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Incidence and Relative Distribution of Distinct Caulimoviruses (Genus *Caulimovirus*, Family *Caulimoviridae*) Associated with Dahlia Mosaic in *Dahlia pinnata*

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ABSTRACT

Pahalawatta, V., R. Miglino, K.L. Druffel, A. Jodlowska, A. R. van Schadewijk, and H.R. Pappu. 200x. Incidence and Relative Distribution of Distinct Caulimoviruses (Genus *Caulimovirus*, Family *Caulimoviridae*) Associated with Dahlia Mosaic in *Dahlia pinnata*. **Plant Dis. xxxx.**

Dahlia mosaic, caused by *Dahlia mosaic virus* (DMV), is one of the most important viral diseases of dahlia. Molecular characterization of DMV showed the association of two distinct caulimoviruses (DMV-D10, DMV-Portland) and a D10-like sequence variant (DMV-Holland) with the disease. Using primers specific to these two viruses and the sequence variant, a polymerase chain reaction-based assay was used to determine their relative incidence in several dahlia samples from the USA and the Netherlands. Testing was done on samples collected in 2005 and 2006 in the USA and in 2006 in the Netherlands. Results indicated the predominance of DMV-D10 over DMV-Portland and DMV-Holland in both USA and the Netherlands. Using conserved regions of the viral genome, primers were designed and used to detect all three sequences. Results suggested that DMV-D10 is predominantly associated with dahlia mosaic but diagnostics should also include testing for DMV-Portland and DMV-Holland.

Dahlia mosaic virus (DMV) of genus *Caulimovirus* and Family *Caulimoviridae* is one of the most important viruses of dahlia (*Dahlia pinnata*) in USA (8) and several parts of the world (1, 3). Symptoms caused by DMV include mosaic, chlorotic vein banding and leaf malformation (7). DMV is geographically widespread but the natural host range is limited only to *Dahlia* spp. A survey by Pappu et al. (8) using a PCR-based detection assay, of dahlias collected from several states in USA, showed a very high incidence of DMV in dahlias. Accurate and reliable detection of the virus is required for developing effective virus elimination programs. Based on the partial molecular characterization of the DMV genome, PCR-based detection of DMV was reported (2, 4, 8).

As part of an ongoing study to determine the sequence variability of the DMV genome, we identified a distinct caulimovirus in dahlia, referred to as DMV-D10 (5) which was found to be distinct from another caulimovirus (DMV-Portland) (11). Partial genomic sequence representing the viral coat protein and reverse transcriptase genes of another isolate, referred to as DMV-Holland, has been reported (4). While our previous survey for the incidence of DMV-D10 showed its widespread occurrence in USA (8), no information is available on the incidence of DMV-Portland. Moreover, no information is available on the incidence of DMV-Holland in Europe or in the USA. Due to the continuous movement of dahlia planting material within USA and between USA and Europe, the relative incidence of these viruses should be ascertained to devise effective and comprehensive detection and virus elimination strategies. In this study, using primers specific to DMV-D10, DMV-Portland, and DMV-Holland, the relative distribution was determined in samples collected from several states in USA and from the Netherlands.

Materials and Methods

Source of dahlia samples. One hundred and nineteen dahlia plant samples collected from commercial nurseries in California, Georgia, Montana, New Mexico, Oklahoma and dahlia varietal trial gardens in Maryland and Washington State in USA during 2005 and 2006 were used in the study. In addition, 41 samples from field-grown dahlia plants from a dahlia varietal trial garden in Lisse, the Netherlands collected in 2006 were also used. Samples were collected at random irrespective of the presence of symptoms suggestive of virus infection.

Total nucleic acid purification. Total nucleic acid was purified according to a modified Dellaporta procedure (9). Approximately 0.1 g (or a quarter-sized leaf piece) was used for DNA extraction.

Primers used in PCR. Several primer pairs encompassing different regions of the viral genomes were designed for the specific detection of the caulimoviruses associated with the disease. One set consisting of three primer pairs, designated DMV-D10, was designed for the specific detection of DMV-D10 (Table 1). A second set, designated DMV-Portland, consisting of six primer pairs (11) and a third set, designated DMV-Holland (4), were designed based on the sequences available in GenBank, for the specific detection of DMV-Portland and DMV-Holland, respectively (Table 1). Two DMV-D10, three DMV-Portland and two DMV-Holland primer pairs were used for exclusive detection of the two viruses and the sequence variant respectively, in the dahlia samples. A primer pair, Den518-F/Den1156C, that could detect DMV-D10, -Holland and

-Portland was designed based on the conserved region of the 1171 bp sequence representing partial open reading frames of coat protein and the reverse transcriptase genes (Figure 1).

PCR amplification. Two µl of 1:5 diluted total nucleic acid from above was used in each PCR reaction containing 2 µl 10X PCR buffer (Invitrogen, Carlsbad, CA), 1.2 µl 2.5 mM dNTP's, 0.8 µl 50 mM $MgCl_2$, 0.6 µl 20 µM primer 1, 0.6 µl 20 µM primer 2, 13.6 µl ddH_2O, and 0.2 µl Taq DNA polymerase (Invitrogen). PCR amplification was performed in a DNA thermal cycler (BioRad, Hercules, CA) programmed for 3 min at 94^{o}C for initial denaturation and 50 cycles each consisting of a denaturation step at 94^{o}C for 30 sec, the required annealing temperature based on the primer pair used at 20 sec and an extension step at 72^{o}C determined based on the size of the amplicon to be synthesized at the rate of 1000 bp of PCR per minute followed by a final extension for 7 min at 72^{o}C. PCR reactions (7.5 µl) were analyzed by agarose gel electrophoresis.

Cloning and sequencing. PCR products of expected size were cloned using pGEM-T (Promega, Madison, WI) or TOPO TA (Invitrogen) cloning kits. Some PCR products were sequenced directly after purification using QIAquick PCR purification kit (Qiagen, Valencia, CA). Nucleotide sequences were determined using the ABI Prism sequencing system at the Molecular Biology Core Laboratory of the Washington State University, Pullman, WA. Recombinant plasmids were sequenced using M13 forward and reverse primers, whereas PCR products were sequenced using the specific primer pairs that were initially used to obtain the PCR product.

Sequence analysis. Sequences were compiled using PC-based AlignX (Vector NTI Suite 9, Informax Inc, Bethesda, MD) or SeqAid (10)software. Multiple alignments were generated using CLUSTAL W (13). Sequences of DMV available in GenBank were used for comparisons. Primers were designed by using Primer Designer version 2.0 (Scientific and Educational Software, Cary, NC).

Results and Discussion

Each of the primer pairs used produced a specific amplicon of expected size. Amplicons resulting from using each of the primer pairs were cloned and sequenced to verify their identity. A majority of the dahlia leaf samples collected in USA (87.4%) and the Netherlands (97.6%) produced amplicons of expected size with the DMV-D10 primers. Results of PCR using the DMV-Portland specific primers showed fewer positives, with 21.8% of the USA samples and 53.6% of the samples from the Netherlands producing amplicons of expected size. In contrast, 97.6% of the samples from the Netherlands produced amplicons of expected size with DMV-Holland primers, whereas only 31.9% of the USA samples tested positive (Table 2). Only 6.7% of the samples from USA tested positive for all three viruses, whereas 36.6% of samples tested from the Netherlands were positive for all three. All samples positive with DMV-Portland were also positive for either DMV-D10 or DMV-Holland primers. The percentage of USA samples positive for both DMV-D10 and DMV-Holland was 28.6% whereas, 21.0% of the samples were positive for both DMV-D10 and DMV-Portland. A

similar trend was seen in the samples from the Netherlands, where 41.6% of the samples were positive for both DMV-D10 and DMV-Holland, while none of the samples was positive for only DMV-D10 and DMV-Portland primers. These results indicate that the most prevalent DMV sequences in dahlia in both USA and the Netherlands were those amplified by DMV-D10 primers.

Primers based on the conserved region among DMV-D10, DMV-Portland and DMV-Holland (Figure 1) were capable of amplifying all three viral sequences associated with dahlia mosaic (Figure 2).

Overall, the data indicate that there is a higher incidence of DMV-D10 in USA, whereas in the Netherlands both DMV-D10 and DMV-Holland appear to be prevalent. It is not surprising that more samples from the Netherlands were positive for DMV-Holland since the sequence from which our primers were derived was obtained from a Dutch isolate (4). These results also highlight the importance of testing dahlia planting material for viruses associated with dahlia mosaic prior to export to other countries in the light of our findings that DMV-D10, DMV-Holland and DMV-Portland sequences seem to be present at a different frequency in dahlias from USA than in the dahlia samples in the Netherlands.

Pairwise comparisons of amino acid sequences representing each open reading frame of DMV-D10 (5) and DMV-Portland (Genbank accessions AY309480, AY309479, AY291588, AY291587, AY291586, AY291585) showed that the sequence identity between DMV-D10 and DMV-Portland ranged from 47% to 73%. This divergence suggests that two distinct caulimoviruses are associated with dahlia mosaic. Resutls show that DMV-D10 is more prevalent than DMV-Portland. The third primer

pair representing DMV-Holland amplifies a part of the coat protein gene and the reverse transcriptase gene (4). The ca. 1.2 kb sequence (5) of DMV-Holland shows 59.1% sequence identity with the corresponding sequence in DMV-D10 and 41.2% identity with that of DMV-Portland. Thus, it appears that there are at least two and quite possibly three distinct caulimoviruses associated with dahlia mosaic. Complete genome characterization of DMV-Holland is needed to establish whether DMV-Holland is a distinct caulimovirus infecting dahlia. Our results also indicate that the co-existence of divergent caulimoviruses in a given dahlia plant is common. However, at present, there is no successful infectivity assay to correlate mosaic symptoms to a particular virus/sequence variant in dahlias.

DMV infection often remains latent and as a result many infected but asymptomatic plants and propagating material is distributed which might have contributed to its widespread distribution in the US (8). At present, PCR-based detection assays are the most reliable method of detecting DMV in dahlias. Considering the diversity in caulimoviruses associated with dahlia mosaic disease and the prevalence of mixed infections, use of primers specific to a single virus is not adequate for disease diagnosis as it may lead to false negatives for other caulimoviruses that may be present.

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Table 1. Primers used for the detection of *Dahlia mosaic virus* (DMV)-D10, DMV-Portland and DMV-Holland in *Dahlia pinnata.*

GenBank Accession	Primer pair	Sequence (5'-3')	T_m (^oC)	Expected size (bp)
	DMV-D10			
	ORF1-START	ATGGATCGTAAAGATT	50	900
	ORF1-END1	CTGTTTTTCTGTGTTTCTACTGG		
	ORF4CP1-1431F[a]	TGCATAAAATGAGTTCTATC	58	480
	ORF4CPI-1926C	TGAACTTGTTCATCATTATC		
	ORF6-START	ATGGAAGAAATTAAGGCGT	60	1280
	ORF6-END1	TTGTCTTCATCCATAAAGCAG		
	DMV-Portland			
AY291585	KORF1-F	ATG AAT ATC TTA GAA AGG AA	50	966
	KORF1-R	CTT AAT CCT TAA GTT ATC AA		
AY291586	Kapht-F	ATG AGT AAT GCT TCA GCA A	56	504
	Kapht-R	TGA CCA TGG CTT CTA ACT GT		
AY291587	KDNAb-F	TGA CCA CCA TCA AAG ACT TA	59	363
	KDNAb-R	TCA TAC TGG AGG CCA TTT TA		
AY291588	Kcp-F	ATG GCC TCC AGT ATG AAA G	53	1476
	Kcp-R	TTA TTC TGT TCC TGA TGA TT		
AY309479	Kpoly-F	ATG TTG TCA CAA CAG ATG AT	50	1830
	Kpoly-R	ATT CTT TTT CAT TAC TAT GG		
AY309480	KIB-F	ATG GAG GAA GAA TTA AAA GC	51	1515
	KIB-R	TTA TAT AGG CAA GTC TTC AG		
	DMV-Holland			
AJ515906	DenORF4-F	TCCAAGCAGACAGACAAACC	56	600
	DenORF4-R1	ATTGCATTTCATTTAGAACA		
AJ515906	DenORF4-F1	CAGCAAGAAACAGGAATTGA	60	600
	DenORF4-R	TTTAGCAGCTTCGACTGTAAA		

[a] Reference (6)

Table 2. Percentage of dahlia samples positive for caulimoviruses associated with dahlia mosaic.

Primer set	USA.	Netherlands
DMV-D10	87.4	97.6
DMV-Portland	21.8	53.6
DMV-Holland	31.9	97.6
DMV-D10+DMV-Portland	21.0	43.9
DMV-D10+DMV-Holland	28.6	97.6
DMV-Portland+DMV-Holland	6.7	17.1
DMV-D10+DMV-Portland + DMV-Holland	6.7	36.6
Negative	4.2	0

Figure 1. Nucleotide sequence alignment of the reverse transcriptase gene of DMV-D10 (unpublished), DMV-Portland (Genbank accession AY309479) and DMV-Holland (Genbank accession AJ515906) using CLUSTAL W (12). * - identical nucleotides. Boxed regions denote the sequence used to design degenerate primer Den518-F[a] and Den1156C[b]. Degenerate primer sequences are given in bold.

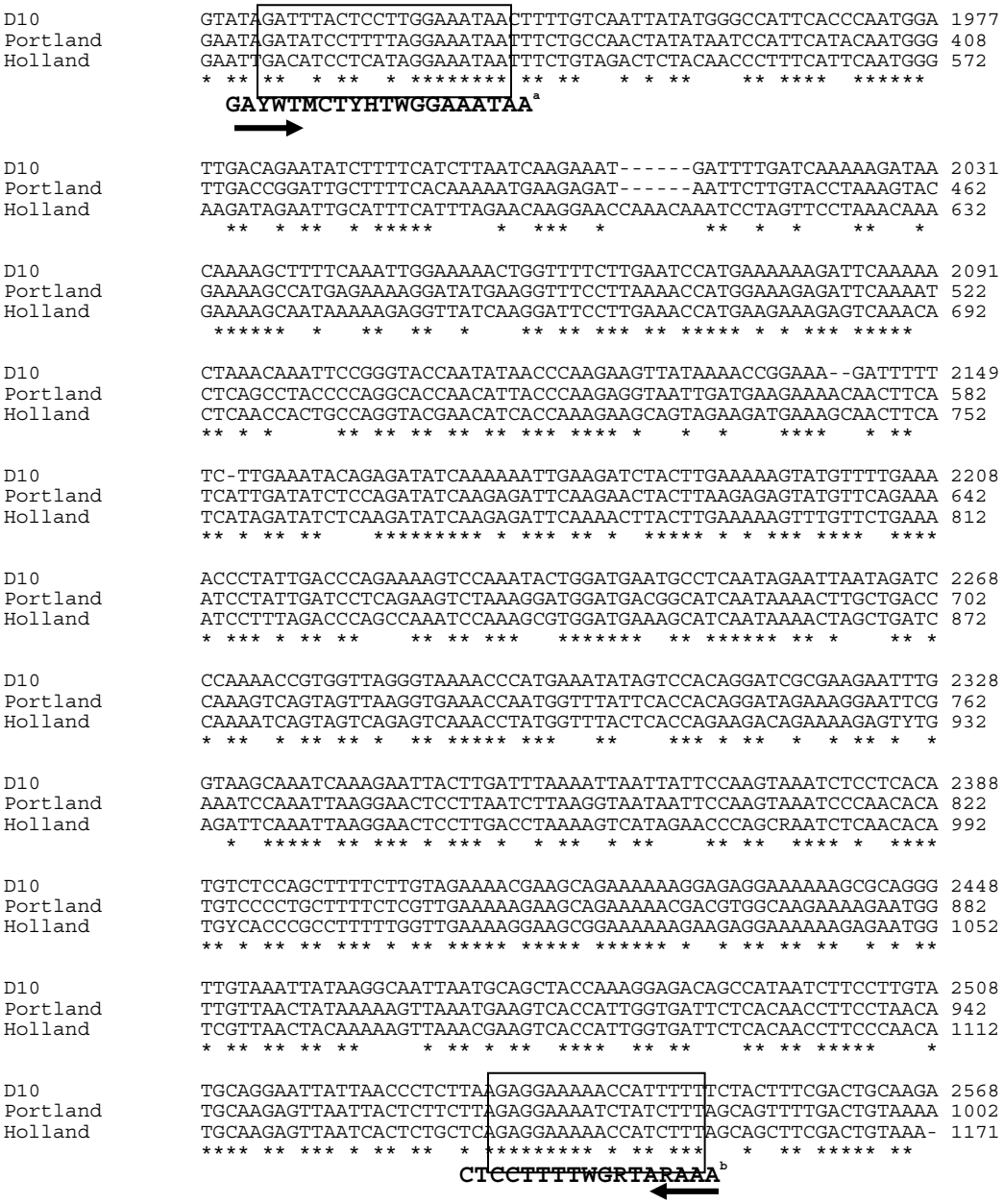

Figure 2. Agarose gel electrophoresis of PCR products from DNA extracts of three dahlia samples amplified with primers specific to *Dahlia mosaic virus* (DMV)-D10 (D), DMV-Portland (P), DMV-Holland (H) and degenerate (Dg) primers that could amplify all three caulimoviruses associated with dahlia mosaic. L – 1 kb ladder.