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Global analysis of population structure, spatial and temporal dynamics of genetic diversity, and evolutionary lineages of *Iris yellow spot virus* (*Tospovirus*: *Bunyaviridae*)

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ABSTRACT

Thrips-transmitted *Iris yellow spot virus* is an economically important viral pathogen of *Allium* crops worldwide. A global analysis of known IYSV nucleocapsid gene (N gene) sequences was carried out to determine the comparative population structure, spatial and temporal dynamics with reference to its genetic diversity and evolution. A total of 98 complete N gene sequences (including 8 sequences reported in this study) available in GenBank and reported from 23 countries were characterized by *in-silico* RFLP analysis. Based on RFLP, 94% of the isolates could be grouped into NL or BR types while the rest belonged to neither group. The relative proportion of NL and BR types was 46% and 48%, respectively. A temporal shift in the IYSV genotypes with a greater incremental incidence of $IYSV_{BR}$ was found over $IYSV_{NL}$ before 2005 compared to after 2005. The virus population had at least one evolutionarily significant recombination event, involving $IYSV_{BR}$ and $IYSV_{NL}$. Codon substitution studies did not identify any significant differences among the genotypes of IYSV. However, N gene codons were minimally positively selected, moderately negatively selected denoting the action of purifying selection, thus rejecting the theory of neutral mutation in IYSV population. However, one codon position (139) was found to be positively selected in all the genotypes. Population selection statistics in the $IYSV_{BR}$, $IYSV_{NL}$ genotypes and in the population as a whole also revealed the action of purifying selection or population expansion, whereas $IYSV_{other}$ displayed a decrease in population size. Genetic differentiation studies showed inherent differentiation and infrequent gene flow between $IYSV_{BR}$ and $IYSV_{NL}$ genotypes corroborating the geographical confinement of these genotypes. Taken together the study suggests that the observed diversity in IYSV population and temporal shift in $IYSV_{BR}$ genotype is attributable to genetic recombination, abundance of purifying selection, insignificant positive selection and population expansion. Restricted gene flow between the two major IYSV genotypes further emphasizes the role of genetic drift in modeling the population architecture, evolutionary lineage and epidemiology of IYSV.

1. Introduction

Thrips-transmitted *Iris yellow spot virus* (IYSV) belongs to the genus *Tospovirus* family *Bunyaviridae*, members of which are serious pathogens of a wide range of crops (Gent et al., 2006; Mandal et al., 2012; Mumford et al., 1996; Pappu et al., 2009; Turina et al., 2012). Initially considered as a monotypic genus consisting of *Tomato spotted wilt virus* (TSWV), the *Tospovirus* genus now consists of more than 29 distinct species and new species are being continuously reported from different parts of the world (Anonymous, 2012; Nichol et al., 2005; Pappu et al., 2009). IYSV has become an increasingly important constraint to the production of bulb and seed onion in growing regions around the world (Gent et al., 2004, 2006; Mandal et al., 2012; Pappu et al., 2009; Turina et al., 2012). The virus was first reported in the 1990s (Hall et al., 1993), but beginning in 2000, the virus has spread rapidly and reports of the virus infections in *Allium* and related species have started appearing from many parts of the world (Cosmi et al., 2003; Coutts et al., 2003; Gera et al., 1998; Hafez et al., 2012; Iftikhar et al., 2013; Lobin et al., 2010; Ravi et al., 2006; Sether et al., 2010).

Onion (*Allium cepa* L.) is an important vegetable crop grown all over the world and is one of the important constituents of daily dietary intake. Onion along with garlic is rich in phosphorus, calcium and several antioxidant compounds, polyphenols such as flavonoids and sulfur-containing compounds (Banerjee et al., 2002; Block et al., 1997; Gorinstein et al., 2005; Horie et al., 1992; Ly et al., 2005; Nuutila et al.,

Abbreviations: IYSV, Iris yellow spot virus; N gene, nucleocapsid gene; S RNA, small RNA.

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2003; Prasad et al., 1995; Suh et al., 1999; Yamasaki et al., 1994). It not only adds taste and flavor to the food but also supplies active medicinal compounds as ingredients that helps to ward off cataract and cardiovascular disease due to its hypocholesterolemic, thrombolitic and antioxidant effects (Block, 1985; Block et al., 1997; Nuutila et al., 2003; Vidyavati et al., 2010).

The genome of IYSV is characterized by three RNAs: large (L), medium (M), and small (S) (Adkins, 2000; Bag et al., 2009, 2010; Cortês et al., 1998, 2002; Goldbach and Peters, 1996; Moyer, 1999, 2000; Sherwood et al., 2000). The negative sense L RNA, in virion-complementary sense codes for the RNA dependent RNA polymerase (RdRp) (Bag et al., 2010; de Haan et al., 1991) while M RNA and S RNA have ambisense genome organization (Adkins, 2000; Bag et al., 2009; Cortês et al., 2002; Moyer, 1999; Nichol et al., 2005; Pappu, 2008; Tsompana and Moyer, 2008). M RNA in the viral sense codes for the non-structural movement protein (NSm) and in the viral complementary sense codes for the glycoprotein precursor (Bag et al., 2009; Cortês et al., 2002) and S RNA codes for one non-structural protein (NSs) and the nucleoprotein (N) (Cortês et al., 1998). The genomic RNAs are tightly bound by the nucleocapsid protein and encapsulated in a lipid envelope (Moyer, 1999, 2000; Sherwood et al., 2000). The complete genome of several tospoviruses have been sequenced for genetic diversity studies but the molecular characteristics of N gene was utilized in studying their genetic relationships (de Avila et al., 1993; Krauthausen et al., 2012; Nischwitz et al., 2007; Pappu et al., 2006).

Among the many tospoviruses known to date, IYSV continues to be an emerging and reemerging pathogen of *Allium*, causing economic losses to the tune of $60–$90 million annually in onion seed and bulb crops (Gent et al., 2006; Mandal et al., 2012; Pappu et al., 2009; Pozzer et al., 1999; Turina et al., 2012). IYSV presents an interesting case of epidemiological intrigue. In the US, while the virus was reported in onion as early as in 1990s, it remained inconsequential with respect to economic damage. However, since 2000, the virus was reported from several states in the US and started to cause significant economic losses. On a global scale, similarly the virus was reported from several countries in Africa, Asia, Australia, and Europe (Ben Moussa et al., 2005; Córdoba-Sellés et al., 2005; Coutts et al., 2003; Gera et al., 1998). With increased incidence and economic impact, research on IYSV was intensified and as a result characterization of IYSV isolates was carried out with subsequent availability of several N gene sequences in GenBank. As a part of our on-going global project to characterize IYSV population at the molecular level, we characterized IYSV at the molecular level in Alliums collected from several countries (Huchette et al., 2008; Iftikhar et al., 2013; Lobin et al., 2010; Pappu and Rauf, 2013; Sether et al., 2010; Ward et al., 2008). Our most recent survey for IYSV included onion bulb and seed crops in several onion-growing provinces of Pakistan and the USA (Iftikhar et al., 2013). With nearly 100 accessions of complete N gene sequences available in GenBank from more than 23 countries, IYSV N gene sequences now represent a large enough and diverse sample for detailed genetic diversity studies on a global scale to better understand genetic drift, population structure and evolutionary lineages of this important emerging viral pathogen.

2. Materials and methods

2.1. Sample collection

Onion plants found with characteristic symptoms associated with IYSV infection such as spindle-shaped straw-colored irregular chlorotic lesions, necrotic to hay-colored spots were collected from thirteen districts of southern and northern Punjab in Pakistan (Chiniot, Faisalabad, Gujranwala, Hafizabad, Jaranwala, Jhang, Jhelum, Layyah, MandiBahauddin, Muzafargarh, Nankana sahib, Sargodha, Sheikhupura) during February 2012 to March 2013. The geographical co-ordinates of the locations where samples were collected are latitude 30.28–31° to 71–73° longitude.

During summer 2011, onion seed and bulb crops showing characteristic symptoms including chlorotic lesions, spindle and long yellow stripes caused by IYSV were collected from the commercial fields in the states of Colorado, Idaho, New Mexico, New York and Washington, USA. The leaf samples were preserved in −80°C until further analysis.

2.2. Enzyme-linked immunosorbent assay (ELISA)

Samples were tested for IYSV using a commercially available ELISA kit (Agdia Inc., Elkhart, USA and LOEWE, Sauerlach, Germany). Samples were considered positive for IYSV if the absorbance values were two times greater than the values of healthy samples.

2.3. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA from the symptomatic, ELISA-positive leaf samples was extracted using the RNeasy Plant Mini kit (Qiagen, Maryland, USA) following the manufacturer's instructions. First strand complementary DNA (cDNA) synthesis was done using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, USA) and IYSV N gene was amplified using forward primer 5′-CTCTTAAACACATTTAACAAGCAC-3′ and reverse primer 5′-TAAAACAAACATTCA-AACAA-3′ flanking the nucleocapsid (N) gene encoded by the small RNA of IYSV. Amplified IYSV N gene fragments were cloned in pGEM-T easy vector (Promega, Madison, USA) and sequenced at ELIM Biopharma (Hayward, USA). At least two clones for each isolate were sequenced. Sequences of IYSV N gene obtained from the samples derived from Pakistan and the USA were annotated and compared to available IYSV N gene sequences. Complete N gene sequences of various IYSV isolates reported across the globe were retrieved from the GenBank for comparative analysis.

2.4. Sequence annotation and analysis

Sequence alignment and phylogenetic trees were generated in MEGA 6 using ClustalW algorithm (Tamura et al., 2013). The phylogenetic tree was constructed using the neighbor joining method (default parameters with 2000 replicates in the bootstrap analysis). To study nucleotide diversity and DNA polymorphism, DnaSP (Librado and Rozas, 2009) was used. The analysis included quantifying the levels of DNA polymorphism such as the number of haplotypes and haplotype diversity in order to analyze the distribution pattern of DNA variation, or to compare alternative evolutionary scenarios.

2.5. In silico RFLP analysis of the nucleoprotein gene

Complete N gene sequences (ORFs) available in GenBank, NCBI, were analyzed for *in silico* RFLP pattern. The RFLP simulation of N gene was carried out using Restriction Mapper Version3 (http://www.restrictionmapper.org/) to perform virtual digest of the gene and to map the sites recognized by restriction enzyme *Hin*f1 (Zen et al., 2005). IYSV isolates could be grouped into IYSV Netherlands ($IYSV_{NL}$) or IYSV Brazil ($IYSV_{BR}$) types (Pozzer et al., 1999) based on *Hin*f1 digestion. The largest size fragment resulting from the digestion is considered for differentiating the genotype of a given isolate into two groups. Those that did not conform to either genotype were considered as "$IYSV_{other}$".

2.6. Temporal analysis of IYSV genotype distribution

IYSV genotypes were analyzed for the temporal shift in two time periods that were arbitrarily chosen—those reported before 2005 and after 2005. The year 2005 bifurcates the periods of study (1997–2013) in to two equal halves (1997–2005 and 2006–2013). For the temporal analysis of IYSV genotypes, date of collection of sample was considered wherever available; otherwise date of submission to GenBank was taken for the analysis of temporal study of population based on *in silico* RFLP.