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Research Papers

Distribution and identification of luteovirids affecting chickpea in Sudan

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Summary. In Sudan yellowing viruses are key production constraints in pulse crops. Field surveys were carried out to identify luteovirids affecting chickpea crops in the major production regions (Gezira Scheme and River Nile State). A total of 415 chickpea plant samples with yellowing and stunting symptoms were collected during the 2013, 2015 and 2018 growing seasons. Serological results (Tissue-blot immunoassays) showed that *Luteoviridae* and Chickpea chlorotic dwarf virus (CpCDV, genus *Mastrevirus*, family *Geminiviridae*) were the most common viruses, with rare infections with Faba bean necrotic yellows virus (FBNYV, genus *Nanovirus*, family *Nanoviridae*). Some samples reacted only with a broad-spectrum luteovirid monoclonal antibody (5G4-MAb), and others showed cross reactions between the specific monoclonal antibodies, suggesting the occurrence of new luteovirid variants. Serological results were confirmed by amplification with reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of the partial coat protein gene. Molecular analyses provided a basic, sufficient and reliable characterization for four viruses affecting chickpea that belong to *Polerovirus* (family *Luteoviridae*). These were Cucurbit aphid-borne yellows virus (CABYV), Pepper vein yellows virus (PeVYV), Pepo aphid-borne yellows virus (PABYV) and Cotton leafroll dwarf virus (CLRDV), that shared high similarity with the type sequences. Phylogenetic analyses also revealed high similarity to luteovirid species. This study has established reliable, rapid and sensitive molecular tools for the detection of luteovirid species.

Keywords. Molecular characterization, sequence alignment, *Polerovirus, Luteoviridae*, Sudan.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an economically important food crop in West Asia and North Africa (WANA) and in semi-arid areas of the world

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(Van der Maesen, 1987). The total world area under chickpea cultivation during the 2018 cropping season was 1.78 million ha with an estimated annual production of 17 million tonnes (FAO, 2018), making chickpea the third most important pulse crop after soybean and common bean. Chickpea is an important source of protein in human diets and plays a significant role in farming systems (Merga and Haji, 2019).

In Sudan, chickpea is the third most economically important food legume crop after faba bean and cowpea, as a cash crop that generates income for farmers and rural communities, and as a significant source of protein for Sudanese people (Mohamed *et al.*, 2015). It is traditionally grown as a winter crop in River Nile State, northern Sudan. However, chickpea production has recently expanded to the central clay plain of central Sudan. The Gezira Scheme is one of the world's largest irrigation systems under one management, centred in the Sudanese state of Gezira, southeast of the confluence of the Blue Nile and White Nile at the city of Khartoum. The major crops in the Gezira Scheme are cotton, vegetable crops, cereals (sorghum and wheat), and currently kabuli type chickpea production is expanding due to its high price and low cost of production. The chickpea area harvested in Sudan during 2018 was 6,716 ha, and yielded 11,698 tonnes (FAO, 2018). The productivity in Sudan is generally low (1.75 t ha^{-1}) (FAO, 2018), partly due to the use of inferior seeds purchased from local markets or imported from neighboring countries. Chickpea fields planted in November each year (early planting) are susceptible to high virus and wilt/root rot infections (Mohamed *et al.*, 2015; 2018). Late planted crops (December/early January) showed low amounts of virus and root rot infections, but are more exposed to heat than early sown crops (Abdelmagid Adlan Hamed, personal communication), and this leads to high amounts of empty pods.

Generally, diseases causing yellowing, stunting and leaf roll symptoms are primarily caused by luteovirids, which are considered the most destructive virus diseases that infect cool season food legumes worldwide (Bos *et al.,* 1988; Makkouk *et al.,* 2003c; 2014; Kumar *et al*., 2008; Kumari *et al.,* 2009). Virus species in the family *Luteoviridae* are transmitted in a circulative, non-propagative manner by specific aphid vectors. These viruses often cause phloem necroses that spread from inoculated sieve elements and cause symptoms by suppressing translocation, reducing plant growth and prompting chlorophyll loss, which results in characteristic yellowing and dwarfing of infected plants. Several members of the *Luteoviridae* have host ranges largely restricted to one plant family, and other members infect plants in several or many families. For instance, Bean leafroll virus (BLRV) and Soybean dwarf virus (SbDV) (*Luteovirus*) infect mainly legumes, whereas Beet western yellows virus (BWYV, *Polerovirus*) infects more than 150 species of plants in over 20 families (Domier, 2011).

Serologically, virus species in the *Luteoviridae* (mainly those in *Polerovirus*) cannot be distinguished using polyclonal antisera (Duffus and Russell, 1975; Govier, 1985) and most monoclonal antibodies (MAbs) (Oshima and Shikata, 1990; Smith *et al.*, 1996), due to cross reactions with non-target species. Furthermore, antibodies for many species within this family are not easily available (D'Arcy *et al.,* 1989; Fortass *et al.,* 1996). Molecular assays are generally more sensitive than serological tests, especially with luteovirids, which are present in lower concentrations than many other plant viruses. Reverse transcription-polymerase chain reaction (RT-PCR) technology provides more sensitive assays which have the potential to identify luteovirid-infected plants more reliably, especially in the early stages of infection, and also helps to improve virus classifications (Lemaire *et al.,* 1995; Hauser *et al.,* 2000; Xiang *et al.,* 2008a, 2008b; Mnari-Hattab *et al.,* 2009; Shang *et al.,* 2009; Knierim *et al.,* 2010). For example, virus isolates previously identified as BWYV have been reclassified as four distinct virus species (BWYV, Beet chlorosis virus (BChV), Beet mild yellowing virus (BMYV), and Turnip yellows virus (TuYV)) on the basis of differences in host range and molecular characterizations (Hauser *et al.,* 2000; 2002; D'Arcy and Domier, 2005). Using molecular techniques, Chickpea chlorotic stunt virus (CpCSV), identified as a new member of *Polerovirus*, has been shown to naturally infect a range of cool-season food legumes, and cause leaf yellowing and plant stunting in Ethiopia and Syria (Abraham *et al.,* 2006) and in many countries in WANA region (Kumari *et al.*, 2007; Asaad *et al.,* 2009). In addition, many virus isolates that were identified as a luteovirid based on their positive reactions with a broad spectrum MAb "5G4" (Katul, 1992) in the past, did not react serologically with the available specific MAbs (Makkouk *et al.,* 1988; Abraham *et al.,* 2008; Mustafayev *et al.,* 2011; Kumari *et al.,* 2017). These viruses remained unidentified due to the lack of specific antibodies or appropriate molecular tools.

Chickpea can be naturally infected with a number of viruses causing yellowing and stunting symptoms (Nene *et al*., 1996; Kumar *et al.,* 2008). However, in Sudan, four viruses have been identified to naturally this host and cause significant economic damage. These are, Faba bean necrotic yellows virus (FBNYV, *Nanovirus*, *Nanoviridae*), Chickpea chlorotic dwarf virus (CpCDV, *Mastrevirus*, *Geminiviridae*), CpCSV and BWYV (Abraham *et al.,* 2009; Makkouk *et al.,* 2003b; 1995; Makkouk, 2020). Cucurbit aphid-borne yellows virus (CABYV) has also been reported by Kumari *et al.* (2018) to infect chickpea in Sudan and cause stunting, yellowing and necrosis. However, that study suggested the presence of other luteovirids in survey samples, but the identity of these was not reported.

Previous studies and surveys conducted in many regions of Sudan have indicated the occurrence of unrecognized viruses with wide distributions and sometimes with high incidence. However, the diversity of luteovirid species infecting cool-season food legume crops in Sudan has not been previously and extensively studied, and information on the incidence of specific viruses affecting these crops is limited. To address this knowledge gap, we carried out field surveys in the main chickpea production areas of Sudan to accurately characterize the identity, diversity, variability and geographic distributions of luteovirid species that affect chickpea, using conventional and molecular analyses.

MATERIALS AND METHODS

Field surveys and serological tests

Field surveys were conducted in the major chickpea production areas in Sudan, including areas of the Gezira Scheme (middle, north and south) and River Nile State (Hudeiba Agriculture Research Station, Berber and Shendi). The 204 chickpea samples collected by Kumari *et al.* (2018) in February 2013 and March 2015 were included in the present study, to investigate luteovirid diversity in addition to the CABYV already reported. A further 211 chickpea samples were collected in February 2018 when the crops were at the flowering/pod setting stage. Shoot samples from a total of 415 chickpea plants with yellowing and stunting symptoms were collected from 35 fields (133 plants from ten fields in 2013; 71 plants from four fields in 2015 and 211 plants from 21 fields in 2018). In each field visited, data on field location, crop condition, growth stage, virus disease symptoms, virus disease incidence and aphid populations were recorded. Virus disease incidence in each field was determined on the basis of visual virus symptoms and the number of infected plants per m^2 at randomly chosen locations in the field, and were grouped into five categories (<1%, 1-5%, 6–20%, 21–50% or >50%). The fresh stem of each sample plant was blotted on nitrocellulose membrane (NCM, 0.45 µm, Bio-Rad, Cat No. 1620115) in ten replicates. The leaves of all collected samples were dried over silica gel or lyophilized for further molecular analyses.

Three replicates of blotted NCMs were tested for the presence of viruses by tissue-blot immunoassay (TBIA; Makkouk and Kumari, 1996), using a broad-spectrum legume luteovirid monoclonal antibody (MAb) (5G4; Katul, 1992), MAb for FBNYV (3-2E9; Franz *et al.,* 1996) and a polyclonal antibody for CpCDV (Kumari *et al.*, 2006).

To identify individual luteovirids, samples that reacted positively with MAb 5G4 in TBIA (23 samples in 2013, 18 samples in 2015, 45 samples in 2018) were retested further, using specific MAbs to BWYV (A5977 from Agdia, USA), BLRV (4B10; Katul, 1992), SbDV (ATCC PVAS-650, USA) and a mixture of three MAbs (1-1G5, 1-3H4 and 1-4B12) produced against an Ethiopian isolate of CpCSV (CpCSV-Eth) and a mixture of three MAbs (5-2B8, 5-3D5 and 5-5B8) produced against a Syrian isolate of CpCSV (CpCSV-Sy) (Abraham *et al.,* 2006, 2009).

Molecular analyses

RNA extraction

Total RNA was extracted from 50 to 100 mg of virus-infected lyophilized tissue following a user-developed protocol using McKenzie lysis buffer (McKenzie *et al.,* 1997) with the RNeasy® Plant Mini Kit (Cat No. 74904, Qiagen). RNAs for all tested samples were stored as solutions in Nuclease free-water at −80°C.

Complementary DNA (cDNA)

Synthesis of cDNA was achieved using the M-MLV Reverse Transcriptase kit (Cat No. 28025013, Invitrogen) as per the manufacturer's instructions, with reverse primer AS3 (Abraham *et al.,* 2008) (Table 1). Three µL of total RNA, 1 µL of 10 µM AS3 primer, 1 µL of Nuclease free water and 1 µL of 10 µM dNTPs (2'-deoxynucleotide 5'-triphosphates) were heated at 65°C for 5 min. The reaction was cooled on ice for 2 min and the following reagents were added: 2 µL 5× First-Strand Buffer, 1 µL 0.1 M DTT and 0.5 µL Nuclease free water. The reaction was incubated at 37°C for 2 min then 0.5 µL of M-MLV RT enzyme was added (final volume 10 µL) followed by a further 50 min at 37°C before deactivating at 70°C for 15 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

The success of reverse transcription was checked by performing a PCR using the generic primer pairs (AS3/Pol3870F) (Sharman *et al.,* 2015) to amplify 370 bp of

Table 1. Luteovirid primer sets used in this study.

Primer pairs	Primer Sequence (5' to 3')	Product Size (bp)	Target virus species[a]	Reference
Generic primers				
AS3	CACGCGTCIACCTATTTIGGRTTITG	370	CLRDV, CpCSV, CABYV, PLRV, BWYV, TuYV, BLRV, CBTV, SbDV	Abraham *et al.*, 2008
Pol3870F	ATCACBTTCGGGCCGWSTYTWTCAGA			Sharman *et al.*, 2015
Specific Multiplex primers				
Master Mix-I				
AS3				
BLRV3589F	CAAGGAGACGTTTACCAGTCGT	551	BLRV	Sharman, unpublished data
BWYV3969F	GTCTCCGARGCCTCTTCCCAA	276	BWYV/TuYV	Sharman, unpublished data
SbDV3731F	CGWGTTTTCRAAGGACGGCA	418	SbDV	Sharman, unpublished data
PBMYV3396F	GGTTGGTTCTTCCAGTCCAAT	838	PBMYV	Sharman *et al.,* 2021
Master Mix-II				
AS3				
CABYV3635F	GAAACCGCCGACGCCCTAAT	474	CABYV	Sharman, unpublished data
CpCSV3705F	AAYARGCGYMCTGTTCAGCGGGC	566	CpCSV	Sharman, unpublished data
Specific Uniplex primer pairs				
Pol3982R	CGAGGCCTCGGAGATGAACT	310	CLRDV	Sharman *et al.,* 2015
CLRDV3675F	CCACGTAGRCGCAACAGGCGT			
PeR	TCGCTTGCCCGCCTTTGGTG	1249	PeVYV	Zhang *et al.,* 2015
PeF	GGAGCGTTGCGGAATGGATGC			

[a] Virus acronyms are CLRDV = Cotton leafroll dwarf virus; CpCSV = Chickpea chlorotic stunt virus; CABYV = Cucurbit aphid-borne yellows virus; PLRV = Potato leafroll virus; BWYV = Beet western yellows virus; TuYV = Turnip yellows virus; BLRV = Bean leafroll virus; CBTV = Cotton bunchy top virus; SbDV = Soybean dwarf virus; PhBMYV = Phasey bean mild yellows virus; PeVYV = Pepper vein yellows virus.

the partial coat protein (*CP*) gene (Table 1), using the My Taq polymerase kit (Cat No. BIO-21108, Bioline). The positive samples with sharp band were processed by Multiplex RT-PCR (MP-PCR) (Murray Sharman, unpublished data), using the generic reverse primer AS3 with species-specific primers for Phasey bean mild yellows virus (PBMYV), CpCSV, BWYV, SbDV, BLRV and CABYV (Table 1), and by following the manufacturer's instructions for the My Taq polymerase kit (final volume 10 μL). Due to the proximity in product sizes for some primers, the MP-PCR amplification mixture was divided in two multiplex master mixes; master mix-I included AS3 with primers BLRV3589F, BWYV3969F, SbDV3731F and PhBMYV3396F and master mix-II consisted of AS3 with CABYV3635F and CpCSV3705F (Table 1). These primers amplify partial *CP* gene. Positive controls for all tested viruses were used in both master mixes as checks to accurately identify PCR products of the different viruses. The PCR for both sets consisted of an initial denaturation of 95°C for 1 min, then 35 cycles (95°C for 30 sec, 62°C for 20 sec, 56°C for 10 sec, 72°C for 30 sec) followed by a final extension of 72°C for 3 min. All PCR products were analyzed on 1.5% agarose gel stained with RedSafe™ Nucleic Acid Staining Solution (20,000×) (Cat No. 21141, iNtRON) with final concentration of 5% in 0.5% TBE (Tris-borate-EDTA) buffer.

In addition to the above primers, two specific uniplex primer pairs targeted Pepper vein yellows virus (PeVYV) (PeF/PeR; Zhang *et al.,* 2015) and Cotton leafroll dwarf virus (CLRDV) (CLRDV3675F/Pol3982R; Sharman *et al.,* 2015) (Table 1) to confirm the sequencing outputs of the DNA fragments generated by AS3/Pol3870F.

DNA sequencing and molecular analysis

PCR amplicons of interest were amplified with total volumes of 50 μL. From each of these, 5 μL was analyzed on agarose gel, and the high-quality products were directly sequenced by the Sanger method following the instructions of a commercial sequencing company (Macrogen). The sequences were compared with available sequences in the GenBank database using the basic local alignment search tool (BLAST; Altschul *et al.,* 1997; 2005). In this study, BLAST search and sequence analyses were carried out based on the greatest similarity of the submitted sequences with the following four GenBank accessions: GenBank accession Nos. KC685313 for PeVYV, KJ789902 for Pepo aphid-borne yellows virus (PABYV), EU871539 for CLRDV and EX398665 for CABYV. Sequences of 24 Sudanese isolates were submitted to the GenBank (see Table 4 for accession numbers).

Sequence assembly and pairwise comparisons were carried out using MEGA-X (Kumar *et al.,* 2018) for the partial *CP* sequence of 18 *Polerovirus* isolates (from 13 countries) from the GenBank database and four Sudanese chickpea isolates representing four polerovirus species identified further in the present study (SuCp122-13: CABYV, SuCp31-15: CLRDV, SuCp29-15: PABYV and SuCp42-13: PeVYV). Sequence alignments were generated under the Hasegawa–Kishino–Yano (HKY) (Hasegawa *et al.*, 1985) model with a bootstrap value of 1000 by MEGA-X. Nucleotide pairwise similarities were calculated using SDTv 1.2 (Muhire *et al.*, 2014).

Phylogenetic analyses of nucleotide and amino acid sequences were carried out using a Clustal_X program after multiple alignment of sequences by neighbour joining algorithms with 500 bootstrap replications (Thompson *et al.,* 1997).

RESULTS

Field distribution and serological tests

The most commonly observed symptoms suggestive of virus infection in chickpea fields were yellowing, stunting, chlorosis and reddening of the leaves and tip wilting (Figure 1). Based on the symptoms observed in the fields, 17% of chickpea fields (one field in 2013 and five fields in 2018) had virus incidence of 5% or less, 31% of fields had incidence of 6–20% (two fields in 2013 and nine in 2018), 29% of fields had incidence of 21–50% (four fields in 2013, two in 2015 and four in 2018), and 8 fields (23%) had virus incidence greater than 50% (three fields in 2013, two in 2015 and three in 2018).

TBIA results from 415 symptomatic plant samples collected during the 2013, 2015 and 2018 growing seasons indicated that CpCDV was the most common virus, with average relative infection rates of 59% of the tested samples in 2013, 89% in 2015, and 17% in 2018. In addition, 21% of tested samples reacted positively with a broad-spectrum legume luteovirid MAb (5G4) (23 samples in 2013, 18 in 2015 and 45 in 2018), whereas FBNYV infection was detected in only one sample during 2013 (Table 2). When 86 samples that reacted positively with 5G4 MAb were further tested using specific luteovirid MAbs, 11 samples reacted with BWYV MAb, 22 samples reacted with CpCSV MAbs, 23 samples reacted with both BWYV and CpCSV MAbs, and the 30 remaining samples reacted only with 5G4 MAb (Table 3).

Molecular analyses

According to TBIA reactions with different MAbs, 36 samples were selected for further molecular characterization (eight samples that reacted only with MAb 5G4, seven that reacted positively with 5G4, BWYV and CpCSV MAbs, and 21 samples that reacted positively with 5G4 samples and CpCSV MAbs). The generic primer pair AS3/Pol3870F amplified the expected product size of 370 bp from 33 chickpea samples out of 36 samples tested (Figure 2-A). The MP-PCRs (set 1 and set 2) results showed presence of CABYV in 12 samples with amplicon size of approx. 474 bp (Figure 2-B). However, there were 21 samples that were positive in generic RT-PCR AS3/Pol3870F but were negative in all MP-PCRs. Thus, all unrecognized samples along with five samples that were amplified with CABYV-specific primer pairs were sequenced by Sanger sequencing.

The sequence analyses confirmed presence of CABYV (five samples) with 96% nt similarity with the

Figure 1. Plants showing yellowing and stunting symptoms in chickpea fields in the Gezira Scheme, Sudan during the 2015 cropping season.

Table 2. Results of serological tests (Tissue blot immunoassay, TBIA) for chickpea samples collected from different regions of Sudan during the 2013, 2015 or 2018 growing seasons.

Year/Region	Number of fields visited	Number of samples tested	Number of samples reacted positively with[a]		
			5G4 (MAb)	FBNYV (MAb)	CpCDV (PAb)
2013					
Gezira Scheme					
North	5	68	6	0	63
Middle	3	32	3	1	14
River Nile State					
Hudeiba Agr. Res. station	1	25	14	0	1
Berber	1	8	0	0	1
2015					
Gezira Scheme					
South	4	71	18	0	63
2018					
Gezira Scheme					
North Gezira	7	58	11	0	2
Middle Gezira	8	81	27	0	21
River Nile State					
Shendi	2	19	7	0	10
Hudeiba Agr. Res. station	1	21	0	0	0
Berber	2	32	4	0	4
Total	34	415	86	1	179

[a] 5G4 (MAb): broad-spectrum legume luteovirid monoclonal antibody (Katul, 1992); FBNYV (MAb): Faba bean necrotic yellows virus (monoclonal antibody) (3-2E9; Franz *et al.,* 1996); CpCDV (PAb): Chickpea chlorotic dwarf virus (Polyclonal antibody) (Kumari *et al.,* 2006).

type reference sequence for CABYV (GenBank accession no. NC_003688), and three luteovirid species were detected for the first time from chickpea in Sudan (all belonging to *Polerovirus*), PeVYV (six samples), PABYV (14 samples) and CLRDV (one sample). Sequences were submitted to the GenBank, and the GenBank accession numbers are shown in Table 4.

When six PeVYV samples and one CLRDV sample were subjected to RT-PCR using specific primer pairs for PeVYV (PeF/PeR; Zhang *et al.,* 2015) and CLRDV (CLRDV3675F/Pol3982R; Sharman *et al.,* 2015) (Table 1), amplicons of the expected sizes (1249 bp for PeVYV and 310 bp for CLRDV) were generated (Figure 2-C and 2-D).

The comparison of detection methods between TBIA and MP-PCR clearly showed that there was greater variation in species detected than indicated by TBIA alone, i.e., the common character between the analyzed samples is that all these samples reacted positively with 5G4 MAb, which means there is no false positive reaction or cross reaction with another family of plant viruses. On the other hand, there was no compatibility between the serological results and molecular characterization. It is obvious that CpCSV and/or BWYV were not detected in any of the samples, despite that most samples reacted with CpCSV MAb mixtures and BWYV MAb due to the serological cross reaction which is common for luteovirids (Oshima and Shikata, 1990; Smith *et al*., 1996) (Table 4)

Pairwise comparisons of CP amino acid sequences of representative isolate for each virus indicated that the virus isolates from Sudan were probably members of recognized *Luteoviridae* species. The nucleotide sequence of the isolate SuCp42-13 showed that it was indistinguishable from PeVYV-Sudan isolate, despite that PeVYV-Sudan was isolated from hot pepper (Table 5). The phylogenetic analysis tree also showed that this isolate was close to PeVYV-Sudan (GenBank acces-

Table 3. Serological results of Tissue blot immunoassay (TBIA) with specific luteovirid monoclonal antibodies (MAbs) for chickpea samples collected from different regions of Sudan during the 2013, 2015 or 2018 growing seasons.

Year	Number of samples reacted with 5G4 MAb[a]	Number of samples reacted positively with MAbs[b]					Unidentified luteovirids
		BWYV	CpCSV	BLRV	SbDV	CpCSV& BWYV	
2013	23	4	6	0	0	9	4
2015	18	0	1	0	0	7	10
2018	45	7	15	0	0	7	16
Total	86	11	22	0	0	23	30

[a] 5G4: broad-spectrum legume luteovirid monoclonal antibody (Katul, 1992).

[b] Monoclonal antibodies used are BWYV: Beet western yellows virus (A5977 from Agdia, USA); BLRV: Bean leafroll virus (4B10; Katul, 1992); SbDV: Soybean dwarf virus (ATCC PVAS-650, USA); CpCSV: a mixture of three MAbs (1-1G5, 1-3H4 and 1-4B12) produced against an Ethiopian isolate of Chickpea chlorotic stunt virus (CpCSV-Eth), and a mixture of three MAbs (5-2B8, 5-3D5 and 5-5B8) produced against a Syrian isolate of CpCSV (CpCSV-Sy) (Abraham *et al.,* 2006, 2009)

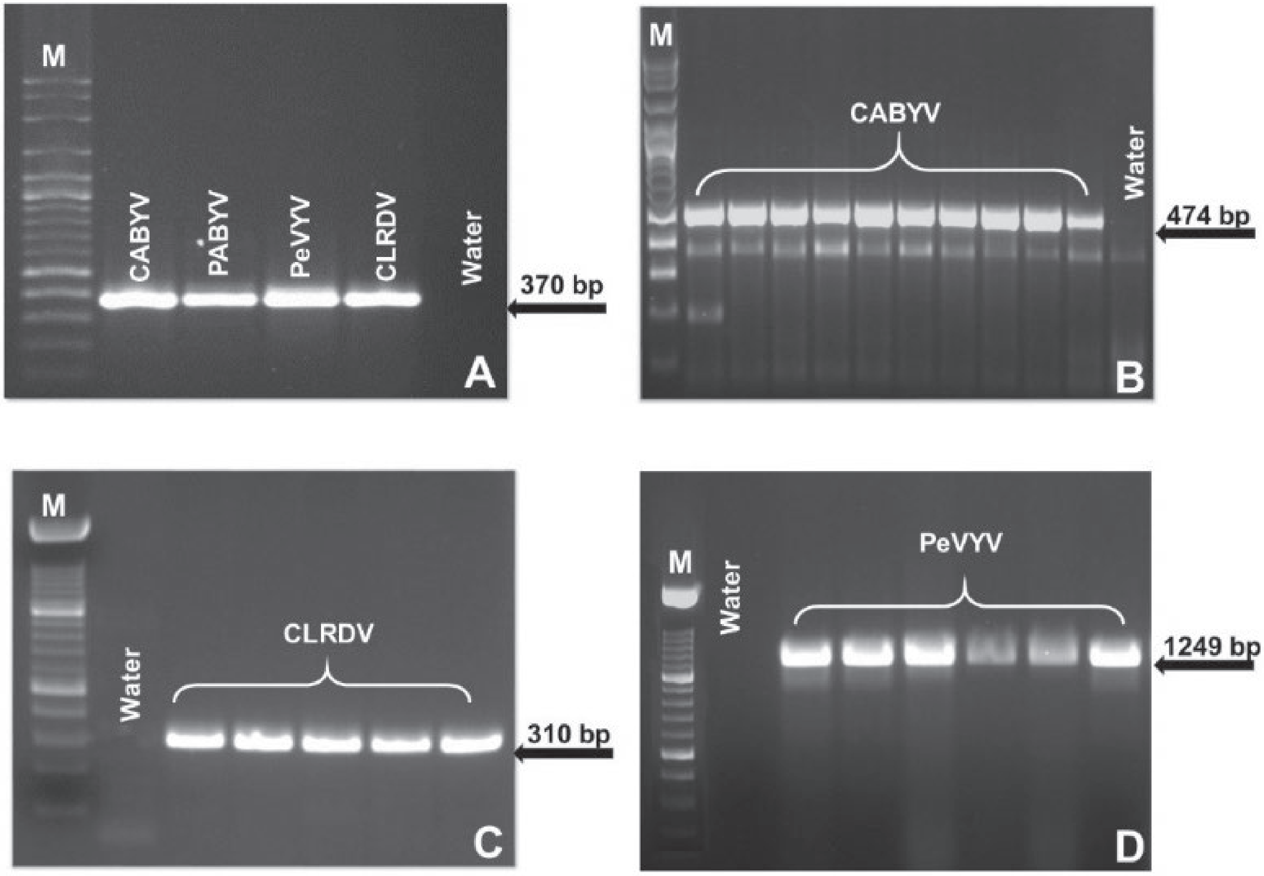

Figure 2. (A) Detection of Cucurbit aphid-borne yellows virus (CABYV), Pepo aphid-borne yellows virus (PABYV), Pepper vein yellows virus (PeVYV) and Cotton leafroll dwarf virus (CLDRV) by RT-PCR using AS3/Pol3870F generic primers; (B) Detection of CABYV by MP-PCR using AS3/CABYV3635F specific primers; (C) Detection of CLRDV by RT-PCR using Pol3982R/CLRDV3675F specific primers (one sample from Sudan and four samples from Uzbekistan were used as positive controls); (D) Detection of PeVYV by RT-PCR using PeF/PeR specific primers. M = DNA Ladder VC 100 bp Plus (Cat No. NL1405, vivantis, Malaysia).

sion no. KC685313) (Figure 3). BLAST analysis of the PCR product generated by AS3/Pol3870F revealed high nucleotide sequence similarity with *Polerovirus* viruses: 95-96% similarity was found with PeVYV (accession no. KC685313) and 93-94% for PABYV (accession no. KJ789902). Similarities of 90% were found for CLRDV (accession no. EU871539) and 90-92% for CABYV (accession no. KX398665) (Table 4). A nucleotide sequence obtained from the isolate SuCp29-15 was also distinct from all other luteovirid sequences. Pairwise comparisons of the predicted CP amino acid sequences showed that isolate SuCp29-15 was close to the PABYV-Cote d'Ivoire isolate (GenBank accession no. KR476816) with 97% similarity (Table 5; Figure 3).

The CP sequence of SuCp31-15 was 96% similar to that of the CLRDV-Brazil isolate, which was the closest phylogenetically. Similarly, isolate SuCp122-13 shared distinct similarity with both CABYV isolates from Tunisia (GenBank accession no. EF187345) and Italy (GenBank accession no. EF029113 (Table 5; Figure 3).

The phylogenetic comparison of the nucleotide sequence of the virus isolates grouped the isolates in distinct clusters depending on identical and different sequences which revealed that the grouping model is typically correlated to the geographical origin of the isolates (Figure 3). This result also was supported by a two-dimensional color-coded matrix of pairwise identity scores (Figure 4) generated by species demarcation tool (SDT) (Muhire *et al.*, 2014), which revealed that the representative isolates have overlapping identity range with *CP* gene from GenBank isolates (59-99%). Despite the fact that some virus isolates were identified from different hosts, the SDT showed similar identity as for isolate SuCp42-13 and reference isolate (PeVYV, GeneBank accession no. KC685313).

Table 4. Designations, geographic origins, comparison and identity with reference GenBank accessions of chickpea luteovirids characterized in this study.

Isolate name[a]	GenBank accession number	Region in Sudan	TBIA reaction with MAbs[b]	Virus sequence Blastn_ Reference GenBank accessions[d]	Blastn similarity %
SuCp10-13	MK461113	North of Gezira Scheme	5G4, CpCSV	PeVYV_ KC685313	96
SuCp14-13	MK461114	North of Gezira Scheme	5G4	PeVYV_ KC685313	96
SuCp42-13	MK461115	Middle of Gezira Scheme	5G4, CpCSV	PeVYV_ KC685313	95
SuCp108-13	MK461116	Hudeiba Agr. Res. Station, River Nile	5G4, CpCSV	PeVYV_ KC685313	96
SuCp111-13	MK461120	Hudeiba Agr. Res. Station, River Nile	5G4	PABYV_ KJ789902	93
SuCp21-15	MK461121	South of Gezira Scheme	5G4, CpCSV, BWYV	PABYV_ KJ789902	94
SuCp22-15	MK461122	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp26-15	MK461123	South of Gezira Scheme	5G4	PABYV_ KJ789902	94
SuCp28-15	MK461124	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp29-15	MK461125	South of Gezira Scheme	5G4, CpCSV, BWYV	PABYV_ KJ789902	94
SuCp30-15	MK461126	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp32-15	MK461127	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp33-15	MK461128	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp34-15	MK461129	South of Gezira Scheme	5G4, CpCSV, BWYV	PABYV_ KJ789902	94
SuCp35-15	MK461130	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp36-15	MK461131	South of Gezira Scheme	5G4, CpCSV, BWYV	PABYV_ KJ789902	94
SuCp37-15	MK461132	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp38-15	MK461133	South of Gezira Scheme	5G4, CpCSV	PABYV_ KJ789902	94
SuCp31-15	MK411565	South of Gezira Scheme	5G4, CpCSV	CLRDV_ EU871539	90
SuCp106-13 [e]	MG933685	Hudeiba Agr. Res. Station, River Nile	5G4, CpCSV	CABYV_ KX398665	91
SuCp110-13	MK461117	Hudeiba Agr. Res. Station, River Nile	5G4, CpCSV	CABYV_ KX398665	91
SuCp117-13	MK461118	Hudeiba Agr. Res. Station, River Nile	5G4	CABYV_ KX398665	90
SuCp122-13 [e]	MG933686	Hudeiba Agr. Res. Station, River Nile	5G4, CpCSV	CABYV_ KX398665	90
SuCp23-15	MK461119	South of Gezira Scheme	5G4, CpCSV, BWYV	CABYV_ KX398665	92

[a] The last two numbers refer to year of collection.

[b] Information of MAbs are given in Table 3. Virus acronym used is CpCSV: Chickpea chlorotic stunt virus; BWYV: Beet western yellows virus.

[c] All samples were amplified only with generic primer pairs (AS3/Pol3870F) except last 5 samples were amplified with AS3/CABYV3635F in addition to generic primer pairs.

[d] Virus acronym used is CLRDV: Cotton leafroll dwarf virus; CABYV: Cucurbit aphid-borne yellows virus; PeVYV: Pepper vein yellows virus; PABYV: Pepo aphid-borne yellows virus.

[e] CABYV isolates reported in Kumari *et al.* (2018).

DISCUSSION

The present study has shown that CpCDV and luteovirids were the most common viruses affecting chickpea crops in Sudan, whereas FBNYV was rare. These viruses have been reported on faba bean and chickpea in many countries in the WANA region (Kumar *et al.*, 2008; Kumari and Makkouk, 2007; Makkouk and Kumari, 2009). CpCDV has been reported on faba bean and chickpea (Makkouk *et al.*, 1995), and FBNYV (Makkouk *et al.*, 2003b) and BLRV (Makkouk *et al.*, 1988) were reported on faba bean in Sudan, based on serological assays using polyclonal antibodies.

Based on serological results, 11 samples reacted positively with BWYV MAb, 22 with CpCSV MAbs, and 23 samples reacted with both BWYV and CpCSV MAbs. However, sequence analyses showed that no samples were infected with either BWYV or CpCSV. This demonstrates that virus identification based solely on serology can be inaccurate due to cross reactions between specific MAbs and a range of viruses in *Polerovirus*. Our approach of initially screening large numbers of symptomatic field samples by serology, followed by molecular confirmation of species from serologically-positive samples, has proved to be useful to accurately identify virus species involved in disease outbreaks.

Although results of serological tests confirmed the growing importance and challenge caused by luteovirids in legume crops in the WANA region, there have been many indications that the use of serological techniques

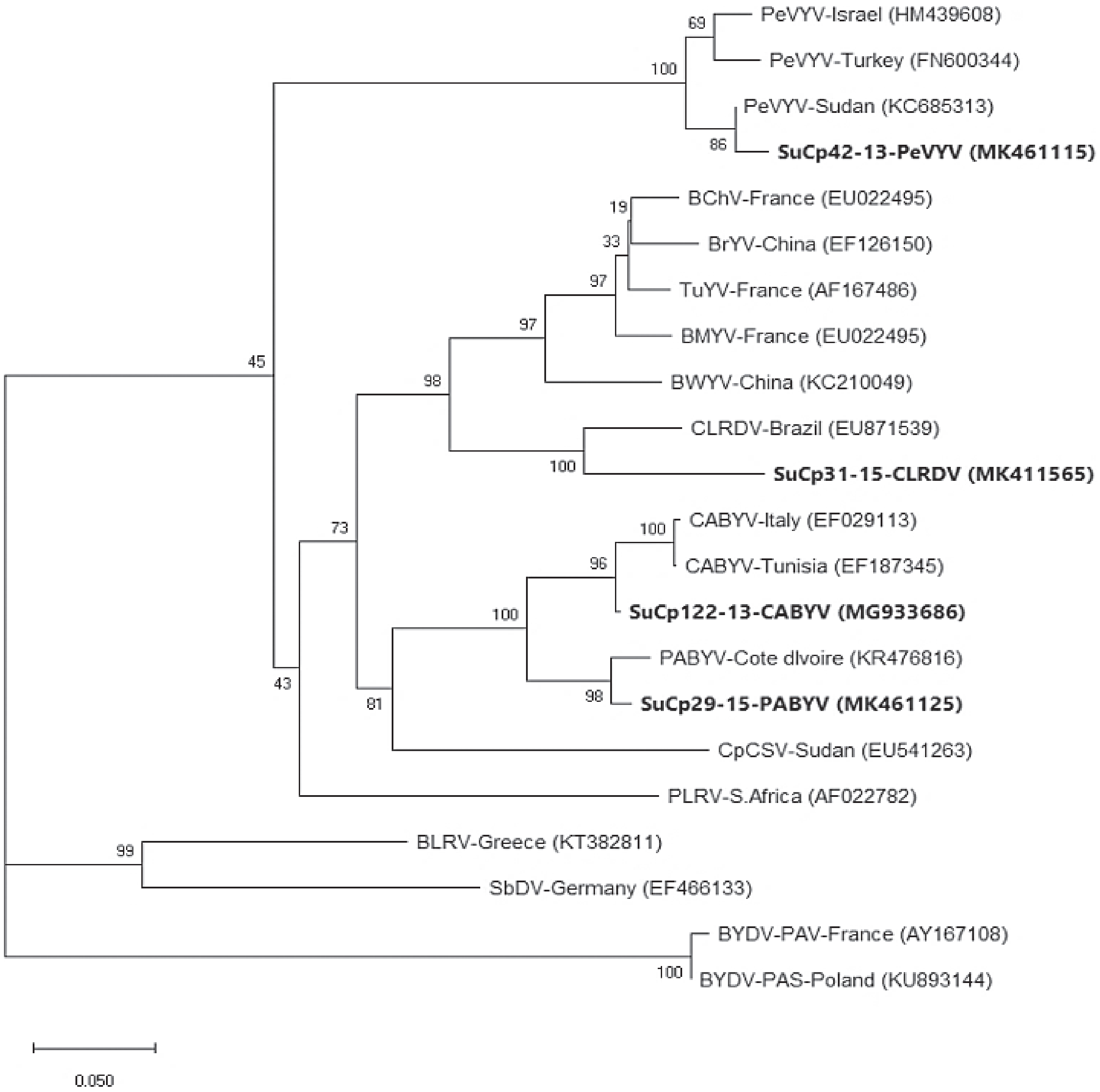

Figure 3. Dendrogram showing the phylogenetic relationships of the predicted partial coat protein amino acid sequences from AS3/Pol3870F fragment of new detected distinct luteovirid isolates with those of other luteovirids from the database. The scale bar represents 0.050 divergence of the Hasegawa-Kishino-Yano dissimilarity index. Bootstrap analysis was carried out with 1000 replicates of the starting tree. Bootstrap values are shown in each branch. Database accession numbers of the luteovirid sequences and the virus acronyms used are presented in Table 5.

are not sufficiently reliable for the identification of luteovirid species, because different luteovirids share a number of epitopes (Martin and D'Arcy, 1990; Fortass *et al.,* 1997; Abraham *et al.,* 2006). However, as Makkouk and Kumari (1996) confirmed, TBIA is a helpful method for easy, rapid and cheap detection of plant viruses, especially in the developing countries, and TBIA can be an important tool for virus detection in large scale surveys. The molecular detection method for CABYV, PABYV, PeVYV and CLRDV diagnoses used in this study showed the RT-PCR analysis is very reliable for detection of these four viruses in symptomatic samples.

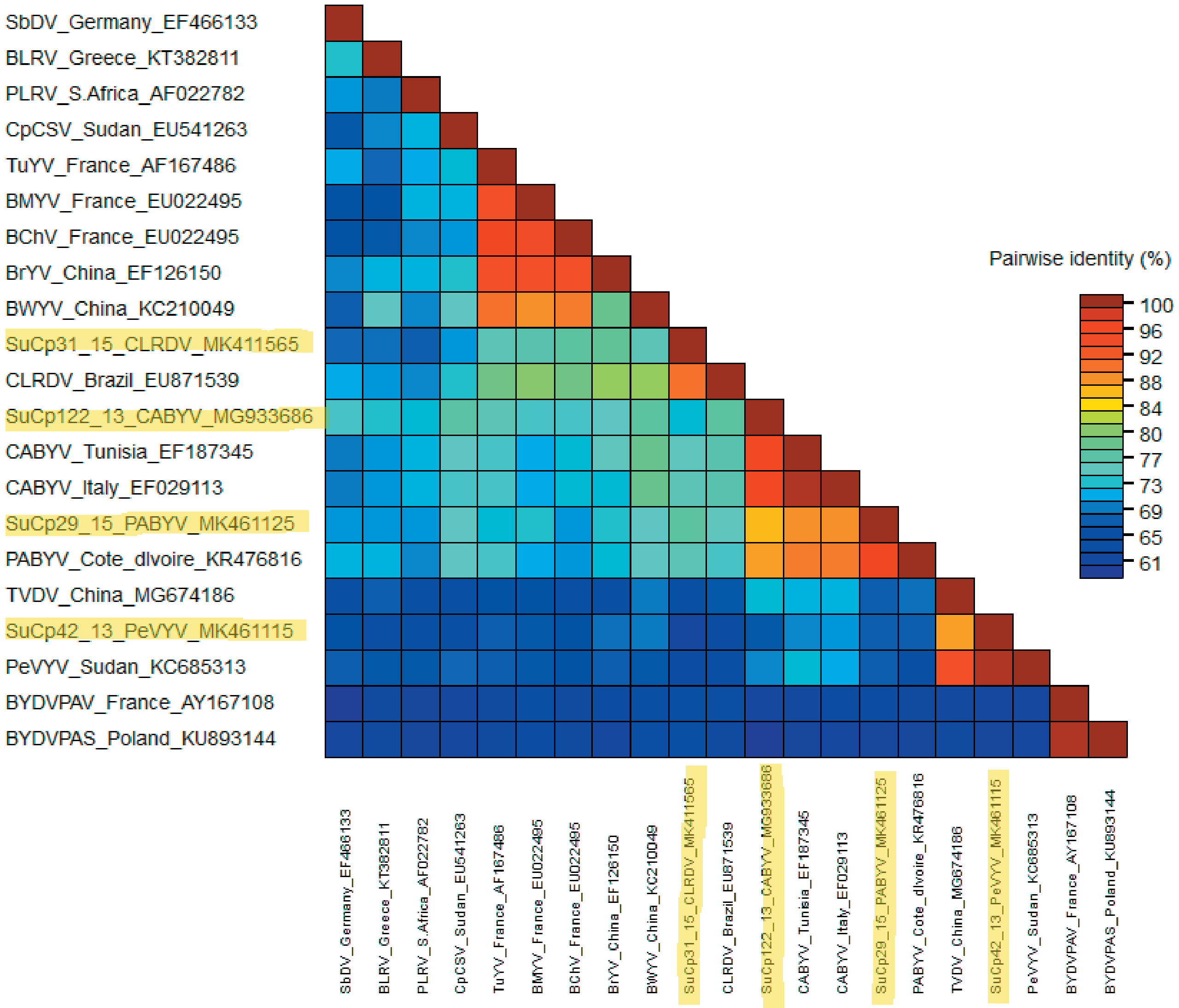

Figure 4. Two dimensional percentage pair wise similarity plot matrix of different selected strains of *Luteoviridae* generated using the Species Demarcation Tool (http://web.cbio.uct.ac.za/SDT). Each coloured cell represents a percentage similarity between two sequences (one indicated horizontally to the left and the other vertically at the bottom) displayed in the colour key. The luteovirid isolates from Sudan are highlighted, and all accessions details used for this study are listed in Table 5.

Therefore, the MP-PCR method, which can rapidly identify luteovirids, is an important tool for identifying and determining the distribution of luteoviruses that affect cool season legumes. Generally, MP-PCR technology, in addition to sensitivity and specificity, has the added benefits of saving time and costs compared with Uniplex RT-PCR (Deb and Anderson, 2008; Murray Sharman, unpublished data).

The field surveys carried out in the present study indicated that *Aphis craccivora* is present in most chickpea fields. *Aphis craccivora* is polyphagous and preferences *Fabaceae* hosts, but other host plant families include *Brassicaceae*, *Cucurbitaceae*, *Malvaceae*, and *Solanaceae*. Crops attacked by this aphid include brassicas, cucurbits, beetroot, peanut, cotton, cowpeas and chickpea. In addition, this aphid is the vector of a number of plant viruses including *Luteoviridae* species. The major crops in Gezira Scheme are cotton, vegetable and chickpeas, and the viruses reported on chickpea in this study also affect cotton and vegetable crops. Further study is therefore needed on behaviour of aphid species in agriculture systems in the Gezira Scheme to use the information for effective management of these viruses. Furthermore, occurrence of these new viruses suggests

Table 5. Pairwise comparisons of the percentage amino acid sequence similarities for partial *CP* gene (AS3/Pol3870F fragment) of four distinct luteovirid sequences amplified from four representative samples with that of other luteovirids from the database and to each other.

Virus species (source)	Accession Number	Sequenced representative Isolates			
		SuCp42-13 (PeVYV)	SuCp29-15 (PABYV)	SuCp31-15 (CLRDV)	SuCp122-13 (CABYV)
Barley yellow dwarf virus-PAS (Poland)	KU893144	43.43	45.83	42.55	50.00
Barley yellow dwarf virus-PAV (France)	AY167108	43.43	45.83	42.55	49.36
Bean leafroll virus (Greece)	KT382811	52.53	58.95	58.06	64.83
Soybean dwarf virus (Germany)	EF466133	57.58	66.32	60.22	65.73
Beet chlorosis virus (France)	EU022495	65.06	75.31	75.00	76.98
Beet mild yellowing virus (France)	EU022496	62.65	74.07	75.00	77.42
Beet western yellows virus (China)	KC210049	64.65	80.00	78.72	75.00
Brassica yellows virus (China)	EF126150	61.46	76.67	79.78	80.45
Carrot red leaf virus (Mauritius)	FJ969849	57.69	59.18	51.11	61.11
Chickpea chlorotic stunt virus (Syria)	EU541270	60.42	79.12	70.00	80.58
Cotton leafroll dwarf virus (Brazil)	EU871539	61.62	78.95	**96.17**	76.97
Cucurbit aphid-borne yellows virus (Italy)	EF029113	61.62	89.36	75.79	**97.93**
Cucurbit aphid-borne yellows virus (Tunisia)	EF187345	60.42	89.01	75.00	**97.24**
Pepo aphid-borne yellows virus (Cote d'Ivoire)	KR476816	62.11	**96.70**	73.33	88.97
Pepper vein yellows virus (Sudan)	KC685313	**100.00**	62.50	60.00	63.16
Potato leafroll virus (South Africa)	AF022782	60.61	65.62	61.70	68.21
Tobacco vein distorting virus (China)	MG674186	74.95	65.62	58.95	65.97
Turnip yellows virus (France)	AF167486	67.47	75.31	75.00	79.84
SuCp42-13-PeVYV (Sudan, this study)	MK461115	-	61.70	59.57	58.89
SuCp29-15-PABYV (Sudan, this study)	MK461125		-	NS*	60.00
SuCp31-15-CLRDV (Sudan, this study)	MK411565			-	75.56
SuCp122-13-CABYV (Sudan, this study)	MG933686				-

* NS: No significant similarity found.

the need for further screening of legume crops, including chickpea, for resistance to luteovirids, and for development of new management strategies to incorporate host resistance as an important component for virus disease control.

Despite the limited number of samples analyzed for sequencing, PABYV sequences were amplified from the majority of the luteovirid-positive samples analyzed (14 samples of 24 sequenced samples), followed by PeVYV and CABYV. This suggests that these viruses are widespread in cool-season food legumes grown in the WANA countries, and are more prevalent than the other luteovirids detected so far from the region, such as BLRV and SbDV (Fortass and Bos, 1991; Tadesse *et al.,* 1999; Abraham *et al.,* 2000; Makkouk *et al.,* 2003a). The observed variability within the sequences together with detection in samples from different locations and different luteovirids, suggest that these viruses have been infecting legumes for many years in Sudan but have remained undetected and/or incorrectly identified as one of the other legume luteovirids, possibly due to the lack of appropriate diagnostic methods. In addition, PeVYV has been previously reported infecting hot pepper (*Capsicum annuum*) in Sudan (Alfaro-Fernándezn *et al.,* 2014). The molecular analysis found that PeVYV chickpea isolate (SuCp42-13, GenBank accession no. MK461115) was almost identical to the Sudanese PeVYV isolated from pepper (GenBank accession no. -KC685313), indicating that both chickpea and pepper isolates are same, or are very similar, but this virus has not been previously recognized in grain legumes due to antibody cross reactions.

Most previous studies have been based on serological tests that are not reliable for the identification of luteovirids to species level. The present study has confirmed the occurrence of CABYV, PABYV, PeVYV and CLRDV in Sudan, using robust molecular techniques. These samples reacted serologically with one or more of antibodies specific to BWYV and CpCSV, suggesting they share a common epitope with these two viruses.

CABYV was first described in 1992 in France (Lecoq *et al.,* 1992), but was later detected infecting cucurbits in many other countries (Kassem *et al*., 2013). In addition to cucurbits, CABYV can infect other crop species, including lettuce (*Lactuca sativa*) and fodder beet (*Beta vulgaris*), as well as some common weeds (Kassem *et al.,* 2013), which are thought to be virus reservoirs. Recently, CABYV was reported to infect faba bean (*Vicia faba* L.) in Turkey (Buzkan *et al.,* 2017), and the present study is the first report of CABYV affecting chickpeas. CABYV is transmitted by *Aphis gossypii* and *Myzus persicae* (Lecoq *et al.,* 1992), and both these vectors have very broad host ranges. Further disease surveillance is required to determine if CABYV is also present in other grain legume production regions of the world.

Cotton blue disease (CBD) was first described in the Central African Republic in 1949 (Cauquil and Vaissayre, 1971), although no causal agent was characterized at the time. CLRDV has now been shown to cause Cotton blue disease from Brazil (Corrêa *et al.,* 2005). Our detection of CLRDV from Sudan is the first confirmation of this virus from Africa, and this suggests that this virus may have been unnoticed on chickpea in Sudan, where cotton cultivation is widespread. Hence, further research is required to outline the life cycle of this virus on cool season and warm season crops. More recently, CLRDV has been reported on chickpea in Uzbekistan (Kumari *et al.,* 2020).

Sharman *et al.* (2015) and Mukherjee *et al.* (2016) indicated that the host range of CLRDV is not well understood, but mainly includes plants in *Malvaceae*, especially *Gossypium* spp. While the main vector of CLRDV in cotton is *Aphis gossypii* (Michelotto and Busoli 2007), this virus is also transmitted by *M. persicae* and *A. craccivora* in chickpea (Mukherjee *et al.,* 2016). In Sudan, cotton and chickpea crops are grown in rotation, and they probably share these viruses and their aphid vectors. This may play a role in the epidemiology of these viruses, allowing them to survive between seasons on alternating crops. The study by Reddy and Kumar (2004) on the host range of the chickpea stunt disease associated virus (CpSDaV), most likely synonymous to CLRDV (Naidu *et al.,* 1997; Corrêa *et al.,* 2005), indicated that CLRDV can infect several grain legume species, many of which are commonly cultivated in Sudan, suggesting that CLRDV may have suitable hosts all year around. Mukherjee *et al.* (2016) studied the genetic similarity between CLRDV and CpSDaV in India, and found that these two viruses are possibly two different strains of the same virus. This information would be helpful for managing these serious diseases, possibly by altering the cropping patterns used by producers.

The present study is the first to report CLRDV and PABYV from crops in Sudan, and is the first report of PeVYV isolated from chickpea in this country. Kumari *et al.* (2018) made the first report of CABYV in Sudan, and the present report has greatly extended the understanding of the diversity, geographical range and incidence of CABYV in Sudan. In addition, this study is the first reliable molecular characterization for these four *Polerovirus* species identified from chickpea samples collected in Sudan. Further field investigations and surveys are required to determine more accurately the ongoing impacts and geographical distribution of these newly detected viruses on chickpea and other grain legume crops in the WANA region. Accurate local knowledge of identity of viruses affecting these crops is essential for breeding for disease resistance and effective crop management.

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