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Full Length Research Paper

Occurrence of begomoviruses in cotton-vegetable agro ecosystem in India

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Begomoviruses are the important pathogens of variety of crop plants and are responsible for causing huge economic losses. It is assumed that cotton vegetable agro eco system plays a vital role in the complex etiology of the diseases caused by begomoviruses. Therefore, the present work was planned to study the relationship among begomoviruses infecting cotton, chilli, radish, tomato and papaya. For this purpose, the symptomatic leaves of these plants were collected and were processed for DNA isolation. The amplified coat protein gene of begomoviruses was cloned and sequenced. The phylogenetic analysis showed that begomoviruses infecting cotton are totally different from the begomoviruses infecting other crops. Based on the coat protein gene sequences, the viruses infecting cotton were identified as cotton leaf curl virus, chilli leaf curl virus in chilli, radish leaf curl virus in radish, tomato leaf curl virus in tomato and papaya leaf curl virus in papaya.

Key words: Begomovirus, cotton, vegetables, variability.

INTRODUCTION

During the last two decades, geminiviruses have emerged as devastating pathogens causing huge economic losses and threats in agricultural production. These viruses form the second largest family of plant viruses, the *Geminiviridae*. Based on the genome organization, insect vector, host range and sequence relatedness, earlier the family *Geminiviridae* was sub-divided into four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus* (van Regenmortel et al., 2000; Fauquet and Stanley, 2003). But recently, three new genera *Becurtovirus*, *Eragrovirus* and *Turncurtovirus* have been created in the family Geminiviridae (Varsani et al., 2014). More than 80% of the known geminiviruses are transmitted by whitefly in a semi-persistent circulative manner but not by sap inoculation (Muniyappa et al., 1991) and infect dicotyledonous plants. They belong to genus Begomovirus (Rojas et al., 2005). These viruses are the important pathogens of a variety of crops like cotton, grain legumes, cassava and vegetables (tomato, chilli, okra and cucurbits) in the tropical and sub-tropical areas (Varma and Malathi 2003; Kang et al., 2004) and are responsible for causing crop yield losses between 20-

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100% (Sastry and Singh, 1973; Singh et al., 1979; Brown and Bird, 1992; Sivalingam et al., 2007).

Begomoviruses are known to induce distinct types of symptoms of leaf curl, vein yellowing, severe reduction in leaf size, downward curling, crinkling of interveinal areas, interveinal and marginal chlorosis, occasional development of enations on the underside of leaves, purple discoloration of the abaxial surface of leaves, shortening of internodes, development of small branches and reduced fruiting. Therefore, most of the diseases caused by begomoviruses have been collectively described as either leaf curl or yellow leaf curl or yellow vein based on their biological properties and subtle differences in symptoms.

Based on phylogenetic studies and genome arrangement, begomoviruses have been divided broadly into two groups: the Old World (OW) viruses (eastern hemisphere, Europe, Africa, Asia) and the New World (NW) viruses (western hemisphere, the Americas) (Rybicki, 1994; Padidam et al., 1999; Paximadis et al., 1999). Begomovirus genomes have a number of characteristics that distinguish OW and NW viruses. All indigenous NW begomoviruses are bipartite, whereas both bipartite and monopartite begomoviruses are present in the OW. The length of the genome of monopartite begomoviruses is about 2800 nucleotides (Navot et al., 1991) while the bipartite begomoviruses contain two nearly equal-sized DNA molecules of size 2600-2800 nucleotides which are designated as DNA-A and DNA-B.

These genomic components of a given single virus differ in their function and nucleotide sequences, except for the sequence of an intergeneric region of about 200 nucleotides called the common region (Lazarowitz et al., 1992). This region encompasses a stemloop that contains the conserved TAATATTAC nonanucleotide sequence, common to all viruses and this sequence helps in the initiation of rolling circle replication of circular DNA. The component, DNA-A encodes for all viral functions necessary for virus replication and encapsidation of viral DNA (Lazarowitz, 1992). The DNA-B encodes for functions associated with virus movement within the plant and symptom expression (Revington et al., 1989). Most of the bipartite begomoviruses require both DNA components for infection but not the coat protein (Padidam et al., 1995) while the monopartite viruses may require the presence of single stranded DNA molecule known as DNA-β. This DNA-β consists of unrelated sequences half the size of their helper begomoviruses (1370 nucleotides), encoding a single gene known as βC1 which is the major pathogenicity determinant of the complex while for replication, encapsidation and movement in plants and for transmission, it depends upon the helper virus that is DNA-A. In addition to these, DNA alpha satellites have also been found to be associated with DNA-A and DNA-β complex (Mansoor et al., 1999).

Begomoviruses are successfully transmitted by silver whitefly (*Bemisia tabaci* Gennadius) in a semi-persistent manner, which has been known to be polyphagous with a very wide host range. The appearance of new biotype of vector (B-biotype) has resulted in the emergence of new begomoviruses and its movement into new regions and high fecundity have increased the incidence of begomoviruses around the world (Brown and Bird, 1992).

Considerable variability has been found in virus isolates from different parts of India (Varma and Malathi, 2003). Several factors including evolution of new variants of the viruses, appearance of efficient vectors, weather events, changing cropping systems, movement of infected planting material and introduction of susceptible plant varieties individually, or in combination, have contributed to the emergence of begomovirus problems around the world.

Begomoviruses have wide host range infecting large number of crops like cotton, cucurbits, okra, tomato, chilli belonging to different families like *Malvaceae*, *Solanaceae*, *Brassiceae* and *Cucurbitaceae* etc. These crops are known to play a very important role in farmer economy. In this region, cotton growing season overlaps with the growing season of vegetables like chilli, tomato, radish, okra etc. Therefore, it is assumed that cotton vegetable agro-ecosystem may play a vital role in the higher incidence and emergence of new begomoviruses. Many workers have studied the variability of begomoviruses on individual crops but very little work has been done in the cotton vegetable agro ecosystem. Considering the occurrence of begomoviruses on these crops and their importance, the present investigations were aimed to study the phylogenetic relationship among begomoviruses infecting cotton, tomato, chilli, radish and papaya growing in the region.

MATERIALS AND METHODS

Sample collection

The infected samples of cotton and three vegetable crops viz., chilli, tomato, radish and one fruit plant papaya showing typical symptoms of begomoviruses like leaf curling, vein thickening, and yellow veins were collected from different locations of Punjab during the year 2010 and 2011.

DNA Isolation

The DNA from these samples was isolated using CTAB method as described by Ghosh et al. (2009) where 850 µl of CTAB buffer containing PVP and mercaptoethanol was added to 100 mg freshly prepared powder of infected leaves and was incubated for 55 min at 65°C in serological water bath. To remove the proteins, 800 µl of chloroform: isoamylalcohol (24:1) was added and kept on the Rocker for 30 min. After centrifugation, an equal volume of ice-cold Isopropanol was added to the supernatant to precipitate the DNA which was dissolved in 1X TE.

Rolling Circle Amplification

The isolated DNA was quantified and the poor quality DNA was

amplified by rolling circle amplification using illustra™TempliPhi 100 Amplification kit (GE Healthcare) according to the manufacturer's instructions using 50 ng of DNA as template. These RCA products were quantified on 1% agarose gel containing ethidium bromide (10 mg/ml).

PCR

For the detection of begomoviruses in the collected samples, universal degenerate primers (Wyatt and Brown, 1996) were used. The PCR was performed in a 25 µl reaction mix where 100 ng of the DNA taken as template was amplified using Green GoTaq (Promega, USA). The reaction mix finally consisted of 200 µM each dNTP, forward and reverse primers (20 pmol), 1.5 mM $MgCl_2$ and 10 X PCR buffer. The PCR cycles set were, initial one cycle at 94°C of 1 min, 52°C of 1:30 min, 72°C of 2 min followed by 35 cycles of denaturation at 94°C (45 s), annealing at 52°C (1 min) and extension at 72°C for 1:30 min; the final extension was given at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel.

Purification of PCR products

For the purification of PCR products, the reaction was run for 50 µl containing same concentrations of the reaction mix as already discussed. The PCR products were purified to remove the primer dimers, extra dNTPs and contaminants present along with the desired amplified product using Wizard SVGel and PCR purification kit (Promega, USA) according to the manufacturer's instructions.

Cloning of amplified product

After confirming the presence of begomoviruses, the amplified coat protein gene of DNA-A was cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning kit by Fermentas, UAB, Luthiana. The transformed cells were plated immediately on pre-warmed LB-amp X-Gal IPTG agar plates which were incubated overnight at 37°C to get the blue white colonies. From the white colonies, the plasmids were isolated and the presence of insert in the isolated plasmids was confirmed by PCR using universal M13 primers (M13F: 5' TGTAAAACGACGGCCAGT 3', M13R: 5' AGGAAACAGCTATGACCATG 3'). The amplified products were visualized on 1% agarose gel stained with ethidium bromide (10 mg/ml) under UV light. The isolated plasmids (100 ng/µl) were submitted to Bioserve Sequencing Pvt Ltd, Hyderabad, along with the M13 primers (10 pmol).

Analysis of sequences

The sequences obtained were submitted to GenBank database. The basic local alignment search tool (BLAST) search analysis of nucleotide sequences of virus isolates was done using BLAST with sequences available in GenBank database (www.blast.ncbi.nlm.nih.gov/html). The nucleotide sequences for all the isolates were multiple aligned using *MegAlign* (Lasergene Core Suit, www.dnastar.com) and the neighbor- joining phylogenetic tree was constructed with Clustal W program using *MEGA 5* software (Tamura et al., 2011) with bootstrap value of 1000. The virus sequences used for phylogenetic analysis have been listed in Table 1.

RESULTS AND DISCUSSION

To study the relationship among begomoviruses infecting cotton and other hosts in Punjab, the infected leaves of cotton showing different kinds of symptoms like vein thickening, leaf curling and enations (Figure 1) were collected from three different locations viz., Muktsar, Faridkot and Ludhiana districts of Punjab state (India).

All these symptoms are the characteristics symptoms resembling with the symptoms of cotton leaf curl disease caused by cotton leaf curl virus as has been previously reported by Watkins (1981) and Hameed et al. (1994). Kapur et al. (1994) described the symptoms of disease as thickening of veins, curling and puckering of leaves and formation of enations on the underside of the leaves.

The suspected hosts of begomoviruses belonging to different families and growing during different seasons other than cotton were sampled from Ludhiana (Table 2). The plants showing typical symptoms (Figure 2) characteristically of begomoviruses were collected. These included chilli, tomato, radish, and papaya. The symptoms of leaf curl, reduction in leaf size and internodal distance were observed on chilli plants. The tomato plants also showed typical leaf curl and reduction in leaf size. The only leaf curl types of symptoms were exhibited by radish plants. The papaya plants showed leaf curl, puckering and reduction in leaf size type of symptoms. Kumar et al. (2012) observed the upward curling, crinkling, puckering, reduction of leaf area along with stunting of whole plant in the plants of chilli infected with leaf curl disease. Leaf curl type of symptoms in radish associated with radish leaf curl disease in Northern parts of India has been reported by Singh et al. (2012).

Detection of begomoviruses

After isolating DNA from the collected samples, it was quantified with the help of NanoDrop. To increase the amount of circular DNA of begomoviruses, the isolated DNA was subjected to Rolling Circle Amplification (RCA) which yielded high quantity of DNA (Figure 3). One µl of this high quantity DNA was dissolved in 9µl of double distilled water which was further used for detecting begomoviruses with PCR.

In the symptomatic samples of cotton collected from different cotton growing areas of Punjab, the association of begomovirus was confirmed with PCR using different universal degenerate primers (Wyatt and Brown, 1996). Amplified products were analyzed on 1.5% Agarose gel stained with Ethidium Bromide, prepared in 1x TAE buffer. These primers amplified the coat protein gene of size 575 bp (Figure 4a). All the symptomatic plants showed the amplification of this region. The same samples were also processed for PCR after their RCA (Figure 4b)

It was observed that the PCR after RCA yielded better amplified products. Polymerase chain reaction has been reported as the quickest way of detecting begomoviruses (Kang et al., 2004), that is why it is being used

Table 1. List of begomoviruses used for phylogenetic analysis.

Accession no.	Name
KM065514	Cotton leaf curl Rajasthan virus
KJ959630	Cotton leaf curl Rajasthan virus
HQ158011	Cotton leaf curl virus isolate Sirsa-Haryana
HQ158010	Cotton leaf curl virus isolate Mohanpura-Rajasthan
HM235774	Cotton leaf curl virus isolate Naruana-Punjab
HM037920	Cotton leaf curl virus isolate Sirsa-UC segment
JF502364	Cotton leaf curl Rajasthan virus isolate In:Abohar5:2010
AY765254	Cotton leaf curl Rajasthan virus - [Sirsa:04]
AJ228595	cotton leaf curl virus
X98995	Cotton leaf curl Multan virus-[Faisalabad1]
AJ002459	Cotton leaf curl Multan virus-[Okra]
KJ649706	Chilli leaf curl India virus
FM210475	Chilli leaf curl virus
HM140365	Chilli leaf curl virus-HD [India:New Delhi:Papaya:2007]
FM21047	Chilli leaf curl virus
DQ376037	Papaya leaf curl virus
AY691901	Tomato leaf curl virus
KC222953	Tomato leaf curl virus
GQ139516	Papaya leaf curl virus
JQ411026	Radish leaf curl virus
GU732204	Tomato leaf curl Pakistan virus
GU732203	Radish leaf curl virus isolate IN:Bih:ok09
AJ436992	Papaya leaf curl virus
JX524172	Tomato leaf curl Karnataka virus
DQ343284	Tomato leaf curl virus
AY375241	Tomato leaf curl Karnataka virus-[tomato:Lucknow]
EU604297	Tomato leaf curl Karnataka virus
U38239	Tomato leaf curl Karnataka virus
FM877858	Chilli leaf curl India virus
HQ630856	Papaya leaf curl virus isolate Lucknow
FJ514798	Tomato leaf curl Karnataka virus – Bangalore [India:Punjab:Mentha:2007],
HM134235	Tomato leaf curl Karnataka virus-B1 [India:Haryana:Papaya:2009]
HM140368	Papaya leaf crumple virus-Nirulas [India:New Delhi:Papaya:2007],

Figure 1. Different types of symptoms observed on cotton plants. **a)** Vein thickening; **b)** enations; c) leaf curling.

Table 2. List of collected samples with their symptoms and locations.

Host plant	Location	Symptoms	Isolate number
Cotton	Muktsar-Bam	Vein thickening	M1
Cotton	Muktsar	Vein thickening, enations, curling, stunting	M2
Cotton	Faridkot-Tehna	Vein thickening and enations	F1
Cotton	Ludhiana	Vein thickening and upward curling	L1
Chilli	Ludhiana	Leaf curl, reduction in leaf size and intermodal distance	LC
Radish	Ludhiana	Leaf curl	LR
Tomato	Ludhiana	Leaf curl and reduction in leaf size	LT
Papaya	Ludhiana	Curling and puckering of leaves and reduction in leaf size	LP

Figure 2. Symptoms observed on a) chilli, b) tomato c) radish and d) papaya.

worldwide for the preliminary detection of the begomovirus. Khan and Ahmad (2005) also used begomovirus specific primer to detect the presence of cotton leaf curl virus in the infected samples of cottonleaves. However, now a days, Rolling circle amplification (RCA) technique is successfully used to increase the virus concentration which helps in avoiding any false negatives and the increased amount of circular viral DNA gives better results in restriction analysis (Johne et al., 2009; Zaffalon et al., 2012).

Characterization of begomoviruses

The purified PCR products were cloned and multiplied in JM109 competent cells. The recombinant plasmids were isolated and obtained sequences were submitted to GenBank under accession numbers KM923991 (M1), KM923992 (M2), KM923993 (F1), KM923994 (L1), KM923995 (LC), KM923996 (LR), KM923997 (LT) and KM923998 (LP). The phylogenetic tree (Figure 5) show that the begomoviruses of each host forms a different clade than the other. That means the begomoviruses infecting cotton are totally different from the begomoviruses infecting other hosts viz., chilli, radish, tomato and papaya. Based on the phylogeny, it is clear that the cotton begomoviruses (KM923991, KM923992, KM923993 and KM923994) belong to cotton leaf curl virus group. The accession number KM923995 (isolate

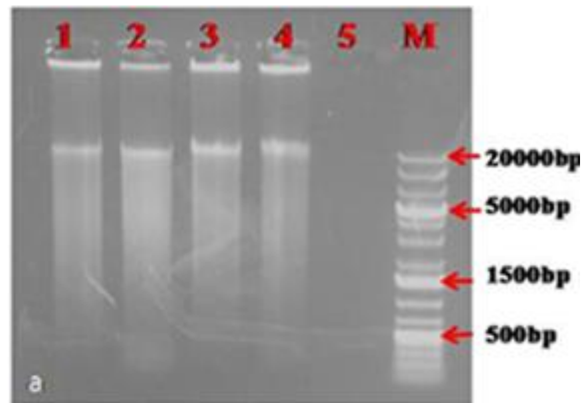

Figure 3. Gel electrophoresis of RCA products (1, 2, 3, 4: RCA products, 5: blank, M: marker).

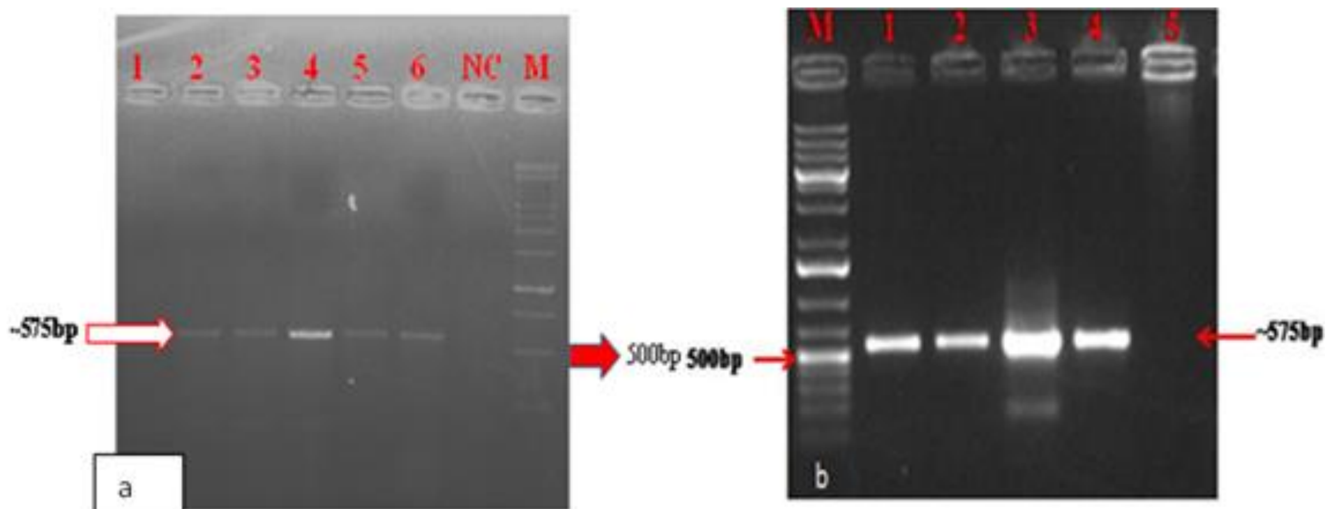

Figure 4. a) Gel electrophoresis of PCR after DNA isolation (1 to 6: infected samples, NC: negative control). **b)** Gel electrophoresis of PCR after RCA.

from chilli) shared maximum identity with chilli leaf curl virus. The radish begomovirus (KM923996) is closely related to Tomato leaf curl virus (Pakistan), acc no. GU732204. KM923997 from tomato forms a clade with tomato leaf curl virus while KM923998 from papaya was identified as papaya leaf curl virus.

Similar kind of variability in begomoviruses infecting different hosts was reported by Rajagopalan et al. (2012) which stated that begomoviruses infecting cotton are different from the begomoviruses of other hosts. Chattopadhyay et al. (2008) demonstrated that chilli leaf curl disease is caused by a complex consisting of the monopartite chilli leaf curl virus and a DNA-β satellite component. In these studies also, chilli leaf curl virus was found in association with chilli leaf curl disease. Singh et al. (2012) reported a radish leaf curl virus in Northern India which shared maximum nucleotide similarity with tomato leaf curl virus.

Conclusion

Based on the coat protein gene sequencing, it is concluded that begomoviruses infecting cotton are totally different from the begomoviruses infecting other crops viz., chilli, radish, tomato and papaya which are assumed to play an important role as an alternate hosts of cotton leaf curl virus. Thus, cotton vegetable agro eco system does not play any role in the complex etiology of the diseases caused by begomoviruses.

Conflict of interests

The authors did not declare any conflict of interest.

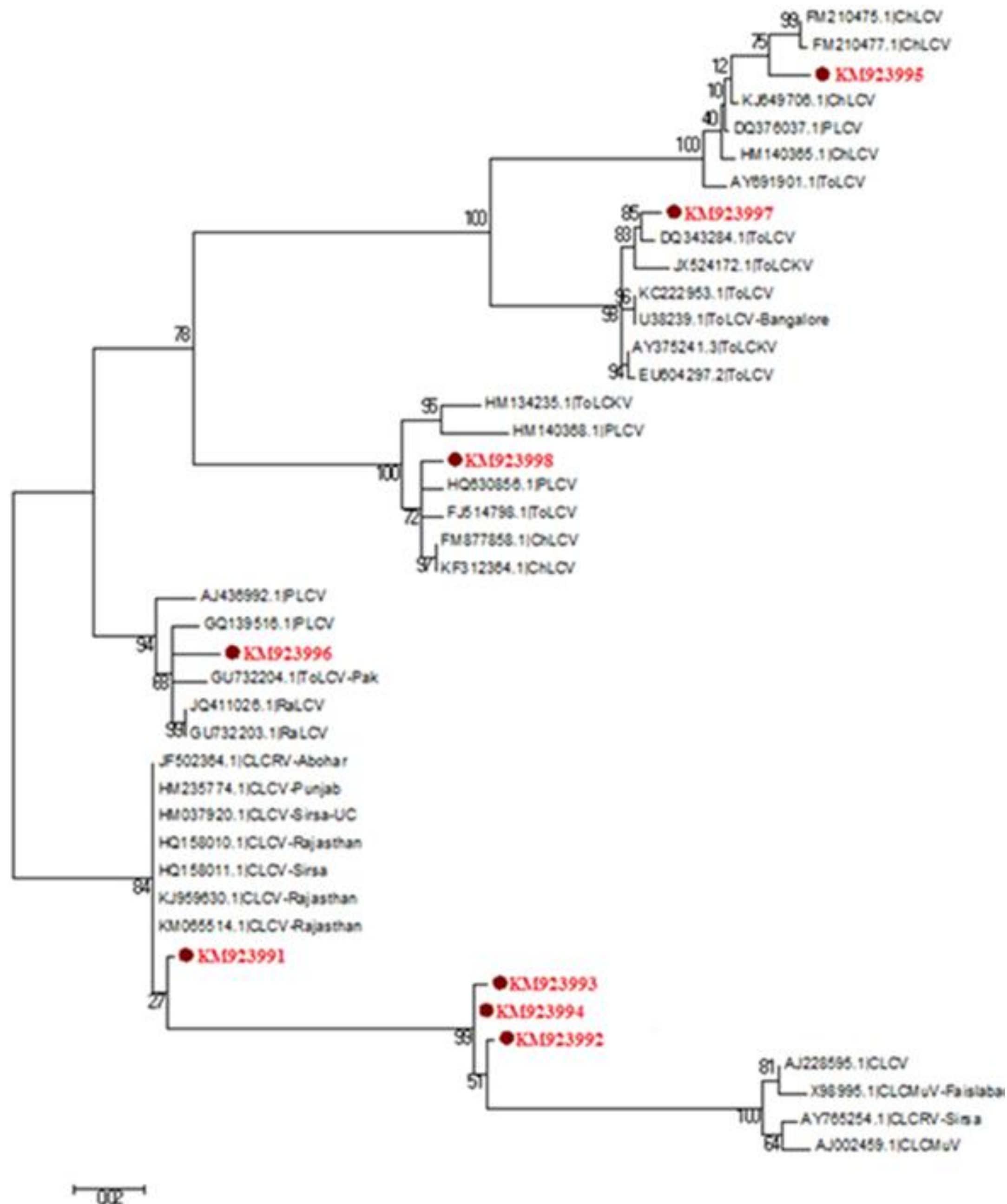

Figure 5. Phylogenetic tree for begomoviruses infecting different hosts.

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