

Research Paper

## Occurrence and molecular characterization of *Cucumber green mottle mosaic virus* in cucurbit crops of KPK, Pakistan

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### Abstract

Field survey of the cucurbit crops revealed a high incidence of *Cucumber green mottle mosaic virus* (CGMMV) in Khyber Pakhtunkhwa Province (KPK), Pakistan. Among the seven districts surveyed, average percent incidence of CGMMV was recorded up to 58.1% in district Nowshera, followed by 51.1% in district Charsada, 40.5% in district Swabi and 37.3% in district Mardan. In Swat and Dir districts average incidence CGMMV was recorded upto 31.2% and 29.4%, respectively. Among the different crops highest incidence in plain areas of KPK was recorded in bottle gourd (59.3%) followed by 56.3% in Squash, 54.5% in Pumpkin, 45.5% in Melon, 41.7% in Cucumber and 29.9% in Sponge gourd. In Northern hilly areas highest incidence of CGMMV (52.9%) was observed in pumpkin, followed by 49.6% in bottle gourd, 47.3% in squash, 45.1% in Melon 42.3% in cucumber and 41.6% in sponge gourd. Little variability was observed in the coat protein amino acid sequence identities of CGMMV Pakistan isolate, when compared with other reported isolates.

**Key words:** cucurbit, CGMMV, incidence, tobamovirus, Pakistan.

### Introduction

Plants of the family *Cucurbitaceae*, including many edible and non-edible species, are native in most countries of the world, especially in the tropics and in areas with relatively warm temperature. Cucurbit fruits have yellow, white or orange flesh, which is rich in carotenoids, the compounds humans need to make vitamin A, and the visual pigment rhodopsin. Cucurbit crops, including summer squash (*Cucurbita pepo*), bottle gourd (*Lagenaria siceraria*), cucumber (*Cucumis sativus*), sponge gourd (*Luffa acutangula*), bitter gourd (*Momordica charantia*) and snake/serpent gourd (*Cucumis melo* var. *flexuosus*), produced throughout Pakistan including the Khyber Pakhtunkhwa (KPK) and are regarded as important summer and winter vegetables.

In KPK cucurbits are grown on 3140 ha (does not include the figures for lufa, long-melon and cucumber at provincial level, as these were not available) with an annual estimated production of 29544 tons, while in Pakistan cucurbit occupies an area of 28600 ha with a total annual pro-

duction of 261,306 tons (MINFAL, 2008). The average yield of such crops (approximately 9 tons/ha) in the region is quite low. A number of biotic and abiotic factors are responsible for this low yield. The cultural practices adapted by the growers, conducive environmental conditions for disease epidemic development, lack of awareness of growers both about viral disease epidemiology and the precautions required for control and the use of uncertified seeds are the major factors that result in high incidence of viral diseases. High population of weeds also plays an important role in virus epidemiology and is a common problem in vegetable growing areas of KPK.

Cucurbit viral diseases are a worldwide problem and induce major economic losses in commercial cucurbit production around the world (Lovisol, 1980). Specially the vector transmitted viruses that may cause losses as high as 100% (Ullman *et al.*, 1991). Almost 35 different viruses have been isolated from *Cucurbitaceae* (Provvidenti, 1996). In Pakistan cucurbit viruses are quite prevalent in the different cucurbit crops of KPK (Ali *et al.*, 2004; Khalid and Ahmad, 1997).

*Cucumber green mottle mosaic virus* (CGMMV) is member of *Tobamovirus* genus, and is commonly reported in different cucurbit crops throughout the world (Nameth *et al.*, 1986; Ullman *et al.*, 1991; Desbiez and Lecoq, 1997; Choi, 2001; Lecoq *et al.*, 2001; 2003; Ali *et al.*, 2004). The virus is also seed transmitted in different cucurbits and is usually present on the surface of the seed as seed contaminant. The very high stable genome coupled with transmission through seeds and contact makes this virus a big menace causing heavy losses across the world wherever cucurbits are grown. Presence of the inoculum right from the beginning of the crop growth results in a very high infection/incidence level and usually it is very difficult for plants to resist infection under such circumstances (Coutts and Jones, 2005). According to a preliminary pilot study CGMMV infection was high in cucurbit crops of KPK (Ali *et al.*, 2004). The present study is first ever effort to determine the detailed incidence and status of CGMMV in different cucurbit crops as well as different agro ecological zones of KPK, to characterize the virus at molecular level and to know the phylogenetic status of the Pakistani isolates.

## Material and Methods

### Field survey

A total of 22 fields in seven districts of the KPK viz; District Peshawar (Tarnab, Budni and Taru), District Nowshera (Jehangira, Nowshera, Pirsabaq), District Mardan (Shelgazi Baba Mumtaz Abad, Takhtbhai), District Swabi (Kalu Khan, Shewi Adda, Tordheri), District Charsada (Sheikhabad, Umerzai, Serdheri), District Swat (Manyar, Bahrain, Kaidam Kalam, Batkhela) and District Dir (Gulabad, Talash, Timergarah, Chakdara) were surveyed to find out the incidence and distribution of CGMMV in Pakistan for two consecutive years (2007-2009) collecting randomly a total of 35 samples from each field. Major crops surveyed for the collection of samples were bottle gourd (*Lagenaria siceraria*), sponge gourd (*Lufa acutangula*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita pepo*), squash (*Cucurbita muschata*) musk melon (*Cucumis melo*) etc. The collected samples were brought to the plant virology laboratory and stored at -80 °C till further processing.

### Disease incidence

All the collected plant samples were tested for the presence of *Cucumber green mottle mosaic virus* (CGMMV) using DAS-ELISA [Double Antibody Sandwiched-Enzyme Linked Immunosorbent Assay (Clark and Adams, 1977). Commercial kits from (Agdia, USA) were used for analysis of the samples. All the buffers, coating antibodies and conjugated antibodies were diluted as per the recommendations of the manufacturer. Following procedure was used to assay the samples. 100 µL of the antibod-

ies, diluted in coating buffer (1:2000 v/v), were coated in each well of the ELISA plates and the plates were incubated at 37 °C for 2 h. After incubation the plates were washed three times with the help of washing buffer. -The collected samples were crushed thoroughly in the extraction buffer (1:5 w/v) and 100 µL of the sap was applied to the wells. The plates were incubated at 4 °C overnight and were washed thrice again with the help of washing buffer. After washing, 100 µL of the diluted conjugate antibodies (1:1000 v/v) were applied to the wells and the plates were incubated at 37 °C for 1 h. Washing was repeated again three times using washing buffer and finally 100 µL of substrate (p-nitro-phenyl phosphate @ 1 mg/mL) were applied to each well. The plates were incubated at about 25 °C (or room temperature) in dark. Data were taken after 30 min and after 60 min.

Disease incidence was calculated using the following formula;

$$\% \text{ Disease incidence} = \frac{Ip}{Np} \times 100$$

where  $I_p$  = number of infected plants and  $N_p$  = total number of plants.

### RT-PCR amplification and sequence analysis of the coat protein gene of CGMMV

The cDNA of the selected positive samples was synthesized using iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions with 3'-end virus-specific primer. CGMMV RNA was isolated by simple direct tube (SDT) method (Suehiro *et al.*, 2005) by crushing the symptomatic leaf (0.1 g) in 1 mL of PBST (PBS + 1%, Tween 20) pH 7.4. 50 µL sap was transferred to a 1.5 mL microfuge tube and incubated at room temperature for 15 min. The tubes were then washed twice with PBST and 30 µL of nuclease free water was added into the tube through pipetting. The contents were placed on hot water bath at 95 °C for 3 min. After heating, 8 µL were transferred to a PCR tube with 0.5 µL reverse transcriptase enzyme, 2 µL of 5x reaction mix and 0.5 µL 3' specific primer, to make a total of 11 µL reverse transcription reaction mixture. cDNA synthesis was performed in three steps including primer annealing at 25 °C for 5 min followed by extension at 42 °C for 30 min and finally treatment of 85 °C for 5 min. This cDNA was then used to amplify the coat protein region of CGMMV, using Super-Hot master mix (Bioron, Germany) in 50 µL PCR mixture containing 1 µL cDNA, 22 µL nuclease free water, 1 µL (25 pmol) each of the CP-F (GATGCATTCTGTTGCAGAGG) and CP-R (TCAACCTCACACACGTAAGA GG) virus specific primers and 25 µL of 2x PCR Super-Hot master mix (1 unit Taq DNA polymerase, 32 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 130 mM Tris HCL with pH 8.8, 0.02% Tween-20, 3 mM MgCl<sub>2</sub> and 0.4 mM each of the dNTPs). After the initial denaturation step at 94 °C for 2 min, PCR was performed for 35 cycles,

each at 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 1 min using MJmini Thermal Cycler, Bio RAD, USA. The (RT) PCR products were separated in 2% agarose gel electrophoresis and detected by ethidium bromide staining under UV light in UVITEC, Gel Doc, UK.

Following PCR amplification, the RT-PCR products of three isolates from different divisions were electro-eluted from the gel using standard procedures (Sambrook and Russell, 2001) and cloned into pUC19 cloning vector for transformation. Competent cells of *Escherichia coli* strain NEB 5- $\alpha$  (New England Biolabs) were transformed with the cloned DNA according to the recommendations of the manufactures. Standard alkali lyses method was used to isolate plasmid DNA (Sambrook and Russell, 2001). Plasmids with the desired DNA insert were selected and sequenced using ABI sequencer. The sequence was analyzed to determine the phylogenetic status of Pakistani Isolate.

Alignment of amino acid (aa) and nucleotide (nt) sequences, representing the coat protein (CP) region of CGMMV-Pak with sequences reported in the data bank (Table 1) were constructed using protein and DNA alignment program in GENETYX Win Version 5.1 (Software Development, Tokyo, Japan). The deduced aa and nt sequences were compared with the sequences reported in the DNA database and the phylogenetic relationship was established using the CLUSTAL-W program (Thompson *et al.*, 1994), by the Neighbor Joining Method. The phylogenetic trees were viewed in Treeview (win32) 1.6.6 program. The amino acid sequence of the Pakistani isolate was compared with the sequences reported elsewhere in the world (Table 1).

## Results and discussion

Field surveys of the cucurbit crops revealed a high incidence of leaf mosaic, mottling, chlorosis and deformation in KPK, Pakistan. The newly growing apical leaves were small with very conspicuous symptoms. *L. siceraria* plants mostly exhibited mosaic symptoms. Interveinal chlorosis and severe mosaic symptoms were observed on *C. sativus*. Squash plants were also showing severe mosaic, mottle, deformation and filiformism. In some of the fields with severe infection leaves were having shoe-string appearance. The prevalence of such symptoms in cucurbit crops have already been reported by Ali *et al.* (2004); Khalid and Ahmed (1997) and Malik *et al.* (2006).

Among the seven districts surveyed, the highest incidence of CGMMV was recorded in Charsada (57.05%) during the final survey of the first season as compared to 29.3% incidence at district Swabi during the earlier survey, the same season. Farmers cultivated different cucurbits in the same fields. Bottle gourd, sponge gourd and squash were often seen cultivated in the same field thereby increasing the crop density making virus transmission more easy

**Table 1** - List of sequences used in the phylogenetic analysis of CGMMV.

Virus acronym/strain	Geographic region	Accession Number
TMV (Out group)	USA	NC 001367
Strain C	Japan	AB015144
Yodo strain	Japan	AB015145
WM strain	Japan	AB015146
SH strain	Japan	D12505
SH strain	Japan	BAA02071
Strain NS	Korea	AJ243831
NS strain	Korea	CAB52287
WM strain	Korea	AF225984
Strain Y	Korea	AJ245440
Indian	India	EU366912
India	India (Rajasthan)	DQ767636
Chinese	China	DQ997778
Russian	Russia	EF521882
GR7	Greece	AJ459423
France	France	CAD22087
Indonesian	Indonesia	AB194531

and quick. Also the cucurbit season lasted a little longer in district Charsada compared to district Swabi which might be responsible for the high incidence. In the early survey of the first season incidence was in the range of 12.50% (cucumber) to 37.50% (pumpkin) which increased up to 66.66% (bottle gourd). The least percent infection was found in district Mardan (46.52%). CGMMV infection during the first survey of the second season ranged from 6.6% in cucumber to 36.6% in squash. Highest average percent incidence of 25.6% was observed in district Charsada followed by 27.4% in district Nowshera, 23.6% in Peshawar, 18% in district Swabi and 15.9% in district Mardan. However, during the late survey of the second season average percent incidence of CGMMV increased up to 58.1% district Nowshera, followed by 51.1% in district Charsada, 40.5% in district Swabi and 37.3% in district Mardan (Table 2).

In Swat and Dir districts the highest incidence of CGMMV during the first season was observed in district Swat where average percent incidence of 31.23% was recorded. In district Dir virus incidence was recorded as 29.37%. Percent incidence in district Swat ranged from 13.3% in cucumber to 56.25% in bottle gourd, while in district Dir percent incidence in different crops ranged from 10% to 53.33% in bottle gourd crop during the first season of crop growth. However, during the second season highest average percent incidence (36.20%) was recorded in district Swat and 27.20% in district Dir (Table 3)

Average percent incidence in the plain areas of KPK was the highest in Pumpkin (35.65%) during the first survey of the first season, followed by 25.80% in Bottle gourd,

**Table 2** - Percent incidence of CGMMV in the plain areas of KPK.

Districts surveyed	1st year		2nd year	
	1 <sup>st</sup> survey	2 <sup>nd</sup> survey	1st survey	2 <sup>nd</sup> survey
District Peshawar Tarnab (Squash)	25	65.6	26.6	56.25
Taru (Squash)	16.6	56.6	19.4	41.4
Budni (Bottle gourd)	23.3	53.14	21.87	48.2
Taru (Sponge gourd)	13.3	26.6	26.6	30.5
Average	19.55	50.49	23.6	44.1
District Nowshera Jehangira (Squash)	13.3	50	36.6	66.6
Pirsabaq (Bottle gourd)	26.6	66.66	23.3	56.6
Nowshera (Cucumber)	12.5	33.33	16.6	46.6
Azakhel (Pumpkin)	-	-	33.3	62.5
Average	17.47	49.99	27.4	58.1
District Mardan Takhtbahi (Bottle gourd)	33.3	53.14	25	40.5
Katlang (Sponge gourd)	-	-	12.5	41.4
Takhtbahi (Sponge gourd)	16.6	33.3	-	-
Mumtaz Abad (Squash)	23.3	53.12	19.6	45.2
Sher Garh (Cucumber)	-	-	6.6	22.1
Average	24.4	46.52	15.9	37.3
District Charsada Serdheri (Bottle gourd)	20	64.5	26.6	53.3
Sheikhabad (M. Melon)	33.3	56.66	23.3	45.4
Umerzai (Cucumber)	23.3	50	33.3	62.5
QaziKhel Jadid (Sponge gourd)	-	-	19.4	43.3
Average	25.53	57.05	25.6	51.1
District Swabi Tordher (M.Melon)	16.6	34.37	19.4	44.9
Kernal Sher Keli (Pumpkin)	37.5	65.62	13.3	27.4
Kalu Khan (Pumpkin)	33.8	43.3	19.4	43.3
Zaida (Sponge gourd)	-	-	20	46.3
Average	29.3	47.76	18	40.5

**Table 3** - Percent incidence of CGMMV in the hilly areas of KPK.

District Swat	1 <sup>st</sup> year		2 <sup>nd</sup> year	
	Kalam (Cucumber)	33.3	Kaidam (Pumpkin)	36.4
Kalam (Pumpkin)	30	Ashoran (Cucumber)	32.3	
Bahrain (Cucumber)	13.3	Laikot (Lagenaria)	33.3	
Batkhela (Lagenaria)	56.25	Batkhela (Luffa)	38.7	
Manyar-Swat (Pumpkin)	23.3	Bahrain (Cucumber)	40.1	
Average	31.23*	Average	36.20	
District Dir				
Talash (Pumpkin)	34.37	Talash (Cucumber)	38.3	
Chakdara (Lagenaria)	10	Chakdara (M. Melon)	36.4	
Talash (Lagenaria)	12.5	Gulabad (Luffa)	28.3	
Temergarah (Lagenaria)	53.33	Temergarah (Squash)	15.3	
Gul Abad (Luffa)	36.66	Chakdara (Lagenaria)	17.4	
Average	29.37	Average	27.20	

24.95% in Melon, 19.55% in Squash, 17.90% in Cucumber and 14.95% in bottle gourd (Table 4). Percent incidence of CGMMV increased rapidly upto 59.34% in Bottle gourd, 56.33% in Squash, 54.46 in Pumpkin, 45.51 in Melon, 41.65 in Cucumber and 29.95 in Sponge gourd during the second survey (Table 4). During the second season highest incidence of CGMMV (52.9%) was observed in pumpkin, followed by 49.6% in bottle gourd, 47.3% in squash, 45.1% in Melon 42.3% in cucumber and 41.6% in sponge gourd (Table 4).

In the northern areas of KPK the highest average percent infection during the first season, was recorded in bottle gourd where 41.66% incidence was recorded, followed by 37.1% in pumpkin, 36.66% in cucumber and 26.66% in sponge gourd. However, during the second year highest incidence (36.9%) was recorded in cucumber, followed by 36.4% in pumpkin, 33.5% in sponge gourd, 25.4% in bottle gourd and 15.3% squash (Table 5)

The most severely infected field was observed at Pirsabaq, where the percent infection was 66.66% (bottle



**Table 4** - Incidence of CGMMV in different crops of the plain areas of KPK.

Crop	1 <sup>st</sup> year		2 <sup>nd</sup> year	
	1 <sup>st</sup> survey	2 <sup>nd</sup> survey	1 <sup>st</sup> survey	2 <sup>nd</sup> survey
Bottle gourd	25.80	59.34	24.1	49.6
Sponge gourd	14.95	29.95	19	41.6
Cucumber	17.90	41.65	19.9	42.3
Squash	19.55	56.33	23.1	47.3
Pumpkin	35.65	54.46	26.3	52.9
Melon	24.95	45.51	21.3	45.1

**Table 5** - Incidence of CGMMV in different crops of the northern hilly areas of KPK.

Crop	1 <sup>st</sup> year	2 <sup>nd</sup> year
Lagenaria	41.66	25.4
Cucumber	36.66	36.9
Pumpkin	37.1	36.4
Luffa	26.6	33.5
Squash	0	15.3

gourd) during the second survey of the first season (Table 2). CGMMV has been reported as the most prevalent virus besides other diseases in cucurbits in many cucurbit growing areas (Yuki *et al.*, 2000; Nameth *et al.*, 1986). The farmers of the plain as well as the northern hilly areas had a little or no knowledge about viral diseases as well as their mode of transmission and spread. During our survey of the cucurbit growing areas of KPK it was observed that majority of the farmers used uncertified seeds available in the open market. These seeds have neither been given any treatment, nor are they tested for the presence of seed transmitted viruses, especially CGMMV and CMV. The contact transmission of the CGMMV coupled with the poor phytosanitary measures, the connecting irrigation channels among the fields and use of contaminated tools exacerbate the situation. High population of weed was found in the fields of district Swabi and Charsada which may serve as alternate hosts of the virus. Also, at the early stage of crop growth most of the cucurbit fields were surrounded by crops like maize, tomato, chilies and tobacco and such a cropping pattern in the area also favours the build-up of virus inoculum. Egg plants, tomato, okra and a number of weed plants are the potential reservoirs of CGMMV which contribute to a great extent for the high incidence of viral diseases (Guerini and Murphy, 1999).

#### Molecular characterization of CGMMV

RT-PCR results revealed the presence of CGMMV in all the samples collected from areas like Tarujaba, Budni, Jehangira, Serdheri, Swat and Chakdara. Using CG-F and CG-R primers, we were able to amplify an expected band of

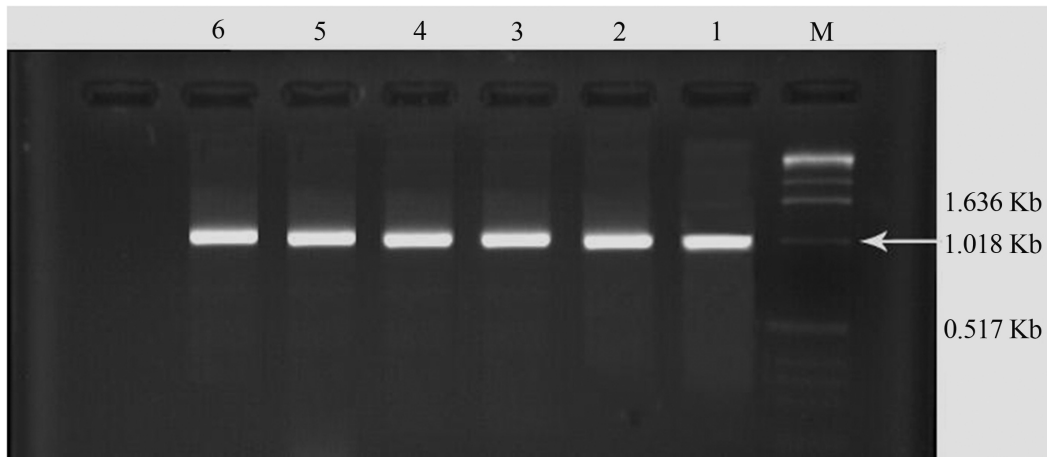
approximately 1 kb comprising the full coat protein and partial movement protein region (Figure 1).

The nt (nucleotide) and deduced aa (amino acid) sequences for the CP gene of the three CGMMV isolates when compared with other reported isolates showed very low diversity. All the three isolates showed 100% amino acid sequence identity among themselves. The aa identities of CGMMV-pk isolates with the isolates reported in the GenBank

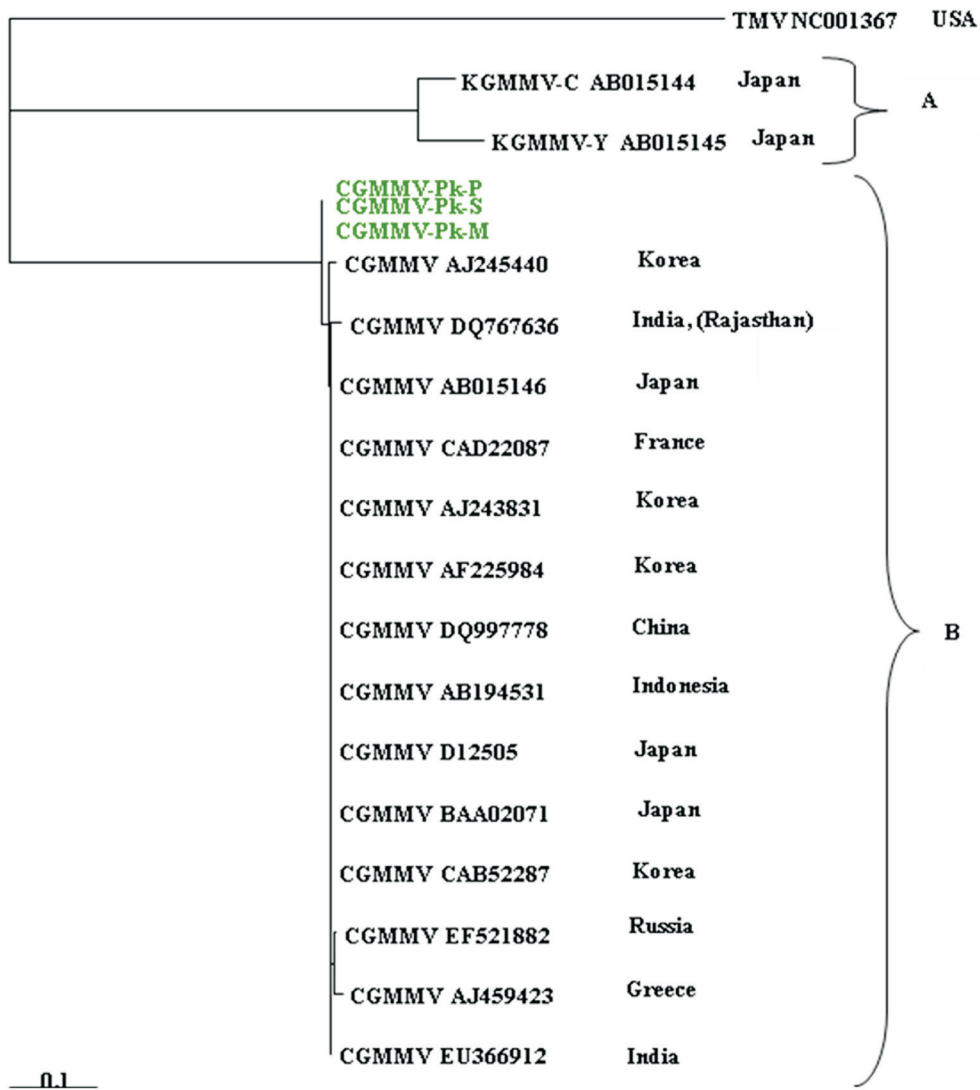
<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein>) were in range of 98.052% (Korea, India and Greece) to 99.351% (Japan, France, Korea, China, Indonesia and India). CGMMV-pk showed 98.01% aa identity with the GR7 strain reported from Greece. The amino acid sequence was highly conserved when aligned in the coat protein region sharing high aa sequence identity with a difference of only two amino acids at position 2 and 13 in N-terminal region of coat protein sequence and 5 amino acids in core coat protein region in between amino acid number 64 to 125 (data not shown). Isolates of CGMMV in particular and tobamoviruses in general are very much conserved and show very little variability (Yoon *et al.*, 2008).

The phylogenetic tree based on CP gene sequence of CGMMV with the other reported sequences resulted in two clusters. All the CGMMV isolates were clustered in a single group (Cluster B). This cluster contains isolates reported from Korea, India, Japan, France, Indonesia, Russia and Greece (Figure 2). Lack of variability in the genome of all these isolates indicates that most of these isolates might have originated from common ancestors or they may be a single strain with recent spread and/or result of high negative selection pressure on CGMMV genome. These results are in agreement with the findings of Yoon *et al.* (2008) who also reported no remarkable diversity among the reported isolates of CGMMV across the world.

Yodo strain and strain-C (reported from Japan) showed high variability and clustered independently, however they were close to each other in the tree (Cluster A; Figure 2). These two isolates were previously grouped as CGMMV but now they are classified as an independent genus and were renamed as *Kuyri Green Mottle Mosaic Virus* (KGMMV). This first ever detailed survey of CGMMV in cucurbit growing areas of the KPK province clearly reflects



**Figure 1** - Agarose (2%) showing the amplified bands of approximately 1 kb encoding coat protein and partial movement protein region in the genome of CGMMV. (1 = Tarujaba, 2 = Budni, 3 = Jehangira, 4 = Serdheri, 5 = Swat and 6 = Chakdara).



**Figure 2** - The phylogenetic relationship of CGMMV isolates reported in the GenBank with Pakistani isolate based on nucleotide sequence of coat protein encoding region using neighbour joining method with a boot strap value of 1000. CGMMV-Pk-S (Swat Isolate; Accession # AB872115), CGMMV-Pk-P (Peshawar Isolate; Accession # AB872114), CGMMV-Pk-M (Mardan Isolate; Accession # AB872113).

the alarming situation, with such a high prevalence of the virus in almost all the districts where cucurbits are grown, and it further emphasizes the need of implementing a quick and effective integrated control management to bring this menace under control.

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