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Analysis of the sequence of a dicot-infecting mastrevirus (family *Geminiviridae*) originating from Syria

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Abstract Chickpea stunt disease (CSD) across southern Asia, the Middle East and North Africa is caused by a number of viruses that include single-stranded DNA viruses of the genus *Mastrevirus* (family *Geminiviridae*). Despite the importance of CSD in reducing chickpea and lentil production, until recently little was known of the nature of the pathogens causing the disease. Sequence characterisation of virus isolates from Sudan and Pakistan showed the viruses concerned to potentially be new mastrevirus species related to *Bean yellow dwarf virus* (BeYDV), a virus known to occur in both southern Africa and southern Asia. Here we have determined the complete nucleotide sequence of a mastrevirus associated with CSD in Syria. This virus represents a proposed new species, closely related to the recently characterised Chickpea chlorotic dwarf Sudan virus and Chickpea chlorotic dwarf Pakistan virus but with the highest sequence identity to BeYDV, for which we propose the name Chickpea chlorotic dwarf Syria virus. In addition the biological integrity of the clone was confirmed by infection of *Nicotiana benthamiana* plants using *Agrobacterium*-mediated inoculation.

Keywords Chickpea stunt disease · Mastrevirus · Geminivirus

Introduction

The viruses causing chickpea stunt disease (CSD) have long been known to be members of the families *Geminiviridae* and *Luteoviridae* [1–5]. CSD occurs across North Africa, the Middle East and the Indian subcontinent [3–5]. Horn et al. [1] demonstrated the presence of ''geminate'' virus particles, a characteristic unique to geminiviruses, in affected chickpea samples originating from India and showed the virus to be transmitted by a leafhopper (then identified as *Orosius orientalis* but now believed to be *O. albicinctus* Distant [6]). This viral causal agent affects several pulse crops including chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.) [3, 4], bean (*Phaseolus vulgaris* L.) and sugarbeet (*Beta vulgaris* L.) in Iran [7] as well as *Phaseolus* bean and other wild legume species (*Accasia* spp, *Cajanus cajan* L., *Dolichus lablab* L., *Rhynchosia minima* L.) in Sudan [8]. Serological analyses showed the virus, for which the name chickpea chlorotic dwarf virus was coined, to be unrelated to *Beet curly top virus* (genus *Curtovirus*, family *Geminiviridae*) and *Tobacco yellow dwarf virus* (TbYDV; genus *Mastrevirus*, family *Geminiviridae*), the only other dicot-infecting, leafhopper-transmitted geminiviruses known at that time.

Recent studies have begun to address the diversity of CSD-associated geminiviruses. The disease in Pakistan and Sudan has been shown to be caused by two closely related viruses (proposed species) in the genus *Mastrevirus* (family *Geminiviridae*) Chickpea chlorotic dwarf Pakistan virus (CpCDPKV) and Chickpea chlorotic dwarf Sudan virus

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(CpCDSDV), respectively ([9]; B. Gronenborn, unpublished). Interestingly a mastrevirus, *Bean yellow dwarf virus* (BeYDV), was also identified in CSD-affected chickpeas in Pakistan. Prior to this BeYDV had only been identified affecting beans in South Africa [10, 11]. In Australia, the country where the first dicot-infecting mastrevirus (TbYDV) was identified [12], a CSD-like disease of chickpea has recently been shown to be caused by three distinct viruses, referred to as Chickpea chlorosis virus-A (CpCV-A), Chickpea chlorosis virus-B (CpCV-B) and Chickpea red leaf virus (CpRLV) [13, 14].

The geminiviruses (family *Geminiviridae*) are a group of phytopathogenic viruses having circular, single-stranded (ss) DNA genomes. Based on their host ranges, genome organisation and vector specificity, the geminiviruses are classified into four genera [15]. Viruses in the genus *Mastrevirus* are transmitted by leafhoppers and infect either monocotyledonous or dicotyledonous hosts [16]. The genomes of mastreviruses consist of a single ssDNA component (~2.6 kb) that encode four proteins; the movement protein (MP) and coat protein (CP) in the virion-sense and the replication-associated protein A (Rep A) and Rep (the only virus encoded protein required for viral DNA replication that is a rolling-circle replication initiator protein) in the complementary-sense [17]. In contrast to geminiviruses in the other genera, for mastreviruses the Rep is expressed from a spliced transcript spanning Rep A and another open reading frame known as Rep B [18].

Here we report the sequence of a mastrevirus associated with CSD from Syria and show it to be distinct from, but closely related to, viruses reported earlier in the region. Additionally the cloned virus was shown to be infectious by agroinoculation to *Nicotiana benthamiana* plants.

Materials and methods

Virus isolate, amplification and cloning

The virus isolate from Syria analysed here has been described previously (given the field isolate code SC3-03 by Kumari et al. [19]) and was isolated from a chickpea field at the ICARDA farm, near Aleppo (36.01°N, 36.56°E). Total nucleic acids were extracted using the method of Doyle and Doyle [20], from powdered leaf tissue stored at room temperature in a dry environment. All circular DNA molecules in the nucleic acid sample were amplified by rolling-circle amplification (RCA), using ϕ29 DNA polymerase (Fermentas, Arlington, Canada), as described previously [21]. The concatameric, RCA-amplified products were then digested with *Eco*RI to yield ~2.5 kb unit-length genomes and ligated into *Eco*RI linearised plasmid pTZ57R (Fermentas).

Sequencing and sequence analysis

A single clone, shown to contain a ~2.5 kb insert upon digestion with *Eco*RI, was selected for further analysis. The plasmid DNA was purified using a GeneJET Plasmid Miniprep Kit (Fermentas) and sequenced commercially by Macrogen (South Korea). Sequence information was assembled using the SeqMan program of the Lasergene sequence analysis package (DNAStar Inc., Madison, WI, USA). ORFs were predicted using ORF Finder run online (NCBI). Sequence alignments and phylogenetic trees were constructed using the neighbour-joining algorithm of CLUSTAL X [22]. Phylogenetic dendrograms were viewed, manipulated and printed using Treeview [23].

Agrobacterium-mediated inoculation

A construct for the *Agrobacterium*-mediated inoculation of the virus clone described here was produced essentially as described previously [24]. The full-length clone was restricted with *Eco*RI and *Xho*I, releasing fragments of 2.8 kb, 2.2 kb and 334 bp, or with *Eco*RI and *Cla*I, releasing fragments sizes of 2.8 kb, 2.2 kb and 357 bp. The two 2.2 kb fragments were gel isolated and ligated, in a single step, into the binary vector pGreen0029 restricted with *Cla*I and *Xho*I to yield a partial, direct repeat of the monomeric clone. The pGreen construct was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and cultures were prepared and inoculated into *Nicotiana benthamiana* plants as described previously [25].

Results

Sequence analysis

The complete sequence of the clone obtained was shown to be 2572 nt in length and has been deposited with the databases under the accession number FR687959. Analysis using ORF Finder showed the clone to potentially encode four open reading frames (ORFs) which are conserved in position and sequence between this and other mastreviruses; two in the virion-sense and two in the complementary-sense. The positions and predicted coding capacities of the ORFs are given in Table 1. In common with a number of mastreviruses, the ORF designated C2 does not contain an ATG start codon. For mastreviruses the product of the C2 ORF is expressed as a fused product with the C1 ORF, following splicing of the messenger RNA, from which the Rep is translated [18, 26]. Consistent with this, the sequence here contains consensus splice donor and acceptor sites which would yield a predicted product of 337 amino acids (Table 2), following splicing of an 86 nt

Table 1 Positions and coding capacity of predicted genes encoded by SYR-2

Gene	Nucleotide coordinates	Coding capacity no. of amino acids (kDa)
MP	131–409	92(10.2)
CP	422–1159	245(27.0)
Rep A	2407–1529	292(33.4)
Rep B	1739–1320	143(16.1)
Rep	2407–1817/1730–120	337(39.5)

intron. SYR-2 also has the characteristic (for mastreviruses) two intergenic regions; the large intergenic region (coordinates 2408–421) that encompasses the virion-sense origin of replication and bidirectional promoter, and the small intergenic region (1157–1531) to which an encapsidated, small DNA primer is annealed that is believed to function in initiation of complementary-strand DNA replication [27].

The Rep of the Syrian virus contains, within its *N*-terminus, an iteron-related domain (IRD) that is the same as those of CpCDSDV, BeYDV, CpCV, CpRLV and TYDV (FRLQ), but is distinct from the IRD of CpCDPKV (FRFQ). The IRD sequences of Rep are predicted to be the sequences that interact with the viral DNA to initiate rolling-circle replication of the genome [28]. Geminivirus Rep proteins are sequence-specific DNA-binding proteins that recognise repeated motifs (known as ''iterons'') adjacent to the nonanucleotide-containing hairpin structure that together form the origin of virion-strand DNA replication. The predicted iteron sequence of the Syrian virus is "TGGAGCCA/T", which is distinct from those of BeYDV and CpCDSDV, which share the iteron sequence ("TGGAGGCA"), and CpCDPKV ("TGGAGACA").

Sequence comparisons with viruses of the genus *Mastrevirus*

Comparison of the sequence of the chickpea virus from Syria with other mastreviruses available in the sequence databases showed it to have the highest levels of sequence identity with dicot-infecting mastreviruses. However, to the dicot-infecting mastreviruses originating from Australia, sequence identities were low (<56%) whereas to those originating from Africa, the Middle East and southern Asia sequence identities were high (>81%; Table 2). Overall the highest nucleotide sequence identity (83.4–83.9%) was to isolates of BeYDV, a dicot-infecting mastrevirus that occurs in both southern Asia and southern Africa [9–11].

Although only full-length nucleotide sequences from the databases were considered in the analyses described above, it is interesting to note that the Syrian virus showed high levels of sequence identity (between 89 and 95%) to the partial sequence of a presumed mastrevirus isolated from sugarbeet in Iran (accession numbers EU034169 and DQ159207). This may suggest that the virus identified here also occurs in Iran and may have a natural host range that includes hosts other than legumes.

A phylogenetic analysis, based upon an alignment of the full-length sequences of all dicot-infecting mastreviruses available in the databases, is shown in Fig. 1. This shows the mastreviruses originating from Australia (TbYDV, CpCV and CpRLCV) to be distinct from the viruses originating from Asia, the Middle East and Africa. The mastreviruses from Asia, the Middle East and Africa fall into three groups corresponding to the one accepted species, BeYDV, and two proposed species, CpCDSDV and CpCDPKV, so far identified in these regions. The sequence of the virus from Syria falls basal to the BeYDV isolates, supporting the sequence similarity analyses which indicated that this species is its closest sampled relative. However, the Syrian virus is as distinct from BeYDV as the other three species are from each other. These groupings all have strong bootstrap support.

Comparison of the predicted amino acid sequences of the gene products encoded by SYR-2 with the homologous products of all other dicot-infecting mastreviruses is shown in Table 2. This showed the virion-sense encoded proteins (CP and MP) to have the highest identity levels to CpCDSDV whereas the complementary-sense encoded proteins (Rep and RepA) showed greater levels of identity to the homologous proteins of BeYDV. This possibly suggested that, in common with many other geminiviruses, the Syrian virus has a recombinant origin. However, RDP analysis using an alignment of all available dicot-infecting mastreviruses identified no significant recombination events involving SYR-2. A closer inspection of the alignment showed the sequence differences between SYR-2 and the closest related mastreviruses (BeYDV, CpCDSDV and CpCDPKV) to be randomly distributed throughout the genome (results not shown). This suggests that the divergence of SYR-2 from BeYDV, CpCDSDV and CpCDPKV has resulted from gradual genetic drift rather than recombination (Fig. 2).

Infectivity analysis

The infectivity of the Syrian virus clone was assessed by *Agrobacterium*-mediated inoculation of a partial repeat construct to *N. benthamiana*. The cloned virus was infectious to *N. benthamiana*, with all of eight inoculated plants being positive for virus infection as indicated by diagnostic PCR with specific primers (results not shown). Symptoms typically appeared within 21 days of inoculation and consisted of yellowing, stunting and crumpling of newly emerged leaves. Plants ceased to grow and rapidly became

Table 2 Highest and lowest percentage nucleotide sequence identities for full-length genome sequences and percentage amino acid identities for the predicted gene products for pair-wise comparisons of the Syrian virus isolated from chickpea with all available dicot-infecting mastrevirus sequences available in the databases

		SYR-2[@]	TYDV (1)[a]	CpRLV (1)[a]	CpCV-B (1)[a]	CpCV-A (1)[a]	CpCDSDV (3)[a]	BeYDV (3)[a]	CpCDPKV (3)[a]
CpCDPKV (3)[a]	Genome	81.5–81.9	54.8–55.5	56.1–56.5	47.6–50.7	47.6–52.4	82.3–82.6	80.7–81.3	97.7–98.2
	MP	84.8	52.2–53.3	62.0–63.0	52.2–53.3	56.5–57.6	87.0–88.0	90.2–92.4	98.9–100
	CP	90.2–90.6	54.3–54.7	72.0–72.4	54.3–54.7	53.5–53.9	90.2–90.6	91.0–91.8	99.6–100
	RepA	85.2–86.3	70.5–70.9	57.5–58.1	64.3–64.7	64.6–65.1	87.3–89.0	87.6–90.8	95.9–97.3
	RepB	82.9	79.2–80.6	72.0	63.6	71.5	82.6–84.0	82.6–84.0	97.9–99.3
	Rep	87.1–87.7	76.3–76.9	67.4–67.6	66.5–66.8	67.6–67.7	87.4–89.2	88.0–89.2	97.6–98.2
BeYDV (3)[a]	Genome	83.4–83.9	54.9–55.4	55.8–56.0	48.3–51.4	48.3–52.4	83.0–84.0	95.8–99.8	
	MP	82.6–85.9	54.3–55.4	60.9–62.0	55.4–56.5	55.4–57.6	85.9–90.2	95.7–98.9	
	CP	91.8	53.9	70.0	52.7	53.1	91.0–91.8	100	
	RepA	86.6–88.4	67.5–68.8	57.2–58.2	64.4–65.4	65.1–66.1	88.4–89.4	94.9–99.3	
	RepB	88.6–89.3	77.8	67.1–67.8	64.3–65.0	69.4–70.8	90.3	95.1	
	Rep	88.9–89.5	75.4–76.0	65.6–66.2	67.7–68.3	68.6–69.8	91.3–93.1	97.3–99.4	
CpCDSDV (3)[a]	Genome	82.7–83.2	54.9–55.0	55.4–55.5	51.1–51.3	51.1–51.9	99.0–100		
	MP	85.9–87.0	55.4	59.8	55.4–56.5	56.7–57.6	87.0–88.0		
	CP	93.1	55.1	70.0–70.8	53.1	52.7	99.2–100		
	RepA	85.6–86.0	69.9–70.2	57.5	63.7–64.0	63.7–64.0	99.3–100		
	RepB	89.3	81.2	69.2	65.7	70.8	100		
	Rep	88.9–89.2	76.0–76.9	66.8–67.0	67.0–67.7	68.5–69.2	98.5–100		
CpCV-A (1)[a]	Genome	48.6–49.1	66.2–71.4	55.2–57.9	74.8	74.8–100			
	MP	53.3	68.6	52.7	84.3	100			
	CP	53.1	86.2	56.0	89.0	100			
	RepA	64.2	67.5	74.0	75.3	100			
	RepB	72.1	70.1	84.6	78.3	100			
	Rep	67.7	74.8	75.7	85.8	100			
CpCV-B (1)	Genome	48.6	66.2	55.2	100				
	MP	52.2	69.6	53.8	100				
	CP	53.9	85.4	57.2	100				
	RepA	66.9	67.8	66.2	100				
	RepB	65.7	69.2	74.1	100				
	Rep	67.1	74.8	70.6	100				
CpRLV (1)[a]	Genome	55.8	50.9	100					
	MP	65.2	51.6	100					
	CP	69.5	56.8	100					
	RepA	57.0	61.0	100					
	RepB	67.9	68.5	100					
	Rep	65.6	69.1	100					
TYDV (1)[a]	Genome	54.8	100						
	MP	51.1	100						
	CP	54.7	100						
	RepA	68.9	100						
	RepB	80.7	100						
	Rep	75.7	100						

[a] The number of sequences available in the databases which were used in the comparisons

The virus acronyms used are Chickpea chlorosis virus-B (CpCV-B), Chickpea chlorosis virus-A (CpCV-A), Chickpea chlorotic dwarf Sudan virus (CpCDSDV), *Bean yellow dwarf virus* (BeYDV), Chickpea chlorotic dwarf Pakistan virus (CpCDPKV), *Tobacco yellow dwarf virus* (TYDV), Chickpea red leaf virus (CpRLV). The virus characterised here is indicated as SYR-2

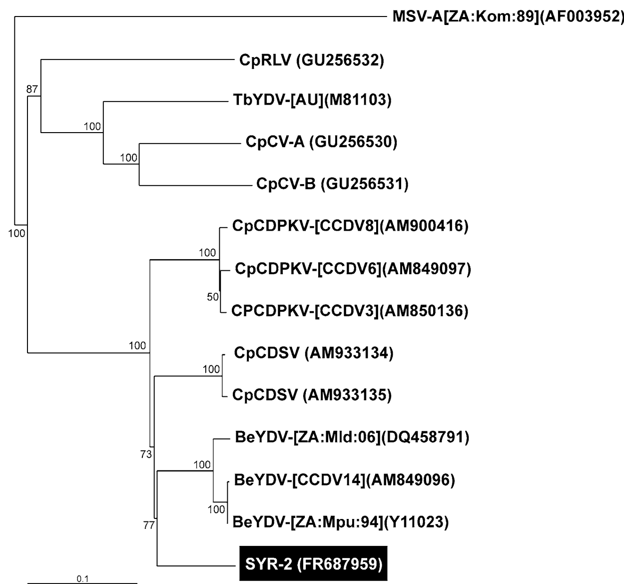

Fig. 1 Neighbor joining phylogenetic dendrogram based upon an alignment of complete nucleotide sequences of all dicot-infecting mastreviruses available in the databases. Vertical branches are arbitrary; horizontal branches are proportional to calculated mutation distance. Values at nodes indicate percentage boot strap values (1000 replicates). The tree was rooted using *Maize streak virus* (MSV) as an outgroup. The virus acronyms used are *Bean yellow dwarf virus* (BeYDV), Chickpea chlorotic dwarf Pakistan virus (CpCDPKV), Chickpea chlorotic dwarf Sudan virus (CpCDSDV), Chickpea chlorosis virus (CpCV), Chickpea red leaf virus (CpRLV) and *Tobacco yellow dwarf virus* (TbYDV). For each virus isolate, the database accession number is indicated. The virus originating from Syria is highlighted

necrotic. Interestingly, tissue at the site of inoculation also became necrotic. This may suggest that, as has been shown for the begomoviruses *Bean dwarf mosaic virus* in bean [29] and *Tomato leaf curl New Delhi virus* in tomato [30], the virus from Syria encodes an avirulence determinant that induces programmed cell death in *N. benthamiana*. Southern blot analysis of nucleic acids extracted from symptomatic *N. benthamiana* plants showed the presence of single- and double-stranded viral DNA forms typical of geminivirus replication (Fig. 3). No hybridization was detected for nucleic acids extracted from a healthy, non-inoculated *N. benthamiana* plant.

Discussion

The cool season legumes, which include chickpea, lentil and faba bean, are a staple food of millions of people, particularly across Africa and Asia [5]. Although a number of different viruses cause disease in these crops, in some areas at least, mastreviruses are believed to be the main biotic constraint to chickpea production [31]. Despite their importance, only recently has the characterisation of these viruses been undertaken, with the identification of two potentially new mastrevirus species in Sudan and Pakistan ([9]; B. Gronenborn, unpublished) and three potentially new species identified in Australia [14]. In view of the vast geographical range where CSD is reported, it would seem likely that further mastrevirus diversity is probably present.

The virus identified in chickpea from Syria is typical of viruses of the genus *Mastrevirus*. It carries four predicted open reading frames that probably encode four products with high degrees of similarity to corresponding proteins found in other dicot-infecting mastreviruses. Characteristically, for mastreviruses, the Rep is translated from a spliced transcript that fuses the RepA and RepB ORFs. Overall the amino acid sequences of the predicted proteins and the complete nucleotide sequence show the highest levels of identity to other mastreviruses identified in southern Asia and North Africa. In most cases, except for the amino acid sequences of the virion-sense genes, the highest levels of sequence identity are to BeYDV.

The species demarcation criteria for mastreviruses presently include the following: a 75% nucleotide sequence identity level cut-off, above which two viruses should be considered strains of a single species; no trans-replication

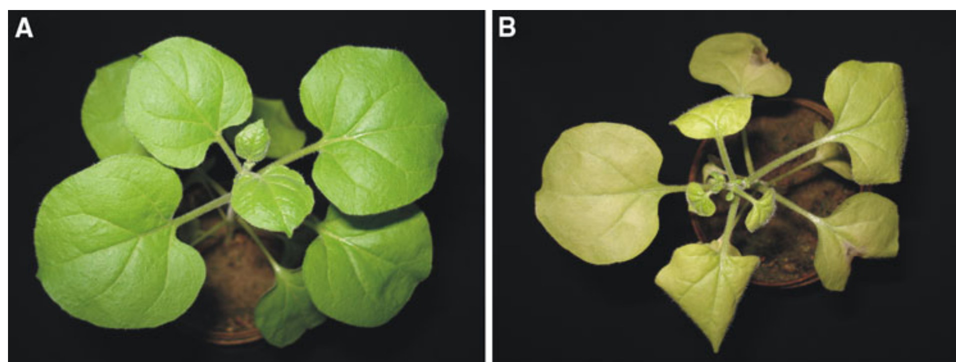

Fig. 2 Symptoms induced following *Agrobacterium*-mediated inoculation of *Nicotiana benthamaina* with a partial direct repeat construct of SYR-2. Shown is a healthy non-inoculated *N. benthamania* plant (**a**) and an *N. benthamania* plant inoculated with SYR-2 (**b**) Note the necrosis at the site of inoculation. The photographs were taken approximately 24 dpi

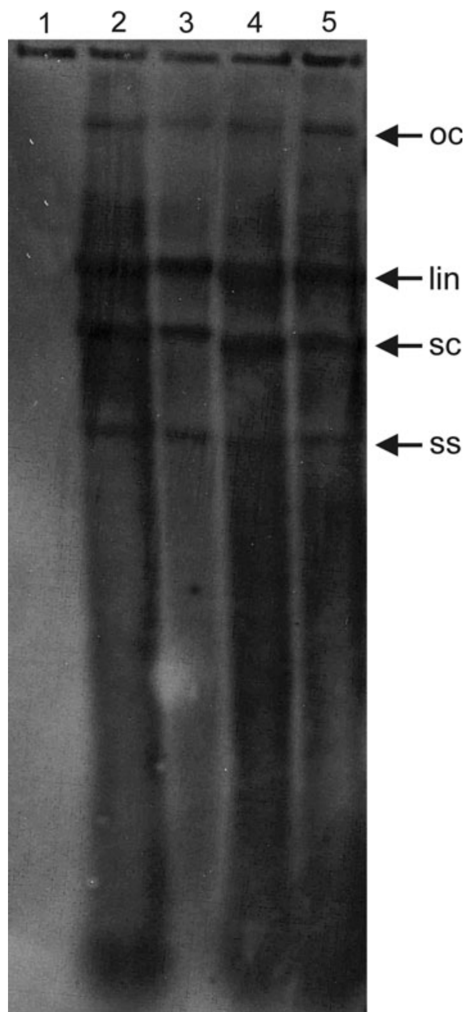

Fig. 3 Southern blot probed with the full-length SYR-2 clone. Nucleic acids were extracted from a healthy, non-inoculated *N. benthamiana* plant (*lane 1*), the chickpea plant from which the SYR-2 clone was obtained (*lane 2*) and three symptomatic *N. benthamiana* plants inoculated with a partial direct repeat construct of SYR-2 (*lanes 3–5*). The *arrows* to the *right* of the blot show the positions of linear (lin), open-circular (oc), single-stranded (ss) and supercoiled (sc) forms of the viral DNA

of genomic components of a second virus; and differences in host range or pathogenicity [32]. The inadequacy of these criteria, which were formulated solely on the properties of the very different African grass-infecting viruses, for many of the recently characterised mastreviruses (both dicot- and monocot-infecting) have been discussed at length in recent publications [9, 14] and there is thus no need to repeat this here. Based on a 75% species demarcation threshold, the virus identified here (together with the proposed species CpCDPKV and CpCDSDV) would be considered an isolate of the species BeYDV. However, if a demarcation threshold at, or closer to, that for begomoviruses (89%) is used, the Syrian virus would be considered a new species, for which we propose the name Chickpea chlorotic dwarf Syria virus (CpCDSYV). The finding that the Syrian virus has iterons that are distinct from the other dicot-mastreviruses and its distinct position in the phylogenetic analysis supports the proposal that this virus be considered a distinct species within the genus *Mastrevirus*.

The available evidence indicates that CSD-causing mastreviruses occur across a wide geographical area, from India to northern Africa. The identification here of further diversity of CSD-associated mastreviruses, together with the earlier evidence, suggests that CSD across this wide area is caused by a complex of closely related species which have diverged from a common chickpea-infecting progenitor and that speciation may be occurring due to geographical separation. Also, the identification of the proposed CpCDSYV as the closest relative of BeYDV that has so far been identified adds weight to the earlier suggestion that BeYDV originated in the Middle East/Asia and was subsequently introduced, possibly by human migration or trade, into southern Africa [9]. However, without further characterisation of African and Middle Eastern isolates, the possibility that the natural geographical host range of BeYDV extends from Asia to southern Africa cannot be ruled out. The availability of clones of BeYDV and its closest relative, CpCDSYV, will also allow us to investigate experimentally the molecular basis for the apparent host range differences between these two species. At this time we are assuming that the BeYDV evolved from a chickpea-infecting virus by adaptation to beans.

The identification of CpCDSYV suggests that further diversity in the complex of mastreviruses that are associated with CSD will likely be present across the geographical range of the disease. Of particular interest would be the nature of the first CSD-associated virus identified, in India, for which sequence information has yet to be obtained. Our present efforts are aimed at determining the diversity of dicot-infecting mastreviruses across the geographical range of CSD, examining the availability and mechanism of natural resistance to these viruses in chickpea as well as investigating pathogen-derived resistance by RNA interference. Specifically it is hoped that additional analyses of chickpea-infecting virus diversity will facilitate the successful design of resistance strategies capable of countering the entire scope of mastreviruses that are associated with CSD.

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