG, as reported in other potyviruses (Atreya *et al.,* 1991; Pappu *et al.,* 1994). In our aphid transmission experiments using *Aphis craccivora* and *Myzus persicae* we could not possibly transmit the virus to any of the three plants. The loss of aphid transmission could be attributed to the single amino acid substitutions in the DAG motif. Moreover we couldn't find any aphids species feeding naturally on the plants in the field. Even though *Aphis craccivora* and *Myzus persicae* are the common aphids for the transmission of the DsMV viruses, further analysis of our isolates using other aphid's species needs to be carried out. Following the aphid transmission motif an unusual and unique stretch of 36 amino acids, which is repetitive and rich in threonine and asparagine is found in all the isolates, followed by several proline residue repeats. The CP of all the three isolates has several threonine and asparagine (NTT, NGT, NNT, NVT NGTS, NVST, and NVS) rich potential N-glycosylation motifs in the central region, including some that cluster among the repeat sequences. Similar motifs were also found in the CP of other DsMV isolates (Pappu *et al.,* 1994). Analysis of the potyviral conserved core CP MVWCIEN motif region shows that, a single base substitution occurs with E/Y in DsMV-Xan1 and with E/D in DsMV-Amp1 and DsMV-Ta1, while the potyviral QMKAA motif was conserved in DsMV-Xan1 and a single base substitution of K/M in DsMV-Amp1 and DsMV-Ta1.

Among the edible aroids of worldwide economic importance, *A. paeoniifolius*, *C. esculenta,* and *X. sagittifolium* play a very important role in the socio-economical and agricultural scenario of India. Since the DsMV infection on the tuber crops results in reduction in the tuber yield, thus becoming major constraints for the farmer, an effective management practice for the virus needs to be developed. As a first step towards development of virus resistant varieties, the molecular characterization of the virus has to be carried out. Moreover there is limited sequence information of DsMV infecting these major edible aroids from India. Hence a comparative analysis of the DsMV viral isolates at the nucleic acid level would facilitate the development of methods for the specific diagnosis, their pattern of infection and ultimately for the development of virus resistant varieties.

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