

1 **Plant Disease: 91:1194-1197 (2007)**

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

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1 **ABSTRACT**

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3 Pahalawatta, V., R. Miglino, K.L. Druffel, A. Jodlowska, A. R. van Schadewijk, and H.R.
4 Pappu. 200x. Incidence and Relative Distribution of Distinct Caulimoviruses (Genus
5 *Caulimovirus*, Family *Caulimoviridae*) Associated with Dahlia Mosaic in *Dahlia*
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9 Dahlia mosaic, caused by *Dahlia mosaic virus* (DMV), is one of the most important viral
10 diseases of dahlia. Molecular characterization of DMV showed the association of two
11 distinct caulimoviruses (DMV-D10, DMV-Portland) and a D10-like sequence variant
12 (DMV-Holland) with the disease. Using primers specific to these two viruses and the
13 sequence variant, a polymerase chain reaction-based assay was used to determine their
14 relative incidence in several dahlia samples from the USA and the Netherlands. Testing
15 was done on samples collected in 2005 and 2006 in the USA and in 2006 in the
16 Netherlands. Results indicated the predominance of DMV-D10 over DMV-Portland and
17 DMV-Holland in both USA and the Netherlands. Using conserved regions of the viral
18 genome, primers were designed and used to detect all three sequences. Results suggested
19 that DMV-D10 is predominantly associated with dahlia mosaic but diagnostics should
20 also include testing for DMV-Portland and DMV-Holland.

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

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

Materials and Methods

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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

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

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5 **PCR amplification.** Two μl of 1:5 diluted total nucleic acid from above was
6 used in each PCR reaction containing 2 μl 10X PCR buffer (Invitrogen, Carlsbad, CA),
7 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
8 primer 2, 13.6 μl ddH₂O, and 0.2 μl Taq DNA polymerase (Invitrogen). PCR
9 amplification was performed in a DNA thermal cycler (BioRad, Hercules, CA)
10 programmed for 3 min at 94°C for initial denaturation and 50 cycles each consisting of a
11 denaturation step at 94°C for 30 sec, the required annealing temperature based on the
12 primer pair used at 20 sec and an extension step at 72°C determined based on the size of
13 the amplicon to be synthesized at the rate of 1000 bp of PCR per minute followed by a
14 final extension for 7 min at 72°C. PCR reactions (7.5 μl) were analyzed by agarose gel
15 electrophoresis.

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

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

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Results and Discussion

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

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

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

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

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

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

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

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

GenBank Accession	Primer pair	Sequence (5'-3')	T _m (°C)	Expected size (bp)
	<u>DMV-D10</u>			
	ORF1-START	ATGGATCGTAAAGATT	50	900
	ORF1-END1	CTGTTTTTCTGTGTTTCTACTGG		
	ORF4CP1-1431F ^a	TGCATAAAATGAGTTCTATC	58	480
	ORF4CPI-1926C	TGAACTTGTTTCATCATTATC		
	ORF6-START	ATGGAAGAAATTAAGGCGT	60	1280
	ORF6-END1	TTGTCTTCATCCATAAAGCAG		
	<u>DMV-Portland</u>			
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		
	<u>DMV-Holland</u>			
AJ515906	DenORF4-F	TCCAAGCAGACAGACAAACC	56	600
	DenORF4-R1	ATTGCATTTTCATTTAGAACA		
AJ515906	DenORF4-F1	CAGCAAGAAACAGGAATTGA	60	600
	DenORF4-R	TTTAGCAGCTTCGACTGTAAA		

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4 ^a Reference (6)

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

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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.

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D10          GTATAGATTTACTCCTTGGAAATAACTTTGTCAATTATATGGGCCATTCACCCAATGGA 1977
Portland     GAATAGATATCCTTTTAGGAAATAATTTCTGCCAATATATAATCCATTCATACAATGGG 408
Holland      GAATTGACATCCTCATAGGAAATAATTTCTGTAGACTCTACAACCCTTTCATTCAATGGG 572
* * * * *
GAYWTMCYHTWGGAAATAAa
      ↓

D10          TTGACAGAATATCTTTTCATCTTAATCAAGAAAT-----GATTTTGATCAAAAAGATAA 2031
Portland     TTGACCGGATTGCTTTTCACAAAATGAAGAGAT-----AATCTTGTACCTAAAGTAC 462
Holland      AAGATAGAATTGCATTTTCATTTAGAACAAGGAACCAAAACAATCCTAGTTCCTAAACAAA 632
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D10          CAAAAGCTTTTCAAATGGAAAAACTGGTTTTCTTGAATCCATGAAAAAGATTCAAAAA 2091
Portland     GAAAAGCCATGAGAAAAGGATATGAAGGTTTTCTTAAAACCATGGAAAAGAGATTCAAAA 522
Holland      GAAAAGCAATAAAAAGAGGTTATCAAGGATTCTTGAACCATGAAGAAAAGAGTCAACA 692
* * * * *

D10          CTAACCAAATTCGGGTACCAATATAACCCAAGAAGTTATAAAACCGGAAA--GATTTT 2149
Portland     CTCAGCCTACCCAGGCACCAACATTACCCAAGAGGTAATTGATGAAGAAAACAACCTCA 582
Holland      CTCAACCACTGCCAGGTACGAACATACCAAGAAGCAGTAGAAGATGAAAAGCAAACCTCA 752
* * * * *

D10          TC-TTGAAATACAGAGATATCAAAAAATGAAGATCTACTTGAAAAAGTATGTTTTGAAA 2208
Portland     TCATTGATATCTCCAGATATCAAGAGATTCAAGAACTACTTAAGAGAGTATGTTTCAGAAA 642
Holland      TCATAGATATCTCAAGATATCAAGAGATTCAAACTTACTTGAAAAAGTTTGTCTGAAA 812
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D10          ACCCTATTGACCCAGAAAAGTCCAAATACTGGATGAATGCCTCAATAGAATTAATAGATC 2268
Portland     ATCCTATTGATCCTCAGAAGTCTAAAAGGATGGATGACGGCATCAATAAAAACCTTGCTGACC 702
Holland      ATCCTTTAGACCCAGCCAAATCCAAAGCGTGGATGAAAGCATCAATAAAAACCTAGCTGATC 872
* * * * *

D10          CAAAACCGTGGTTAGGGTAAAACCCATGAAATATAGTCCACAGGATCGCGAAGAATTGG 2328
Portland     CAAAGTCAGTAGTTAAGGTGAAAACCAATGGTTTATTACCACAGGATAGAAAAGGAATTGG 762
Holland      CAAAATCAGTAGTCAGAGTCAAACCTATGGTTTACTCACCAGAAGCAGAAAAGAGTYTG 932
* * * * *

D10          GTAAGCAAATCAAAGAATTACTTGATTTAAAATTAATTATTCCAAGTAAATCTCCTCACA 2388
Portland     AAATCCAAATTAAGGAACTCCTTAATCTTAAGGTAATAATTCCAAGTAAATCCCAACACA 822
Holland      AGATTCAAATTAAGGAACTCCTTGACCTAAAAGTCATAGAACCAGCRAATCTCAACACA 992
* * * * *

D10          TGTCTCCAGCTTTTCTTGTAGAAAACGAAGCAGAAAAAAGGAGAGGAAAAAAGCGCAGGG 2448
Portland     TGTCCCTGCTTTTCTCGTTGAAAAAGAAGCAGAAAAACGACGTGGCAAGAAAAGAAATGG 882
Holland      TGYCACCGCCTTTTGGTTGAAAAGGAAGCGGAAAAAAGAGAGGAAAAAAGAGAAATGG 1052
* * * * *

D10          TTGTAAATTATAAGGCAATTAATGCAGCTACCAAGGAGACAGCCATAATCTTCTTGTA 2508
Portland     TTGTTAACTATAAAAAGTTAATGAAGTCACCATGGGTGATTCTCACAACTTCCCTAACA 942
Holland      TCGTTAACTACAAAAGTTAAACGAAGTCACCATGGGTGATTCTCACAACTTCCCAACA 1112
* * * * *

D10          TGCAGGAATTATTAACCTCTTAAGAGGAAAAACCATTTTTTCTACTTTCGACTGCAAGA 2568
Portland     TGCAAGAGTTAATTAATCTTCTTAGAGGAAAATCTATCTTTAGCAGTTTTCGACTGTAAA 1002
Holland      TGCAAGAGTTAATCACTCTGCTCAGAGGAAAAACCATTTTTAGCAGCTTTCGACTGTAAA 1171
* * * * *
CTCCTTTTWGRTARAAAb
      ↑

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

