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Molecular characterization and Phylogenetic Analysis of Viruses Associated with Garlic and Onion in Punjab, India

Irfan Khan[1]* and Abhishek Sharma[2]
[1]Department of Plant Pathology,
Punjab Agricultural University, Ludhiana (Punjab), India.
[2]Department of Vegetable Science,
Punjab Agricultural University, Ludhiana (Punjab), India.

(Corresponding author: Irfan Khan)*
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ABSTRACT: Garlic and onion grown in Punjab, India are generally infected with several viruses, including the common alliumviruses of the genera Allexivirus, Potyvirus and Carlavirus, and these were responsible for drastic yield losses. *Onion Yellow Dwarf Virus* (OYDV), *Shallot Latent Virus* (SLV) and *Garlic Virus D* (GarV-D) were identified by RT-PCR assay using virus-specific primer sets. BLASTn analysis of sequences confirmed the presence of OYDV and GarV-D, SLV in infected onion and garlic samples, respectively. During BLASTn analysis, OYDV isolate (KP862051) shared 98-99% identity with OYDV isolates (KP862053, KP862052) from Ludhiana and 97% similarity with OYDV isolate (FR833734) from New Delhi. Whereas in the case of garlic viruses, SLV (KR074209) had 91% similarity to SLV isolate (EF600902) from New Delhi, and GarV-D isolate (KR045606) showed a maximum resemblance of 99% with GarV-D isolate (HQ724840) from Spain. OYDV isolate (KP862051) shared 79% nt identity with Rajasthan isolate (EU045556), and 72.9% with New Delhi isolate (FR873734), GarV-D isolates (KR045606) shared a maximum 99.5% nt similarity with Spain isolate (HQ724840) and SLV isolate (KR074209) shared 90.8 nt similarities with New Delhi isolate (EF600902). Phylogenetic analysis of nucleotide sequences revealed that Indian isolates of OYDV and GarV-D have the closest phylogenetic relationship with Indian and abroad isolates; however, SLV isolate (KR074209) was found to be significantly divergent from abroad isolates. Phylogenetic analysis confirms the evolutionary origin of OYDV and SLV from Indian ancestors and GarV-D origin from Spain isolates. Present investigation unravels the virus diversity, subgrouping, and evolutionary history within the alliumviruses in India.

Keywords: Alliumviruses, Characterization, Phylogenetic, Onion and Garlic.

INTRODUCTION

India's important allium crops are garlic (*Allium sativum* L.) and onion (*Allium cepa* L.). Both crops are used as spices and food throughout the world. They are known to be attacked by fungi, bacteria, viruses and phytoplasmas. Among these, virus diseases are of great importance due to agamic propagation of these crops. Viruses belong to genera Allexivirus, Carlavirus, Potyvirus, Potexvirus, and Tospovirus and are common in Allium species (Mohammed *et al*., 2013; Maliogka *et al.,* 2006; Prajapati *et al.,* 2022). Potyviruses cause globally severe economic losses on edible Allium crops (Bagi *et al.,* 2012; Lunello *et al.,* 2007). There are 176 virus species in the Potyviridae family, 146 of which are in the Potyvirus genus, and OYDV is one of them. A wide variety of hosts, including significant crops, are infected by potyviruses (Adams *et al.,* 2011). In India, a disease in an onion seed crop causing symptoms similar to OYDV was reported by Dhingra and Nariani (1963), but its aetiology could not be ascertained. Later in 1997, Ahlawat and Varma reported mixed infection of OYDV with mite-borne Rymovirus in India (Hoa *et al.*, 2003). There have also been reports of OYDV in the United States, Turkey, Iran, Sudan, Serbia, Poland, Italy, Nepal, Greece, Egypt, Brazil, the Czech Republic, and Argentina (Verma *et al.,* 2015). OYDV has a 10,538 nucleotide-long genome that is a monopartite, linear, positive sense, single-stranded, and belongs to the family Potyviridae (Chen *et al.,* 2003). SLV and GarV-D have also been recorded in garlic from India (Khan *et al.,* 2016; Majumder *et al.,* 2008). GarV-D, a member of the Allexivirus genus and family *Alphaflexiviridae*, is filamentous, (+) single-stranded RNA, 12 nm wide and 800 nm long. Six open reading frames translate genes (Bereda *et al.,* 2015). SLV (genus Carlavirus, family *Betaflexiviridae*) genome is linear, single-stranded, positive sense RNA and 8363 nt long (Chen *et al.,* 2001). Mixed infections of OYDV, SLV, *Garlic common latent virus* (GarCLV) and allexiviruses have been recorded from Indian garlic accessions (Majumder and Baranwal 2014). Similarly, mixed infections of OYDV and allexivirus were detected in Onions from India (Kumar *et al.,* 2010).

There are several viruses under the genus have been reported, including Allexivirus; *Garlic virus B* (GarV-B) and *GarV-D* from the USA (Gieck *et al.,* 2009), *Garlic virus A* (GarV-A) from India (Gawande *et al.,* 2015), *Garlic virus X* (GarV-X) from India and Brazil (Baranwal et al., 2011; Oliveira *et al.,* 2014a), *Shallot virus X* (ShV-X) from India and Sudan (Hamed and Menzel 2012; Majumder *et al.,* 2007). Large-scale routine testing of alliumviruses by Enzyme-linked immunosorbent assay (ELISA) is currently the main diagnostic tool. However, producing highly specific antisera against these viruses is difficult because of multiple infections of allium crops by these viruses. Due to the lack of a differential host, the isolation of these viruses is also difficult. Consequently, reverse transcription polymerase chain reaction (RT-PCR) assays were used to detect potyviruses, carlaviruses and allexiviruses in garlic and onion samples. RT–PCR is another method for detecting these viruses, which is 10^2-10^4 times more sensitive than ELISA (Dovas *et al*., 2001). As trading leads to the spread of these two crops across the country, the chances of spreading the virus from producing area to a new place are quite high. Since no systematic study on prevalent viruses of onion and garlic under Punjab conditions had been attempted earlier, the present investigation was carried out to characterize viruses infecting onion and garlic and their phylogenetic relatedness with other allium viruses and each other.

MATERIAL AND METHODS

RNA extraction and multiplication by RT-PCR. Virus-like symptoms on onion and garlic plants were observed, and leaf samples from healthy and infected plants (ten garlic and eight onion samples each) were collected for further analysis. The total RNA was isolated from 100 mg of leaf tissue from naturally infected and asymptomatic plants using the FastRNA Pro Green kit (MP Biomedicals, USA) as per the manufacturer's procedure. The first strand of cDNA was prepared with the Revert Aid first-strand cDNA synthesis kit (Thermo Scientific). Reaction mixture (20µl) made up of 5µl of total RNA (700 to 1000 ng), 1µl reverse primer OYDV 3R/DCPR/SLV R (20 pM) (Chodorska *et al.,* 2014; Kumar *et al.,* 2010; Majumder *et al*., 2008), 4µl reaction buffer (5X), 1µl ribolock RNase inhibitor (20/µl), 2µl of dNTP mix (1 mM), 1µl M-MuLV reverse transcriptase (200U /µl) and 6µl. Nuclease-free water. According to the manufacturer's protocol, the reaction mixture was incubated in the thermal cycler at 42°C for 45 minutes before being heated at 70°C for 10 minutes. PCR was carried out in a 25 µl reaction volume comprising 2 µl of cDNA, 5 µl of reaction buffer (5X), 1.5mM Mgcl2, 0.2mM dNTP mix, 20 pmol/ml of OYDV 3F/3R, DCPF/R, SLV F/R primer pairs, and 1U DNA polymerase. OYDV's partial Nib and coat protein gene and partial coat protein gene of GarV-D and SLV were amplified using the PCR cycling profile previously described (Chodorska *et al.,* 2014; Kumar *et al*., 2010; Majumder *et al*., 2008). The PCR results were assessed on a 1% agarose gel, stained with ethidium bromide, and examined under UV illumination with a 100bp DNA marker (Promega, USA).

Cloning and sequencing of PCR product. Following the manufacturer's instructions, the RT-PCR product was ligated into the pGEM-T easy vector cloning system (Promega, Madison, WI, USA). Competent Escherichia coli (strain DH5) cells were transformed, clones were chosen on Luria agar plates treated with ampicillin (100 g/ml), and the presence of the insert was verified by PCR using the same primer as stated for RT-PCR. Positive clones were sequenced by Eurofins Genomics India Pvt Ltd. in Bangalore. In the ABI 3730Xl DNA Analyzer, bidirectional sequencing was performed using M13 primers.

Sequence and phylogenetic analysis. The sequence data were analysed to eliminate ambiguity, assembled into constitutive sequences, and deposited in the National Centre for Biotechnology Information (NCBI) GenBank. The obtained isolates' sequences were examined using the BLASTn tool and compared to known corresponding sequences of the same viral species in the GenBank database. Lasergene 8.0.2 software was used to calculate the nucleotide identities (nt) of sequenced genes (DNASTAR, Inc., Madison, WI, USA). The phylogenetic relationships were analyzed using Version 6.0 of the Molecular Evolutionary Genetics Analysis (MEGA) programme (Tamura *et al.,* 2013). Phylogenetic trees were created using the neighbour-joining method with 1000 bootstrap replicates and the Tamura 3 parameter model. For tree roots, OYDV (KP862053) and GarV-D (KR045606) sequences were used.

RESULTS AND DISCUSSION

The present study focused on characterization and phylogenetic analyses of viruses infecting garlic and onion in Punjab. The common symptoms noticed during the investigation were plants with crinkling of older leaves, yellow stripping on leaves and stunting of plants. Crinkling of leaves was more common in onion as compared to garlic. These symptoms were similar to previous reports of a viral infection complex comprising allexivirus and other poty- and carlaviruses. Similar symptoms were recorded during 2007-08 from New Delhi (Kumar *et al.,* 2010). Mosaic and stripe symptoms were observed on stalks of flowers (Hoa *et al.,* 2003). RT-PCR was performed, for molecular detection of allium viruses, using total RNA extracted from onion and garlic samples. Electrophoresis of PCR products resulted in the expected size amplicons of 500 bp (Fig. 1) from onion (100% positive), 500 bp (Figure 2) (100% positive) and 300 bp (Fig. 3) (80% positive) from garlic samples, confirms the association of OYDV and GarV-D, SLV in infected onion and garlic samples, respectively. RT-PCR results revealed that garlic and onion were infected with viruses. Similarly, OYDV has

been detected based on RT–PCR technique in garlic and onion (Arya *et al.,* 2006). LYSV, Garlic latent virus and allexiviruses from Korea (Koo *et al.,* 2002) and OYDV, LYSV, GarCLV, allexiviruse (GarV-A, GarV-B, Garlic virus C, GarV-X) from Sudan (Mohammed et al., 2013) were recorded.

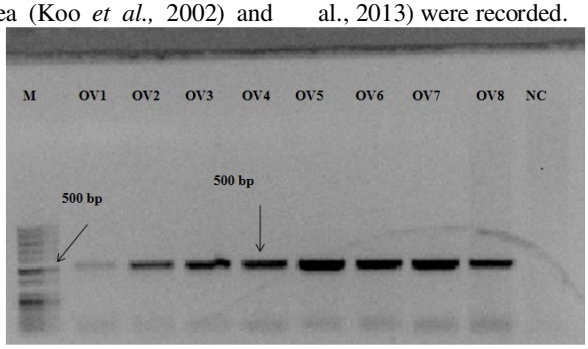

Fig. 1. Agarose gel electrophoresis of RT-PCR products showing expected size ~500bp amplicon using OYDV 3F/3R primer pair. (OV1 to OV8- Infected onion samples, NC-Negative control, M- 100bp ladder).

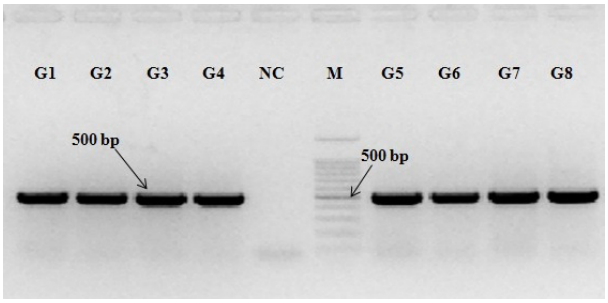

Fig. 2. Agarose gel electrophoresis of RT-PCR products showing expected size ~500 bp amplicon using DCPF/PR primer pair. (G1 to G8- Infected garlic samples, NC- Negative control, M- 100bp ladder).

The primer pair OYDV 3F/3R covers Nib and Coat protein, and DCPF/R, and SLV F/R amplify partial coat protein. These amplicons were cloned and sequenced for molecular characterisation of these viruses. Following analysis, the consensus sequence data were deposited to GenBank with Accession numbers KP862051, KR045606 and KR074209. BLASTn tool was performed to score the nucleotide sequence identity of sequences under study with the publically available sequences. During BLASTn analysis, OYDV isolate (KP862051) shared 98-99% identity with OYDV isolates (KP862053, KP862052) from Ludhiana and 97% similarity with OYDV isolate (FR833734) from New Delhi. Whereas in the case of garlic viruses, GarV-D isolates (KR045606) shared a maximum identity 99% with GarV-D isolate (HQ724840) from Spain and SLV (KR074209) shared 91% identity with SLV isolate (EF600902) from New Delhi. The pairwise sequence identities of sequences (KP862051, KR045606 and KR074209) with respective sequences of selected isolates of OYDV, GarV-D and SLV were generated at nucleotide (nt) level using Lasergene 8.0.2 software package. The sequence (KP862051) shared 79% nt identity with Rajasthan isolate (EU045556), and 72.9% with New Delhi isolate (FR873734). GarV-D isolate (KR045606) shared a maximum 99.5% nt similarity with Spain isolate (HQ724840), and SLV isolates (KR074209) shared 90.8 nt similarities with New Delhi isolate (EF600902). RNA viruses have high variation potential because of their ability to produce large populations and their high mutation and recombination rates. Ecological factors such as virus-vector relationship, host range and geographic distribution influence the genetic diversity among different viruses (Bereda *et al.,* 2015).

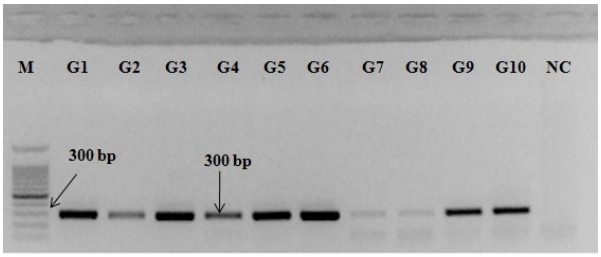

Fig. 3. Agarose gel electrophoresis of RT-PCR products showing expected size ~300bp amplicon using SLV F/R primer pair. (G1 to G10- Infected garlic samples, NC- Negative control, M- 100bp ladder).

Phylogenetic analysis of the nucleotide sequence of the virus (KP862051) with other OYDV isolates available in NCBI database revealed that these sequences were clustered in two clades (designated as clade I and clade II) (Fig. 4). This sequence (KP862051) formed a distinct clade I with other isolates and showed it is the closet phylogenetic relationship with two Indian OYDV isolates (FR833734, EU04556) and one isolate (GQ475404) from Italy. A sequence (EU045557) retrieved from GenBank showed common ancestry with the Australian isolate (HQ258894) and Spain isolates (JX429964). These two isolates (HQ258894, JX429964) were common ancestors in evolution process of OYDV Indian isolate RR1 (Verma *et al.,* 2015). It indicates that both OYDV Jammu isolate (EU045557) and isolate RR1 originated from the same ancestors. Phylogenetic tree placed sequence (EU045557) in clade II instead of placing it in clade I along with Indian isolates.

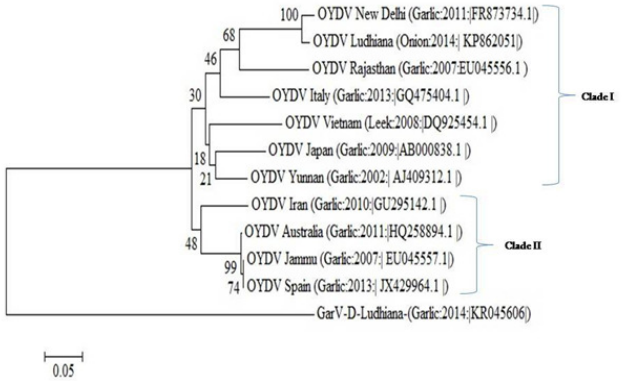

Fig. 4. Neighbor-joining relationship phylogram of the partial coat protein nucleotide sequences of OYDV isolates retrieved from Genbank and OYDV (KP862051) isolate of this study. Phylogram was generated using MEGA 6 with 1,000 bootstrap replicates (placed at the nodes on the tree). The corresponding sequence of GarV-D (KR045606) was used as an out-group. The scale units (0.05) are substitutions per site.

This divergence confirmed the correlation between geographical origin and phylogenetic clustering. This may be due high variability and rapid evolution of this virus. This variability among the viruses may result from a mixed infection of the same host, favouring the interaction between close or distinct related species (Mohammed *et al.,* 2013). A similar analysis was conducted for GarV-D (KR045606) and SLV (KR074209). The sequence (KR045606) clustered with viruses originating from Europe, Asia, Australia, and South America and depicted closet phylogenetic lineage with GarV-D isolate (HQ724840) from Spain (Fig. 5). Similarly, Brazilian isolates have also shown evolutionary relation with isolates from Australia and Korea (Oliveira *et al.,* 2014b). By phylogenetic analysis, sequence (KR074209) was found significantly divergent in partial coat protein gene from isolates of the same species obtained from GenBank. The reason for this divergence may have had a different evolutionary process of SLV Ludhiana isolate (G1) origin. This result is in agreement with evolutionary history reported by other researchers (Mohammed *et al.,* 2013). The phylogenetic tree of sequence (KR074209) clearly showed that all isolates had the same phylogenetic lineage as Indian isolates (Fig. 6).

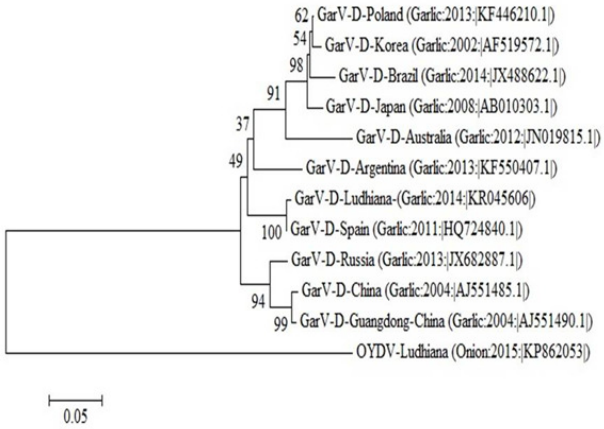

Fig. 5. Neighbor-joining relationship phylogram of the partial coat protein nucleotide sequences of GarV-D isolates retrieved from Genbank and GarV-D (KR045606) isolates of this study. Phylogram was generated using MEGA 6

with 1,000 bootstrap replicates (placed at the nodes on the tree). Corresponding sequence of OYDV (KP862053) was used as an out-group. The scale units (0.05) are substitutions per site.

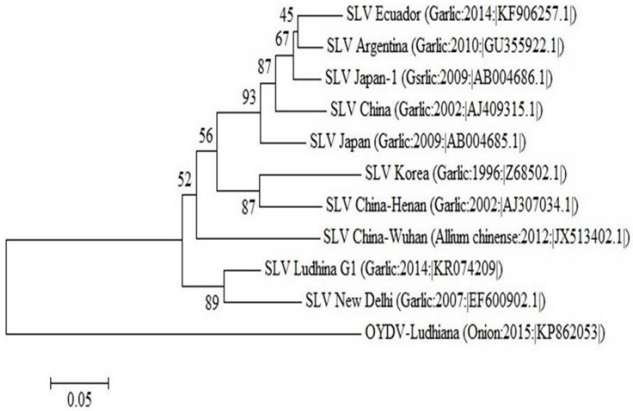

Fig. 6. Neighbor-joining relationship phylogram of the partial coat protein nucleotide sequences of SLV isolates retrieved from Genbank and SLV (KR074209) of this study. Phylogram was generated using MEGA 6 with 1,000 bootstrap replicates (placed at the nodes on the tree). Corresponding sequence of OYDV (KP862053) was used as an out-group. The scale units (0.05) are substitutions per site.

GarV-D, OYDV, and SLV have been identified as the viruses associated with samples of garlic and onions based on their closest phylogenetic relationships and highest nucleotide identities. Phylogenetic analysis confirms the evolutionary origin of OYDV and SLV from Indian ancestors and GarV-D origin from Spain isolates. Phylogenetic analysis of the Indian isolates provided updating on viral species subgrouping and evolutionary process. Present investigation unravels the virus diversity, subgrouping, evolutionary history within the alliumviruses in India.

CONCLUSIONS

GarV-D, OYDV, and SLV have been identified as the viruses associated with samples of garlic and onions based on their closest phylogenetic relationships and highest nucleotide identities. Phylogenetic analysis confirms the evolutionary origin of OYDV and SLV from Indian ancestors and GarV-D origin from Spain isolates. Phylogenetic analysis of the Indian isolates provided updating on viral species subgrouping and evolutionary process. Present investigation unravels the virus diversity, subgrouping, evolutionary history within the allium viruses in India.

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Conflict of Interest. None.

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