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Phylogenetic Analysis of *Iris yellow spot virus* Isolates from Onion (*Allium cepa*) in Georgia (USA) and Peru

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Abstract

Iris yellow spot virus (IYSV) was first observed in sweet onions in Georgia (USA) in 2003 in the Vidalia region. The virus had been reported in the onion-growing regions in western USA several years before being detected in Georgia in the east. Although symptoms were observed on onions in Peru several years earlier, the presence of IYSV was not confirmed in Peru until after the virus was detected in Georgia. We characterized nine isolates of IYSV recovered from sweet onions in both Georgia (four isolates) and Peru (five isolates) by sequencing the nucleocapsid (*N*) gene and compared those sequences with sequences available in GenBank. Sequence divergence between IYSV isolates from Georgia and Peru was low with 1.1%, and comparisons with IYSV isolates from other regions showed divergence of up to 11.4%. Bootstrap analysis indicated with a high degree of confidence that the Georgia and Peruvian isolates fell into the same clade and were different from known isolates from western USA that fell into sister clades. The high degree of similarity between Georgia and Peruvian isolates suggests that gene flow occurred from Peru into Georgia.

Introduction

Iris yellow spot virus (IYSV) is a *Tospovirus* in the family *Bunyaviridae* (Moyer, 1999). It was first observed in the USA in Idaho in 1989 (Hall et al., 1993) and then in the Netherlands in 1992, where the virus was described and characterized (Cortês et al., 1998). In recent years, IYSV was reported from Australia (Coutts et al., 2003), Brazil (Pozzer et al., 1999), Chile (Rosales et al., 2005), Guatemala (Nischwitz et al., this study), India (Ravi et al., 2006), Israel (Gera et al., 1998), Japan (Doi et al., 2003), La Reunion Island (France) (Robène-Soustrade et al., 2006), Peru (Mullis et al., 2006), Spain (Córdoba-Sellés et al., 2005) and parts of western USA (Schwartz et al., 2002; Creamer et al., 2004; Du Toit et al., 2004; Crowe and Pappu, 2005; Pappu et al., 2006a). Among the states in eastern USA, the most recent report of IYSV came from New York (Hoepting et al., 2007). The biology, epidemiology and impact of IYSV were recently reviewed by Gent et al. (2006).

In the Vidalia region of Georgia, onions are a high-value specialty crop that generate a farmgate value of approximately $90 million annually. The varieties grown in Georgia are a Granex-type sweet onion that is cultivated as a short-day winter crop, whereas in the western states, long-day varieties are grown during the summer. To make efficient utilization of equipment and labour, onion growers in Georgia either import onions and repack them for market during the off season or are involved directly in the production of onions in South America.

In Georgia, IYSV was observed for the first time in 2003 which coincided with the initial identification of IYSV as the causal agent of an epidemic on onions (*Allium cepa*) in Peru where the symptoms had been observed for 2–3 years (D. Burrell, personal observation). In Georgia and Peru, the virus causes irregularly shaped, grey-to-bleached white, necrotic lesions and tip dieback on onion leaves. The foliar blight leads to reduced photosynthesis with subsequent reduction of yield as well as the creation of sites for ingress by opportunistic pathogens and secondary colonizers. Significant yield reductions for colossal and jumbo-sized onions were observed in Colorado (Gent et al., 2004), where the IYSV symptoms reported appear to be similar to those observed in Georgia with one exception, namely the presence of diamond-shaped lesions. The primary economic impact of IYSV is reduced yield. Large amounts of photosynthates are transported into the bulbs from leaves (Khan and Asif, 1981). Leaf area is lost due to necrosis after infection with IYSV

resulting in fewer photosynthates that can be translocated. This can lead ultimately to smaller bulb sizes.

Unlike other geographic regions affected, IYSV in Georgia is more of a problem in seedbeds and in the early part of the growing season before the onset of cold weather (C. Nischwitz, R. Gitaitis, S. W. Mullis, personal observation). The greatest apparent economic impact in Georgia comes from increased stand losses after transplanting. Since 2003, there has been an increase in early-season stand losses which coincides with the arrival of IYSV. Although stand losses following transplantation have occurred prior to the arrival of IYSV, they have usually been associated with freezing temperatures. Since 2003 stand losses are not associated with freezing temperatures or any pathogen other than IYSV. Furthermore, surviving plants in fields so affected always had a significant number of plants testing positive for IYSV (C. Nischwitz and R. Gitaitis, personal observation).

The close timeline of the first occurrences of IYSV in Georgia and Peru and the similarity of symptoms in both Georgia and Peruvian onions have led us to the hypothesis that IYSV gene flow could have occurred between Peru and Georgia. The objective of this study was to determine the phylogenetic relationship of Georgian IYSV isolates to isolates from Peru and other parts of the USA and the world to determine the possible origin of IYSV in Georgia.

Materials and Methods

Virus detection

Symptomatic onion leaves were collected throughout the Vidalia region from seedling stage until harvest in October 2005 until April 2006. Sap was extracted from approximately 1 g of fresh leaf tissue using a sap extractor and stored in 1 ml of extraction buffer [for 1 l of buffer: 1 packet of 20x phosphate buffered saline with Tween (PBST) concentrate (Agdia Inc., Elkhart, IN, USA), 1.3 g sodium sulphite (anhydrous), 20 g polyvinylpyrrolidone molecular weight 24 000–40 000 Da, 2 g chicken egg albumin, 20 g Tween-20, 1 l double-distilled water; pH 7.4]. The extracted leaf tissue consisted of symptomatic and surrounding asymptomatic tissue. The extract was analysed using double-antibody enzyme-linked immunosorbent assay (DAS-ELISA) for IYSV following the manufacturer's protocol (Agdia Inc.). Onion leaf samples were collected in three regions (Casma, Supe and Ica) in Peru in 2005 and analysed in Peru as mentioned above. ELISA-positive samples from Georgia and Peru were spotted onto Flinders Technology Associates (FTA) cards (Whatman, Brentford, UK) to release the RNA from plant cells and to bind it to the FTA matrix. Plant tissue was macerated in PBS buffer and 25 μl of the extract was spotted onto the card. The RNA was eluted from the FTA cards following the manufacturer's protocol for reverse transcription-polymerase chain reaction (RT-PCR) directly from card punches using primers specific to the nucleocapsid gene of IYSV (Du Toit et al., 2004). Symptomatic onion tissue from Guatemala was directly applied to FTA cards in Guatemala without prior ELISA testing and analysed using RT-PCR. The reaction mixture for the RT-PCR with a total volume of 50 μl contained 25 μl AccessQuick (Promega, Madison, WI, USA), 1 μl RT-transcriptase (5 units/μl) (Promega), 3 μl of IYSV-F primer (10 μmol/μl), 3 μl of IYSV-R primer (10 μmol/μl), 1 μl Q solution (Qiagen, Valencia, CA, USA), 1 μl of bovine serum albumin (BSA) (25 000 ppm), 2 mm diameter punch-out of FTA card and nuclease-free distilled water was added to obtain the total final volume. RT-PCR failed to amplify the target sequence in some Georgia samples with AccessQuick and were processed as follows: Reverse transcription was conducted using the 'Enhanced First Strand Avian Synthesis Kit' according to the manufacturer's protocol (Sigma-Aldrich Corp., St. Louis, MO, USA) followed by PCR. The 50 μl of PCR reaction consisted of 25 μl of PCR Mastermix (Promega), 1 μl of Q solution (Qiagen), 1 μl of BSA (25 000 ppm), 2 μl of DNA, 3 μl of IYSV-F primer (10 μmol/μl), 3 μl of IYSV-R primer (10 μmol/μl) and 15 μl of water. Reverse transcription was conducted at 45°C for 45 min, followed by 94°C for 2 min. For PCR, the denaturing, annealing and extension times and temperatures were: 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, followed by 72°C for 5 min. Electrophoresis of amplified PCR products was conducted on a 0.8% agarose gel in a 1x TBE buffer and visualized with 0.01% ethidium bromide under UV light. The PCR products from some of the IYSV isolates were cloned into EZ competent cells (Qiagen) using a pDrive cloning vector before sequencing, others were sequenced directly. The PCR products were sequenced in both directions. Sequencing was done by University of Georgia Sequencing and Synthesis facility (Athens, GA, USA) and University of Florida Interdisciplinary Center for Biotechnology Research (Gainesville, FL, USA). Sequences of the *N* gene of IYSV isolates from Georgia (USA), Peru and Guatemala were compared with those from GenBank (Benson et al., 2004) using BLAST (Altschul et al., 1990) search. Accession numbers are listed in Table 1.

Phylogenetic analysis

ClustalW (Thompson et al., 1994) was used for sequence alignment and corrected by sight. PAUP*4.0b.10 (Swofford, 2003) was used for phylogenetic analyses. Phylogenetic trees were created using parsimony analysis. Bootstrap analysis (2000 replications) was performed for the parsimony tree by using stepwise addition with the tree-bisection-reconnection (TBR) branch-swapping option. There were 554 constant characters, 135 parsimony-informative and 111 parsimony-uninformative sites. All characters were considered unordered, of equal weight and gaps were treated as missing data.

Results and Discussion

Results from a GenBank BLAST search indicated that the highest nucleotide homology (97%) of IYSV iso-

Table 1
Plant host, geographic origin and GenBank accession numbers of *Iris yellow spot virus* isolates used in phylogenetic analysis

GenBank accession no.	Isolate name	Host	Location	Publication
AB121026	T3	Lisianthus	Shizuoka, Japan	Doi et al., 2003
AB121025	T2	Lisianthus	Shizuoka, Japan	Doi et al., 2003
AB180918	Sg1	Lisianthus	Saga, Japan	Unpublished data
AB180919	SgOniD1	Onion	Saga, Japan	Unpublished data
AB180920	CbOniB2	Onion	Chiba, Japan	Unpublished data
AB180921	SgA	Lisianthus	Saga, Japan	Unpublished data
AB180922	CbAlsD1	Alstroemeria	Chiba, Japan	Unpublished data
AB181370	Als1	Alstroemeria	Japan	Unpublished data
AF001387		Iris	The Netherlands	Cortês et al., 1998
AF067070		Onion	Brazil	Pozzer et al., 1999
AF271219		Lisianthus	Israel	Gera et al., 1998
AY341825	Vic-98	Onion	Victoria, Australia	Coutts et al., 2003
AY345226	NSW	Onion	New South Wales, Australia	Coutts et al., 2003
AY345227	Vic-1	Onion	Victoria, Australia	Coutts et al., 2003
AY377428		Leek	Slovenia	Unpublished data
AY538778		Leek	Western Australia	Smith et al., 2006
AY556424		Onion	Western Australia	Smith et al., 2006
DQ150107		Onion	Chile	Rosales et al., 2005
DQ270004		Onion	India	Ravi et al., 2006
DQ233468		Onion	Grant Co., Washington, USA	Pappu et al., 2006b
DQ233469		Onion	Pasco, Washington, USA	Pappu et al., 2006b
DQ233470		Onion	Grant Co., Washington, USA	Pappu et al., 2006b
DQ233471		Shallot	Grant Co., Washington, USA	Pappu et al., 2006b
DQ233472		Onion	Nampa, Idaho, USA	Pappu et al., 2006b
DQ233473		Onion	New Plymouth, Idaho, USA	Pappu et al., 2006b
DQ233474		Onion	Parma, Idaho, USA	Pappu et al., 2006b
DQ233475		Onion	Imperial Valley, California, USA	Pappu et al., 2006b
DQ233476		Onion	Lancaster Co., California, USA	Pappu et al., 2006b
DQ233477		Onion	Weld Co., Colorado, USA	Pappu et al., 2006b
DQ233478		Onion	Davis Co., Utah, USA	Pappu et al., 2006b
DQ233479		Onion	Jefferson Co., Oregon, USA	Pappu et al., 2006b
DQ658242		Onion	Texas, USA	Miller et al., 2006
DQ838584	Ica	Onion	Ica, Peru	This Study
DQ838585	Supe1	Onion	Supe, Peru	This Study
DQ838586	Supe2	Onion	Supe, Peru	This Study
DQ838587	Casma1	Onion	Casma, Peru	This Study
DQ838588	Casma2	Onion	Casma	This Study
DQ838589	Guat1	Onion	Guatemala	This Study
DQ838590	Guat2	Onion	Guatemala	This Study
DQ838591		Onion	Georgia	This Study
DQ838592		Onion	Georgia	This Study
DQ838593		Onion	Georgia	This Study
DQ838594		Onion	Georgia	This Study

lates from Georgia were with IYSV sequences from Japan, Brazil and Idaho, US. Genetic variation in the *N* gene of *Tomato spotted wilt virus* (TSWV), another Tospovirus, was found to be linked to the geographic origin (Pappu et al., 1998; Tsompana et al., 2005). Nucleocapsid gene sequences of TSWV clustered by geographic origin indicating that some TSWV isolates found in North Carolina originated from the Netherlands. Smith et al. (2006) made conclusions based on the *N* protein gene that Australian IYSV isolates did not originate in Australia. In our study, we used the *N* gene sequences of the Georgia IYSV isolates to test the hypothesis that IYSV found in Georgia was more closely related to IYSV strains from Peru rather than to IYSV strains from the western US. Maximum parsimony and bootstrap analysis resulted in eight distinct clades, three of which consisted of single isolates. The phylogenetic analysis placed the IYSV isolates from Peru and Georgia into a distinct and well-supported clade (clade 6) (Fig. 1). The Guatemalan isolates clustered with isolates from western USA, Japan and Chile in a sister clade (clade 5) to the Georgia–Peru IYSV clade. The phylogenetic results are further supported by calculation of the divergence in *N*-gene nucleotide sequences of Georgia and Peru strains compared with strains from other parts of the world. Isolates from Georgia and Peru had the lowest percent divergence with an average of 1.2%. The average divergence between Georgia strains and strains from other South and Central American countries, Europe, Australia, Japan and western USA was higher – 2.1%, 11.5%, 11.4%, 6.8% and 3.4%, respectively. The average divergence of Peruvian isolates compared with the other South and Central American, European, Australian, Japanese isolates and isolates from western USA – 2.2%, 11.4%, 11.1%, 6.7% and 3.4%, respectively – was similar to the divergence rate of the Georgian isolates, supporting the hypothesis that IYSV gene flow occurred from Peru to Georgia. Even though Georgia onion growers import onion bulbs from South and Central American countries in addition to Peru, the results of the phylogenetic analysis

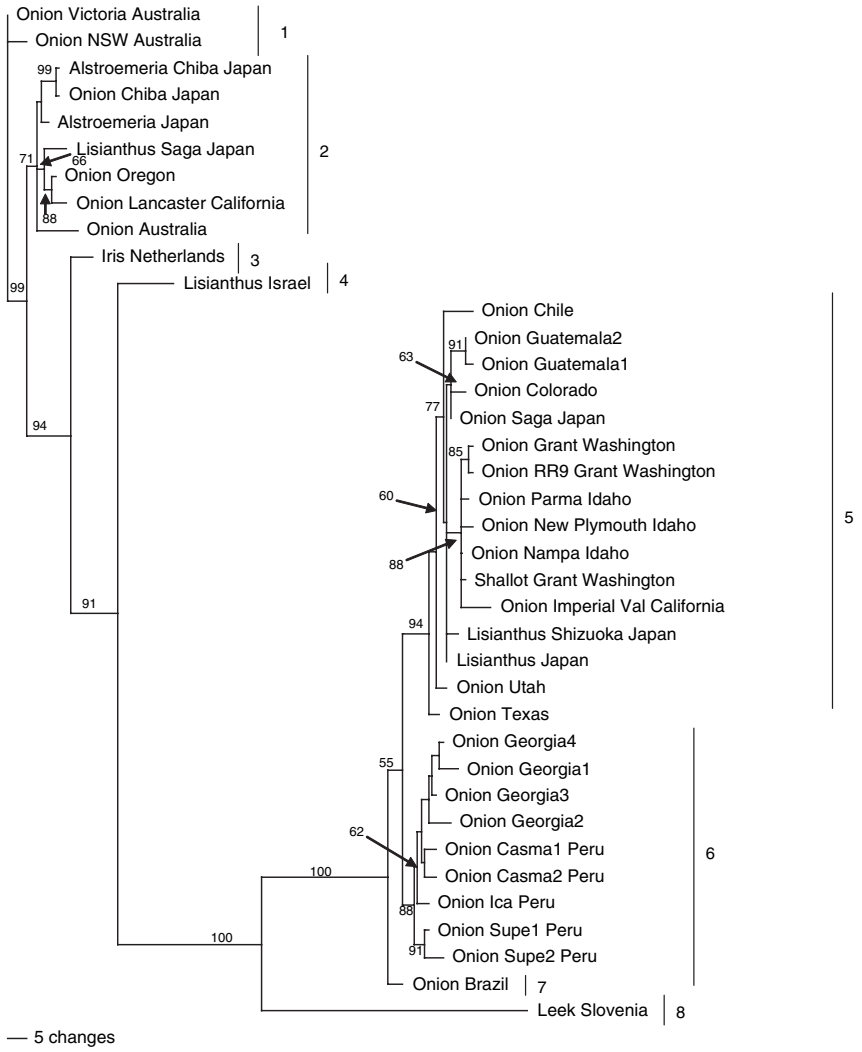

Fig. 1 Maximum parsimony tree based on nucleotide sequences of the partial *N*-gene (800 bp) of *Iris yellow spot virus* obtained from heuristic parsimony search and bootstrap analysis. Bootstrap values greater than 50% are shown at the nodes based on 2000 replications. Gaps were treated as missing data

show that similar IYSV strains are found in Georgia and Peru. Georgia and Peruvian strains are more distantly related to all other known IYSV strains whose sequences are deposited in GenBank. It is possible that the gene flow occurred through viruliferous onion thrips (*Thrips tabaci*) found in imported Peruvian bulbs or by onion thrips feeding on infected Peruvian onion bulbs. Onion thrips are rarely found on onions in this region compared with other onion-growing areas of the world. Live onion thrips were found in imported Peruvian onions in Georgia in the fall of 2003 when IYSV was first detected (Mullis et al., 2004). Georgia onion growers import onions from Peru and other South and Central American countries and sort, grade and repack them into retail-sized bags or boxes. However, the culls are discarded in fallow fields that are often close to (approximately 1 km) onion seedbeds. Onion thrips, *Thrips tabaci*, the vector of IYSV, have been found inside discarded Peruvian onions (A. Sparks, personal observation). At this point we can only speculate that thrips could emerge when the discarded onion bulbs sprout and then feed on the onion leaves. They could then easily move on to the seedlings in the seedbeds, colonize them and transmit the virus.

The predominant species of thrips colonizing Georgia onions is *Frankliniella fusca* (tobacco thrips) and we find very few onion thrips in the fields, yet large numbers of onion plants are infected with IYSV. Further research is needed on the epidemiology of IYSV to determine if other thrips species can transmit the virus to onion, and to contrast and compare symptomology of isolates of IYSV on onion cultivars.

Finally, we do not yet know the cause for the lack of diamond-shaped lesions on both Peruvian and Georgian onions, that are observed on onions in other parts of the world but they could be due to different IYSV strains in Georgia and Peru compared with the strains in other parts of the world. Environmental conditions occurring in Georgia during the winter months may allow for a variation in symptom development in Georgia but as Peru has a climate similar to the summer conditions in parts of western USA, this aspect is inconsistent.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipmon DJ. (1990) Basic local alignment search tool. *J Mol Biol* **215**:403–410.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. (2004) GenBank: update. *Nucleic Acids Res* **32**:D23–26.

Córdoba-Sellés C, Martínez-Priego L, Muńoz-Gómez R, Jordá-Gutiérrez C. (2005) *Iris yellow spot virus*: a new onion disease in Spain. *Plant Dis* **89**:1243.

Cortês I, Livieratos IC, Derks A, Peters D, Kormelink R. (1998) Molecular and serological characterization of *Iris yellow spot virus*, a new and distinct tospovirus species. *Phytopathology* **88**:1276–1282.

Coutts BA, McMichael LA, Tesoriero L, Rodoni BC, Wilson CR, Wilson AJ, Persley DM, Jones RAC. (2003) *Iris yellow spot virus* found infecting onions in three Australian states. *Austral Plant Path* **32**:555–557.

Creamer R, Sanogo S, Moya A, Romero J, Molina-Bravo R, Cramer C. (2004) *Iris yellow spot virus* on onion in New Mexico. *Plant Dis* **88**:1049.

Crowe FJ, Pappu HR. (2005) Outbreak of *Iris yellow spot virus* in onion seed crops in central Oregon. *Plant Dis* **89**:105.

Doi M, Zen S, Okuda M, Nakamura H, Kato K, Hanada K. (2003) Leaf necrosis disease of lisianthus (*Eustoma grandiflorum*) caused by *Iris yellow spot virus*. *Jpn J Phytopathol* **68**:181–188.

Du Toit LJ, Pappu HR, Druffel KL, Pelter GQ. (2004) *Iris yellow spot virus* in onion bulbs and seed crops in Washington. *Plant Dis* **88**:222.

Gent DH, Schwartz HF, Khosla R. (2004) Distribution and incidence of *Iris yellow spot virus* in Colorado and its relation to onion plant population and yield. *Plant Dis* **88**:446–452.

Gent DH, du Toit LJ, Fichtner SF, Mohan KS, Pappu HR, Schwartz HF. (2006) *Iris yellow spot virus*: an emerging threat to onion bulb and seed production. *Plant Dis* **90**:1468–1480.

Gera A, Cohen J, Salomon R, Raccah B. (1998) Iris yellow spot tospovirus detected in onion in Israel. *Plant Dis* **82**:127.

Hall JM, Mohan K, Knott EA, Moyer JW. (1993) Tospoviruses associated with scape blight of onion (*Allium cepa*) seed crops in Idaho. *Plant Dis* **77**:952.

Hoepting C, Schwartz HF, Pappu HR. (2007) First report of *Iris yellow spot virus* in onion in New York. *Plant Dis* **91**:327.

Khan AA, Asif MI. (1981) Studies on the translocation of 14C-labelled photosynthates in onions. *J Hortic Sci* **56**:113–116.

Miller ME, Saldana RR, Black MC, Pappu HR. (2006) First report of Iris yellow spot virus on onion (*Allium cepa*) in Texas. *Plant Dis* **90**:1359.

Moyer JW. *Tospoviruses* (*Bunyaviridae*). In: Granoff A, Webster RG. (eds), *Encyclopedia of Virology*, San Diego, CA, Academic Press, 1999, pp. 1803–1807.

Mullis SW, Langston DB Jr, Gitaitis RD, Sherwood JL, Csinos AS, Sparks AN, Torrance RL, Cook MJ IV, (2004) First report of Vidalia onion (*Allium cepa*) naturally infected with *Tomato spotted wilt virus* and *Iris yellow spot virus* (Family *Bunyaviridae*, Genus *Tospovirus*) in Georgia. *Plant Dis* **88**:1285.

Mullis SW, Gitaitis RD, Nischwitz C, Csinos AS. (2006) First report of onion (*Allium cepa*) naturally infected with *Iris yellow spot virus* in Peru. *Plant Dis* **90**:377.

Pappu HR, Pappu SS, Jain RK, Bertrand PF, Culbreath AK, McPherson R, Csinos AS. (1998) Sequence characteristics of natural populations of Tomato spotted wilt Tospovirus infecting flue-cured tobacco in Georgia. *Virus Genes* **17**:169–177.

Pappu HR, Hellier BC, Dugan FM. (2006a) Wild *Allium* spp as natural hosts of *Iris yellow spot virus*. *Plant Dis* **90**:378.

Pappu HR, du Toit LJ, Schwartz HF, Mohan SK. (2006b) Sequence diversity of the nucleoprotein gene of Iris yellow spot virus (genus *Tospovirus*, familiy *Bunyaviridae*) isolates from the western region of the United States. *Arch Virol* **151**:1015–1023.

Pozzer L, Bezerra IC, Kormelink R, Prins M, Peters D, de O Resende R, de Ávila AC. (1999) Characterization of a tospovirus isolate of *Iris yellow spot virus* associated with a disease in onion fields in Brazil. *Plant Dis* **83**:345–350.

Ravi KS, Kitkaru AS, Winter S. (2006) *Iris yellow spot virus* in onions: a new tospovirus record from India. *Plant Pathol* **55**:288.

Robène-Soustrade I, Hostachy B, Roux-Cuvelier M, Minatchy J, Hédont M, Pallas R, Couteau A, Cassam N, Wuster G. (2006) First report of *Iris yellow spot virus* in onion bulb- and seed-production fields in Réunion Island. *Plant Pathol* **55**:288.

Rosales M, Pappu HR, López L, Mora R, Aljaro A. (2005) *Iris yellow spot virus* in onion in Chile. *Plant Dis* **89**:1245.

Schwartz HF, Brown WM Jr, Blunt T, Gent DH (2002) *Iris yellow spot virus* on onion in Colorado. *Plant Dis* **86**:560.

Smith TN, Jones RAC, Wylie SJ. (2006) Genetic diversity of the nucleocapsid gene of *Iris yellow spot virus*. *Austral Plant Path* **35**:359–362.

Swofford DL. *PAUP*. Phylogenetic Analysis using Parsimony (*and Other Methods)*, Version 4. Sunderland, MA, Sinauer Associates, 2003.

Thompson JD, Higgins DG, Gibson TJ. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acid Res* **22**:4673–4680.

Tsompana M, Abad J, Purugganan M, Moyer JW. (2005) The molecular population genetics of the *Tomato spotted wilt virus* (TSWV) genome. *Mol Ecol* **14**:53–66.