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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.

2.7. Recombination detection analysis

Potential recombination events were detected by Recombination Detection Program-4 (RDP 4 Beta 4.16) (Martin et al., 2010). All the complete N-gene sequences available in GenBank were used in the analysis and the sequence alignment was carried out using ClustalW in MEGA 6 (Tamura et al., 2013) and the aligned sequences were used for recombination detection studies. For identifying recombination events, step-down correction with the highest acceptable p-value setting of 0.05 was used along with other default settings for all of nine methods (RDP, Chimaera, BootScan, 3Seq, GENECONV, MaxChi, SiScan and LARD, PhylPro) available in the RDP 4 (Martin et al., 2010).

2.8. Population selection studies and neutrality tests

Codon-based maximum likelihood methods including SLAC (single like ancestor counting), FEL (fixed effects likelihood), and REL (random effects likelihood) were used to calculate the mean rates of non-synonymous (dN) and synonymous substitutions (dS). The dN/dS ratio in every codon in the alignment was calculated in order to estimate the selection pressure on the N gene belonging to various genotypes like $IYSV_{BR}$, $IYSV_{NL}$ and $IYSV_{other}$ using DATAMONKEY server (http://www.datamonkey.org). To test the theory of neutral evolution test statistics like Tajimas's D (Tajima, 1989), Fu & Li's D and Fu & Li's F (Fu, 1997; Fu and Li, 1993) were determined employing DnaSP software.

2.9. Genetic differentiation and gene flow estimates

In order to estimate genetic differentiation within the populations of IYSV genotypes, nucleotide test statistics such as Ks, Kst (Kst value close to zero indicates no differentiation) and Snn (Snn value close to one indicates differentiation) (Ks, Kst and Snn: Hudson's statistic of genetic differentiation) (Hudson, 2000) and haplotype statistics such as Hs and Hst (Hudson et al., 1992a, 1992b) were computed using DnaSP. The software was further used to study the extent of gene flow between the IYSV populations by estimating statistic Fst (absolute value close to zero indicates free gene flow, whereas value close to one indicates infrequent gene flow) (Hudson et al., 1992b).

3. Results

3.1. Symptomatology

Symptoms in commercial onion fields surveyed included spindle-shaped straw-colored irregular chlorotic lesions, necrotic to hay-colored spots, long yellow stripes. Symptoms were found on both onion seed and bulb crops. Symptomatic plants were predominantly noticed in Faisalabad, Gujranwala, Nankana, and Sheikhupura districts in Pakistan. IYSV infection was confirmed by ELISA. Out of 13 districts, samples from 5 districts, Faisalabad, Gujranwala, Nankana, Sargodha and Sheikhupura were found positive. Viral genome from samples from two of these districts (Faisalabad and Nankana) were cloned and sequenced. Virus isolates were transferred to and maintained in indicator hosts, *Datura stramonium* and *Nicotiana benthamaiana* by mechanical inoculation.

3.2. Molecular characterization

The IYSV N gene was cloned and sequenced from three isolates from the Punjab province of Pakistan and 5 isolates collected from the USA in 2012. The N gene of these isolates was 822 nt long and potentially coded for a 273-amino acid protein. Sequences of N gene reported in this study were submitted to GenBank (KF171103, KF171104, KF171105 from Pakistan, and JQ973065, KF263484, KF263485, KF263486 and KF263487 from the USA).

3.3. Restriction fragment length polymorphism

The *in silico* RFLP of N gene was carried out to determine the relative distribution of the two previously described IYSV genotypes, their distribution pattern across the geographic regions, hosts and over a 20 year time period. The restriction enzyme *Hinf*1 was found to delineate IYSV N gene sequences into two genotypes: Netherlands ($IYSV_{NL}$) and Brazil ($IYSV_{BR}$) based on the RFLP pattern. The *Hinf*1 restriction recognition sites on the ORFs of various isolates revealed 6 different types of restriction pattern. The frequency of the restriction site in the known N gene sequences varied from four to nine. Two thirds of the sequences had five to seven HinfI site (67 accessions out of 98). Though frequency of *Hinf*I recognition site in N gene varied with isolates, the largest size fragment among the resultant fragments in the restriction digestion is an indicator of the genotype. The largest fragment of size 486 bp and 308 bp differentiates the genotypes into $IYSV_{NL}$ and $IYSV_{BR}$ respectively. The *Hinf*1 restriction pattern of N gene divided the 98 accessions almost equally (46% as NL and 48% as BR) and the remaining 6% of the accessions could not be placed in either category and were considered $IYSV_{other}$ (Fig. 1).

The geographical distribution of IYSV genotypes was assessed and the Asian isolates were predominantly of $IYSV_{BR}$ (72%) genotype, while 21% of the accessions belonged to $IYSV_{NL}$. Interestingly, isolates reported from North America were predominantly of the $IYSV_{NL}$ type (Fig. 2). Interestingly, IYSV genotypes generally were confirmed in their incidence to a particular geographic region for example $IYSV_{BR}$ genotypes were reported only from Asia but not from Europe. Similarly the isolates that did not belong to either genotype ($IYSV_{other}$) were reported only from Asia and Europe (Fig. 2).

Among the hosts from which the various isolates were reported, onion (*A. cepa*) was the most commonly reported host of IYSV, while other crops included *Allium tuberosum*, *Allium sativum*, *Allium ampeloprasum*, and *Eustoma russellianum* (Fig. 3). Among the isolates characterized from infected onions, the relative incidence of NL and BR types was about equal: 45% $IYSV_{BR}$ and 47% $IYSV_{NL}$.

3.4. Sequence diversity, DNA polymorphism and phylogeny of the N gene

The N gene sequences from this study and all available complete N gene sequences were used for determining the genetic diversity, polymorphism and phylogenetic analysis. Nucleotide diversity (π) of $IYSV_{BR}$ was slightly higher than that for $IYSV_{NL}$ (0.04194 and 0.03133, respectively). However it was notably higher in $IYSV_{other}$ N gene sequences (0.08297) (Table 1) suggesting that $IYSV_{other}$ is more diverse than the $IYSV_{NL}$ and $IYSV_{BR}$ as number of polymorphic sites (S) of $IYSV_{other}$ genotype are 136 in 6 isolates (Table 1).

The phylogenetic tree based on the N gene sequences showed clustering of IYSV genotypes into two distinct nodes one each representing $IYSV_{BR}$ and $IYSV_{NL}$ (Fig. 4) with a few exceptions. The type isolate of

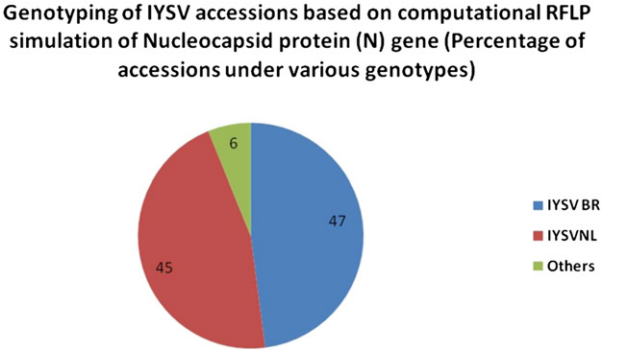


Fig. 1. Genotyping of *Iris yellow spot virus* (IYSV) accessions based on *in silico* RFLP simulation of nucleocapsid (N) gene (percentage of accessions under various genotypes).

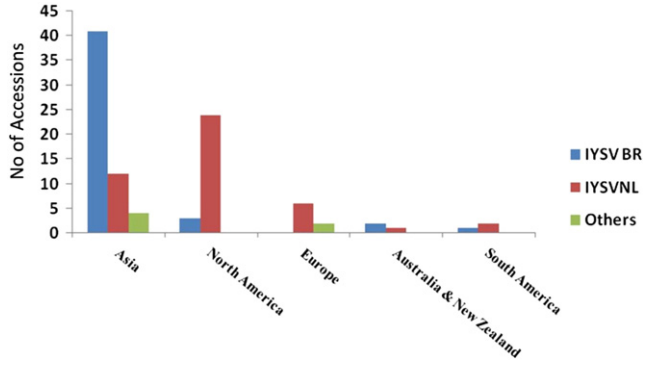


Fig. 2. Geographical distribution of various *Iris yellow spot virus* genotypes.

$IYSV_{BR}$ (AF067070) is found in the $IYSV_{NL}$ node. With the exception of a few NL genotypes (AF271219, AM900393 and AF001387), all $IYSV_{BR}$ genotypes grouped together. Four among the six isolates that belonged to $IYSV_{other}$ also grouped with $IYSV_{BR}$ (Fig. 4).

The nucleotide sequence identity studies revealed that the three isolates from Pakistan reported here (KF171104, KF171103, KF171105) had 99% sequence identity with an isolate from Chile (DQ150107), whereas two of the Pakistan isolates (KF171104, KF171103) exhibited 99% identity with USA isolates (KF263486, JQ973065) while one isolate (KF171105) showed 98.7% sequence identity with both the USA isolates. The isolates from Pakistan (KF171104 and KF171103) also showed 98.9% sequence identity with another isolate from USA (KF263487). The isolates reported from Washington State, USA (KF263486) showed 100% identity with an IYSV isolate of Japan (AB180921). Other IYSV isolates from different states of USA including JQ973065, KF263487, KF263485, and KF263484 showed 99.7%, 99.8%, 99.3% and 99.6% sequence identity with a Japanese isolate (AB180921), respectively.

3.5. Temporal shift in IYSV genotype

Analysis of available sequences was carried out to determine if there was a potential temporal shift in the IYSV genotypes. For this study, two time periods, before 2005 and after 2005 were arbitrarily selected. It was found that prior to 2005, the relative proportion of $IYSV_{NL}$ was higher compared to $IYSV_{BR}$. However, after 2006 the reversal was seen with a greater percentage of $IYSV_{BR}$ (Fig. 5a). Prior to 2005, no $IYSV_{other}$ genotype was observed. Interestingly, however, more of the 'other' genotypes were reported post-2005. One noteworthy observation from the temporal studies was the three-fold increase in $IYSV_{BR}$ between the two periods (before and after 2005), whereas for the same period, $IYSV_{NL}$ genotypes had a two-fold increase (Fig. 5b).

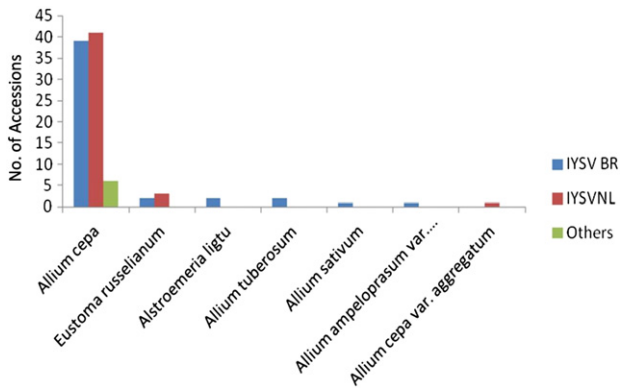


Fig. 3. Host distribution of various *Iris yellow spot virus* genotypes.

Table 1
Genetic diversity of the nucleocapsid gene in various *Iris yellow spot virus* genotypes and the population as a whole.

Genotype	N	*S*	π	Hd
$IYSV_{NL}$	44	117	0.03133	0.996
$IYSV_{BR}$	47	232	0.04194	0.998
$IYSV_{other}$	6	136	0.08297	1.000
$IYSV_{All}$	97	319	0.07712	0.999

N, number of isolates; *S*, number of polymorphic (segregating) sites; Hd, haplotype diversity; π, nucleotide diversity within species. IYSV Netherlands ($IYSV_{NL}$); IYSV Brazil ($IYSV_{BR}$); IYSV that belong to neither genotype ($IYSV_{other}$); entire IYSV population ($IYSV_{all}$).

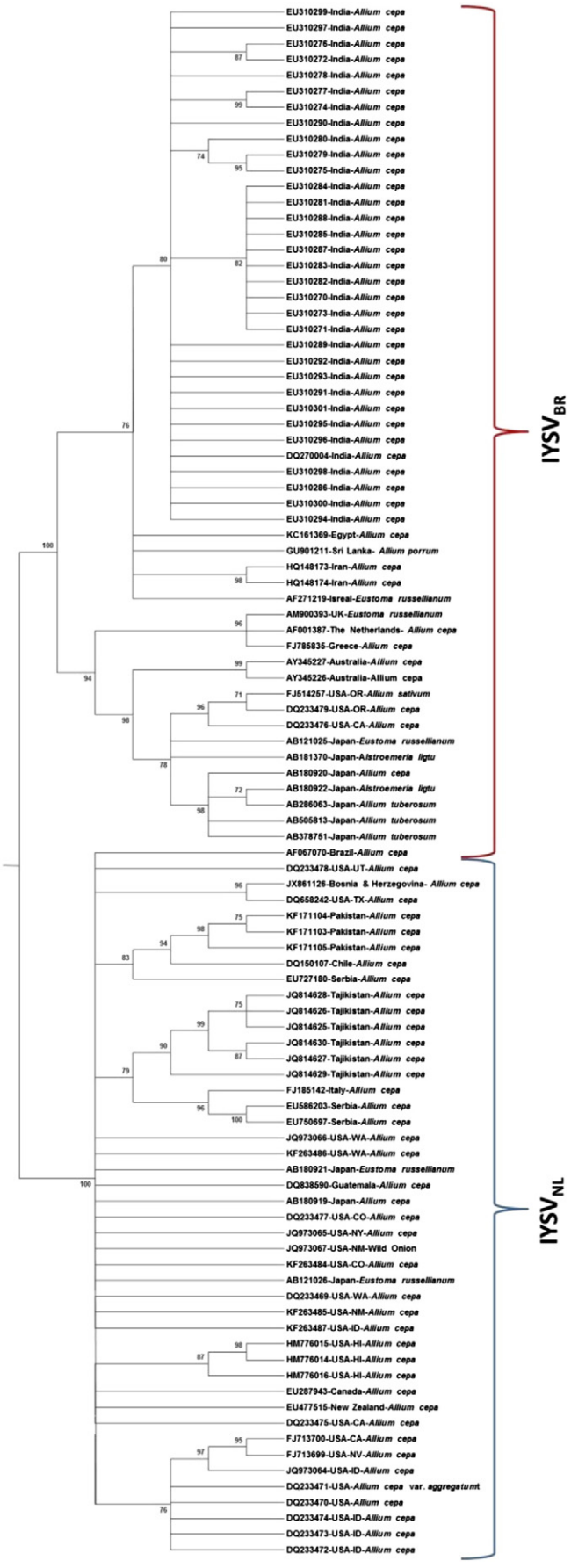
3.6. Recombination detection

In order to examine any potential recombination events among various isolates of IYSV N gene, Recombination Detection Programme 4 (RDP 4) was used. It revealed one potential recombination event despite the relatively small length of nucleotide sequence under study. The recombination event was detected by SiScan and 3Seq algorithms. The IYSV isolate from *Allium* in Brazil, the type isolate for $IYSV_{BR}$ genotype, appears to be the consequence of recombination from this event. As recombination involves two parents, RDP identified the IYSV isolate from the USA, (DQ233478) as a minor parent in this putative recombination event. Interestingly, the parent isolate is an $IYSV_{NL}$ genotype hence the $IYSV_{BR}$ genotype could have been evolved potentially from the NL genotype and thus the event could be evolutionarily noteworthy. The other major parent involved in this putative recombination event turned out to be the IYSV isolate infecting onion from Australia (AY345227). RDP predicted two recombination break points for the event. The probable start break-point for the recombination is between nucleotides 1–44 and the end break-point is recognized at nt position 759 (Fig. 6).

3.7. Population selection and test of neutrality

Population selection studies provide a list of gene codons in an alignment that are under positive or negative selection pressure and thus could shed light on the molecular evolution patterns in the N gene. The mean dN/dS (dN—rate of non synonymous substitutions and dS—rate of synonymous substitutions) for N gene accessions belonging to the BR genotype were found to be 0.198 and did not have a single positively selected codon site. However, 25 negatively selected codon sites were identified using SLAC methodology (Table 2). The same data set when analyzed by FEL, revealed one positively selected codon site (codon no. 139:AGC/ACC) against 66 negatively selected sites. The mean difference between dN and dS (dN–dS) for the N gene sequences belonging to the BR genotypes based on REL analysis was found to be −0.817 suggesting that all the codon sites are under purifying selection acting against deleterious non-synonymous substitutions (Table 2).

Similarly, the mean dN/dS of N-gene sequences of NL genotype was found to be 0.197 and the data set did not reveal any positively selected codons. However, 15 negatively selected codon sites were identified from SLAC analysis. The same data set when analyzed by FEL revealed a positively selected codon site (GAC) at the same place where BR genotypes also exhibited positive selection, along with 59 negatively selected codon sites. The dN–dS for the N gene sequences belonging to NL genotypes based on REL analysis were found to be −0.797. REL analysis also did not identify any positively selected codon sites as against 105 negatively selected sites. The mean dN/dS for sequence accessions belonging to neither BR nor NL genotypes was found to be 0.171 with 3 negatively selected codon sites and not a single positively selected site have been identified by SLAC. Similarly, FEL analysis revealed 39 negatively selected codon sites. The dN–dS was revealed to be −0.810, with one positively selected site (codon 270). Taken together, the results revealed that the codons were generally negatively selected except codon sites (codon no. 139) both in $IYSV_{BR}$ (AGC/ACC) and $IYSV_{NL}$ (GAC)



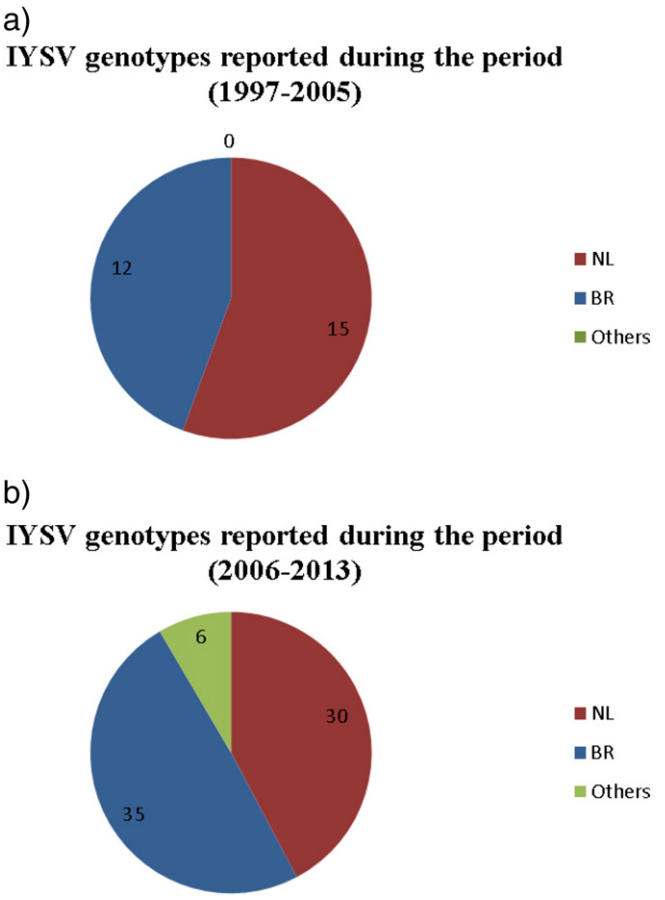


Fig. 5. Temporal shift in genotypes of *Iris yellow spot virus* (IYSV). a) IYSV genotypes reported during the period (1997–2005). b) IYSV genotypes reported during the period (2006–2013).

and codon 270 (GAC) in $IYSV_{other}$ which was found to be positively selected in both virus genotypes. Thus, positive selection of codons (at codon positions 139 and 270) indicates that the replacement substitutions increase the fitness of the N protein codon 139 (AGC/ACC/GAC) and 270 (GAC) in the IYSV population. Negative selection functioning at other sites tends to remove such substitutions from the N gene. Further, the number of negatively selected codon sites in BR is higher than in NL genotypes based on SLAC analysis, suggesting the dominant influence of purifying selection in $IYSV_{BR}$ genotype compared with $IYSV_{NL}$.

The population statistic parameters, however, revealed no significant differences between the two genotypes and the overall population. The statistically significant and insignificant negative values of Tajima's D in $IYSV_{BR}$ and $IYSV_{NL}$, respectively, suggest the dominance of purifying selection and population expansion operating in those genotypes (Table 3). The test statistic Fu and Li's D and F also revealed the same characteristic feature for the $IYSV_{BR}$ and $IYSV_{NL}$ genotypes underlining the principle of operation of purifying selection and population size expansion. The statistically significant negative value of Fu's F further strongly denotes the expansion observed in $IYSV_{BR}$ population. $IYSV_{other}$ genotype revealed positive values for both statistical parameters Tajimas's D and Fu &Li's D suggesting a decrease in population size and balancing selection.

Fig. 4. Phylogenetic tree of nucleotide sequences of the nucleocapsid gene of *Iris yellow spot virus* isolates available in GenBank. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Each IYSV isolate is indicated by its GenBank accession number, place of origin and host.

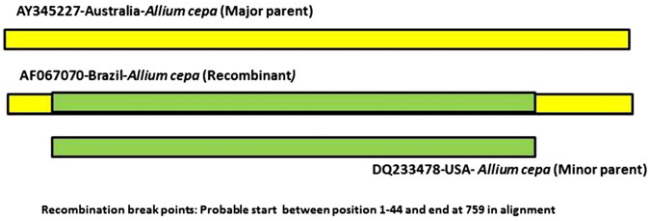


Fig. 6. Recombination events within N gene of various accessions as detected by RDP v4. Recombinant AF067070-Brazil-*Allium cepa* type isolate of $IYSV_{BR}$ genotype is a product of recombination involving DQ233478-USA-*Allium cepa* as the minor parent. Though the start break-point could not be identified with certainty, RDP detected nucleotide positions 1–44 as a probable break-point with accession AY345227-Australia-*Allium cepa* as a possible major parent.

3.8. Genetic differentiation

The inherent genetic differentiation between the IYSV genotypes was evaluated by estimating both haplotype-based statistic (Hs and Hst) and nucleotide-based statistic (Ks, Kst, Snn) (Table 4). The statistically significant test values for Ks, Kst and Z obtained when IYSV populations were compared among themselves revealed the existence of strong genetic differentiation. Despite the insignificant test statistical value (Snn) obtained in comparison studies, its value close to one denotes the genetic differentiation between the IYSV genotypes. The parameter values for Kst in the comparisons also revealed that $IYSV_{BR}$ is relatively least differentiated from $IYSV_{other}$ (Kst value of 0.01838*) compared with $IYSV_{NL}$ genotypes (Kst value of 0.06260*). The computed Fst value of 0.68 between $IYSV_{BR}$ and $IYSV_{NL}$ population indicates restricted gene flow between the populations thus sharing of genetic information between these two populations is infrequent. While $IYSV_{NL}$ exhibited a moderate gene flow with $IYSV_{other}$, $IYSV_{BR}$ exhibited relatively unrestricted gene flow with $IYSV_{other}$.

4. Discussion

IYSV is a major constraint to onion production in many onion-growing regions of the world. Economic loss due to IYSV infection varies with climate, production practices, vector thrip populations, onion cultivars and virus strains (Fournier et al., 1995). Knowledge on the population diversity, spatial and temporal dynamics of IYSV population could be useful in designing appropriate disease control measures. A previous study showed that IYSV isolates from some western US states grouped distinctly from those from other parts of world including Australia, Brazil, Japan, and the Netherlands (Pappu et al., 2006). Similarly, phylogenetic analysis of N gene from IYSV isolates prevalent in Georgia, the USA and Peru revealed that they are related to one another and divergent from those reported from the western states of the US. This study suggested that the gene flow occurred from Peru into Georgia, the USA as the former region is known to have IYSV infection even before its detection in Georgia (Nischwitz et al., 2007).

Computational RFLP simulation studies revealed the categorization of IYSV genotypes into two groups, $IYSV_{BR}$ and $IYSV_{NL}$. Interestingly, all the known N gene accessions were grouped about equally into the two genotype groups. However, few accessions fell into neither category. Interestingly, the $IYSV_{other}$ genotype was reported only from Asia and Europe. The plausible role for recombination in the evolution of $IYSV_{other}$ genotypes could not be disregarded as it tends to rearrange the genomic regions leading to genetic diversity. Considering the relatively short stretch of sequence (822 nt encoding 273 amino acids) that was used for identifying potential recombination events, it was surprising to see that even one such event might have taken place as RDP showed one possible recombination event. The recombination event involving $IYSV_{NL}$ and $IYSV_{BR}$ is evolutionarily significant as it could have been the basis for the evolution of the BR from the NL genotype.

Among the codons of N gene analyzed as a whole population, 19% of the codon positions were identified to be negatively selected by SLAC methodology (Table 2), whereas 33% of the codon positions were detected to be negatively selected in FEL methodology. Further, the test statistic value dN–dS is negative throughout indicating the abundance of synonymous codon substitutions and in turn negative selection. This moderate level of negative selection of codons operating in N gene population denotes the action of purifying selection within the IYSV population. In addition, selection analysis involving all the genotypes and population as a whole revealed that the statistic value dN/dS was found to be less than one (ranging from 0.171 to 0.214) (Table 2). It indicates that while some non-synonymous substitutions could be deleterious, others are neutral thereby strengthening the action of purifying selection. Purifying selection does not mean absence of positive selection, however the latter was not enough to overcome the effects of the former. The population selection study identified a few positively selected codons in the N gene population as a whole or

Table 3
Summary of neutrality tests in *Iris yellow spot virus* (IYSV) population.

Genotypes	Tajimas's D	Fu & Li's D	Fu & Li's F
$IYSV_{BR}$	−1.47873	−3.60071**	−3.35619**
$IYSV_{NL}$	−1.58197	−0.81168	−1.30718
$IYSV_{other}$	0.57659	0.87044	−0.88804

** - statistically significant at $P < 0.01$.

Table 2
Summary of codon substitution studies in nucleocapsid gene of *Iris yellow spot virus* (IYSV) genotypes.

Genotype	Positively selected codon positions	Amino acid substitutions	No. of negatively selected codons	ω = dN / dS	dN–dS
$IYSV_{NL}$	139[b]	Asp-Asn	15[a]	0.197	−0.797
		Asp-ser	59[b]		
		Asp-Val	105[c]		
$IYSV_{BR}$	139[b]	Ser-Thr	25[a]	0.198	−0.817
		Ser-Asp	66[b]		
$IYSV_{other}$	270[c]	–	3[a]	0.171	−0.810
			39[b]		
$IYSV_{All}$	109[b]	Leu-Ile	52[a]	0.214	–
		Ile-Phe	90[b]		
	139[a,b]	Asp-Asn			
		Asp-ser			
		Asp-Val			
		Ser-Thr			
		Ser-Asp			

dN, the number of non-synonymous substitutions per non-synonymous site; dS, the number of synonymous substitutions per synonymous site.
ω — Ratio of dN/dS from SLAC (single like ancestor counting) methodology, dN–dS obtained from REL (random effects likelihood).
[a] Codons identified by SLAC at a cut off p-value 0.1.
[b] Codons identified by FEL at a cut off p-value 0.1.
[c] Codons identified by REL at a cut off Bayes factor value 50.

Table 4
Genetic differentiation and gene flow of the nucleocapsid gene between different *Iris yellow spot virus* genotypes.

Genotypes	H_s	H_{st}	χ^2	P value	K_t	K_s	K_{st}	S_{nn}	Z	F_{st}
$IYSV_{BR}$ vs $IYSV_{NL}$	0.99700	0.00154	91.000	0.3083	62.97485	3.12600*	0.18548*	0.98901	6.65663*	0.68268
$IYSV_{BR}$ vs $IYSV_{other}$	0.99830	0.00025	53.000	0.3592	39.69303	3.39689*	0.01838*	0.92453	6.15872*	0.11744
$IYSV_{NL}$ vs $IYSV_{other}$	0.99614	0.00060	50.000	0.3175	36.92490	2.98441*	0.06260*	0.98000	5.96030*	0.37528

H_s, H_{st}: Haplotype based statistic to estimate genetic differentiation.
K_s, K_{st}, S_{nn}, Z: Nucleotide based test statistic to estimate the genetic differentiation (Kst value close to zero indicates no differentiation; Snn value close to one indicates differentiation).
F_{st}: Statistic estimates the extent of gene flow between various genotypes (value close to zero indicates free gene flow or panmixis value close to one indicates that genotypic groups are closed to gene flow).
* - statistically significant at $P < 0.05$.

within the genotypes [codon position 139 (AGC/ACC/GAC) in all genotypes and overall population, 270 (GAC) in $IYSV_{other}$ and 102 (ATT/CTT) in overall population] (Table 2). Among the positively selected codon position, 139 is biochemically significant as amino acid substitutions reveal a change from negatively charged amino acid (Asp) to uncharged R groups (Asn, Ser) and to aliphatic R group (Val).

The role of positive selection in amino acid codons has been ascribed to the ability of *Tomato spotted wilt virus* (TSWV) to break the host resistance against gene *Sw-5* in tomato (Sundararaj et al., 2014). On similar lines, the role of negatively selected amino acid codons in functional properties and their importance to virus survival have been discussed in case of *Tomato mosaic virus* (Rangel et al., 2011) and to a lesser extent in *Fig mosaic virus* (FMV) (Walia et al., 2014). However, most of the N gene amino acid codons are free from any selection hence they evolve neutrally. This neutral mode of evolution observed in IYSV population has also been previously observed with the populations of *Cucumber mosaic virus* (Davino et al., 2012). Findings from our genetic differentiation studies are in accordance with the phylogenetic analysis of N gene sequences of various IYSV isolates, wherein $IYSV_{BR}$ and $IYSV_{NL}$ genotypes formed two distinct groups. The genetic differentiation and the absence of gene flow between $IYSV_{BR}$ and $IYSV_{NL}$ are also further corroborated from the geographical confinement of these genotypes as North America had predominantly the NL genotype, whereas Asia had the BR genotype. The relatively frequent gene flow between $IYSV_{BR}$ and $IYSV_{other}$ when compared with $IYSV_{NL}$ further explains the presence of $IYSV_{other}$ in Asia and Europe only. In summary, a global analysis of IYSV N gene sequences revealed important characteristics of the virus population structure, spatial and temporal distribution patterns and provided insights into the evolution of the virus.

5. Conclusions

Global analysis of IYSV N gene sequences revealed that a vast majority of the isolates could be grouped almost equally into two distinguishable genotypes ($IYSV_{BR}$ and $IYSV_{NL}$). Temporal shift in the IYSV genotypes showed a greater incremental incidence of $IYSV_{BR}$ genotype when compared with $IYSV_{NL}$ on a time scale prior to and after the year 2005. Furthermore, recombination detection analysis found an evolutionarily significant putative recombination event involving $IYSV_{BR}$ and $IYSV_{NL}$ suggesting evolution of the BR genotypes from NL. Analysis of codon selection and population genetic parameters revealed the action of purifying selection and population expansion in IYSV population. A restricted gene flow was observed between the genotypes which could be due to the geographical confinement of these genotypes. The observed genetic diversity in IYSV population could be attributed to the combined action of genetic recombination, purifying selection and genetic drift. The results of the study thus provide important insights into to the population architecture, evolutionary lineage and epidemiology of IYSV.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.06.036.

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