

SHORT COMMUNICATION

CHARACTERIZATION OF IRIS YELLOW SPOT VIRUS ISOLATES FROM ONION CROPS IN NORTHERN ITALY

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SUMMARY

During spring and summer of 2007 and 2008, a number of onion fields in Emilia Romagna region (northern Italy) showed various virus-like symptoms. DAS-ELISA carried out in 2007 and early 2008 for *Impatiens necrotic spot virus* (INSV), Iris yellow spot virus (IYSV) and *Tomato spotted wilt virus* (TSWV) showed the occasional presence of TSWV, whereas a number of samples also reacted weakly with IYSV antiserum. In a number of TSWV-negative samples, electron microscopy of leaf extracts revealed the presence of tospovirus-like particles. Western blot analysis on the same set of samples gave positive results with antisera against Tomato fruit yellow ring virus (TFYRV) and IYSV, two serologically related virus species. Sequence analysis of RT-PCR fragments confirmed that the virus in the onion samples was IYSV with approximately 98% identity at the amino acid level with reported Serbian and Spanish sequences of the same virus. Phylogenetic analysis confirmed that the Italian IYSV isolates belong to a newly defined southern European clade. Our data suggest that IYSV is made up of a heterogeneous and serologically distinct group of isolates.

Key words: Tospoviruses, Iris yellow spot virus, onion, diagnosis, ELISA, PCR

Iris yellow spot virus (IYSV), genus *Tospovirus*, family *Bunyaviridae*, causes large economic losses in a wide variety of plant hosts (Gent *et al.*, 2006; Pappu *et al.*, 2009). It has typical tospovirus genome organization with three single-stranded RNA segments of negative or ambisense polarity (Pappu, 2008; Tsompana and Moyer, 2008). IYSV is transmitted by *Thrips tabaci* (Nagata *et al.*, 1999; Kritzman *et al.*, 2001). The virus causes important problems in a number of monocot hosts, and among these, the disease caused to onions is the most

severe. Initial reports of IYSV were from the Treasure Valley of the northwestern USA (Hall *et al.*, 1993), Brazil (Pozzer *et al.*, 1999), the Netherlands (Cortes *et al.*, 1998) and Israel (Gera *et al.*, 1998; Kritzman *et al.*, 2000). IYSV is now present in Australia (Coutts *et al.*, 2003), India (Ravi *et al.*, 2006), Peru (Mullis *et al.*, 2006) and Chile (Rosales *et al.*, 2005). There are recent reports from Canada (Hoepting *et al.*, 2008), New York State (Hoepting *et al.*, 2007) and South Africa (du Toit *et al.*, 2007). In Italy, IYSV has been reported, based only on a very weak DAS-ELISA result, which was not confirmed by further assays (Cosmi *et al.*, 2003). In Europe, IYSV was first recorded from Iris in the Netherlands (Cortes *et al.*, 1998), and epidemics in onions and leeks have been confirmed in Slovenia (Mavric and Ravnikar 2001), Spain (Cordoba-Selles *et al.*, 2005; Cordoba-Selles *et al.*, 2007) and Serbia (Bulajic *et al.*, 2008). Onion infections with IYSV have also been reported from France and Germany (Leinhos *et al.*, 2007; Huchette *et al.*, 2008) but, at the moment, molecular features are not available in the databases.

In 2007 and 2008, surveys of onion bulb and seed production fields in the Emilia-Romagna region suggested that tospoviruses could be responsible for symptoms observed on some plants (Vicchi *et al.*, 2008). Initial serological tests for IYSV were inconclusive, but showed the occasional presence of *Tomato spotted wilt virus* (TSWV). In 2008, six samples were collected from farms in the Bologna and Ravenna provinces of Emilia Romagna. Four of these samples showed the diamond-shaped chlorotic symptoms typical of IYSV (Fig. 1). Electron microscopy of leaf extracts from each of the six samples showed tospovirus-like particles in four samples only.

DAS-ELISA testing of these samples for *Impatiens necrotic spot virus* (INSV), Polygonum ringspot virus (PolRSV) and TSWV as described by Ciuffo *et al.* (2008) gave no positive response. However, when DAS-ELISA was performed using a kit to IYSV (Loewe, Germany) the four samples containing tospovirus-like particles reacted positively (Table 1). Similar results were obtained when a kit by DSMZ (Germany) was used. However, the infected/healthy (I/H) value for two isolates (Cip5 and Cip6) was lower than that obtained



Fig. 1. Diamond-shaped chlorotic lesions on scapes in onion plants infected by the Italian Iris yellow spot virus isolates.

with the Loewe kit (Table 1). DAS-ELISA using the A426 antiserum, prepared in our institute from purified nucleocapsids of isolate PV-0528 of IYSV following protocols described (Ciuffo *et al.*, 2008), showed similar results as with the DSMZ kit (Table 1).

Since no consistent results could be achieved with DAS-ELISA, samples were analyzed by Western blotting (Turina *et al.*, 2006) using antisera to IYSV (Loewe) and to Tomato fruit yellow ring virus (antiserum AE508 form DSMZ). TFYRV, also known as Tomato yellow ring virus (TYRV), is a virus from Iran related in sequence to IYSV (Hassani-Mehraban *et al.*, 2005; Winter *et al.*, 2006). None of the tested samples reacted with

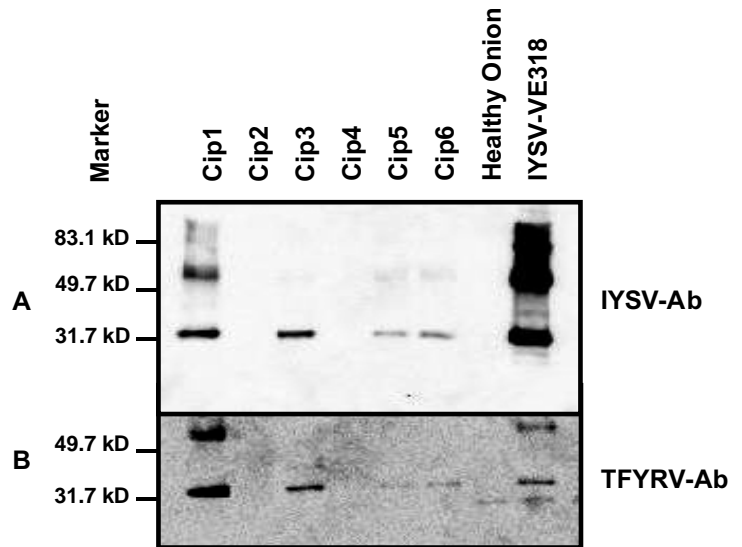


Fig. 2. Western blot detection of the nucleocapsid protein of Iris yellow spot virus (IYSV) using the LOEWE antibody (panel A) and Tomato fruit yellow ring virus (TFYRV) (panel B) on field-collected onion samples. The positive control was diluted 1/10 in healthy onion sap. Cip1 to Cip6 are the six onion samples from different fields in Emilia-Romagna region. M= protein molecular weight marker. Ponceau staining of the membrane was done to ensure that equal amount of protein was loaded.

IgGs to TSWV or INSV (not shown), however, positive reaction was observed with those samples that were positive for IYSV in DAS-ELISA and contained tospovirus-like particles (Fig. 2). DAS-ELISA negative samples were also negative in Western blotting.

The upper band of *ca.* 60 kDa, present in all samples but clearly visible in Cip1, may be due to polymerization of the nucleoprotein. As to the 31.7 kDa N protein, the bands obtained with the two antisera were of differ-

Table 1. Results of double antibody sandwich (DAS)-ELISA using different antisera IYSV VE318 is an isolate obtained from DSMZ, originally from the Netherlands. TFYRV VE334 is an isolate of Tomato fruit yellow ring virus from DSMZ. Shadowed I/H values are considered positive.

Samples	TSWV		INSV		PolRSV		IYSV-DSMZ		IYSV-A426		IYSV/Loewe	
	ABS	I/H	ABS	I/H	ABS	I/H	ABS	I/H	ABS	I/H	ABS	I/H
On01	0.09	0.5	0.08	1.2	0.09	1.5	0.50	6.3	0.66	9.4	0.57	8.14
On02	0.07	0.4	0.07	1.0	0.06	1.0	0.08	1.0	0.10	1.4	0.05	0.71
On03	0.10	0.6	0.07	1.0	0.10	1.7	0.61	7.5	0.35	5.0	0.65	9.28
On04	0.07	0.4	0.07	1.0	0.06	0.9	0.07	0.9	0.07	0.9	0.06	0.85
On05	0.12	0.7	0.10	1.4	0.08	1.3	0.23	2.8	0.08	1.2	0.52	7.42
On06	0.14	0.8	0.11	1.6	0.09	1.5	0.57	7.0	0.08	1.2	0.55	7.85
Healthy Onion	0.17	1.0	0.07	1.0	0.06	1.0	0.08	1.0	0.07	1.0	0.07	1.00
IYSV VE318	0.09	0.5	0.08	1.1	0.06	1.0	2.30	28.6	0.42	6.1	2.10	30.00
TSWV P105	2.83	16.9	0.07	1.0	0.06	1.0	0.08	1.0	0.08	1.1		
INSV glox1	0.29	1.7	2.63	37.3	0.06	1.1	0.08	0.9	0.07	1.1		
PolRSV Plg3	0.08	0.5	0.09	1.2	2.39	39.5	0.09	1.1	0.09	1.3		
TFYRV VE 334	0.07	0.4	0.08	1.1	0.53	8.8	0.08	1.0	0.08	1.2		

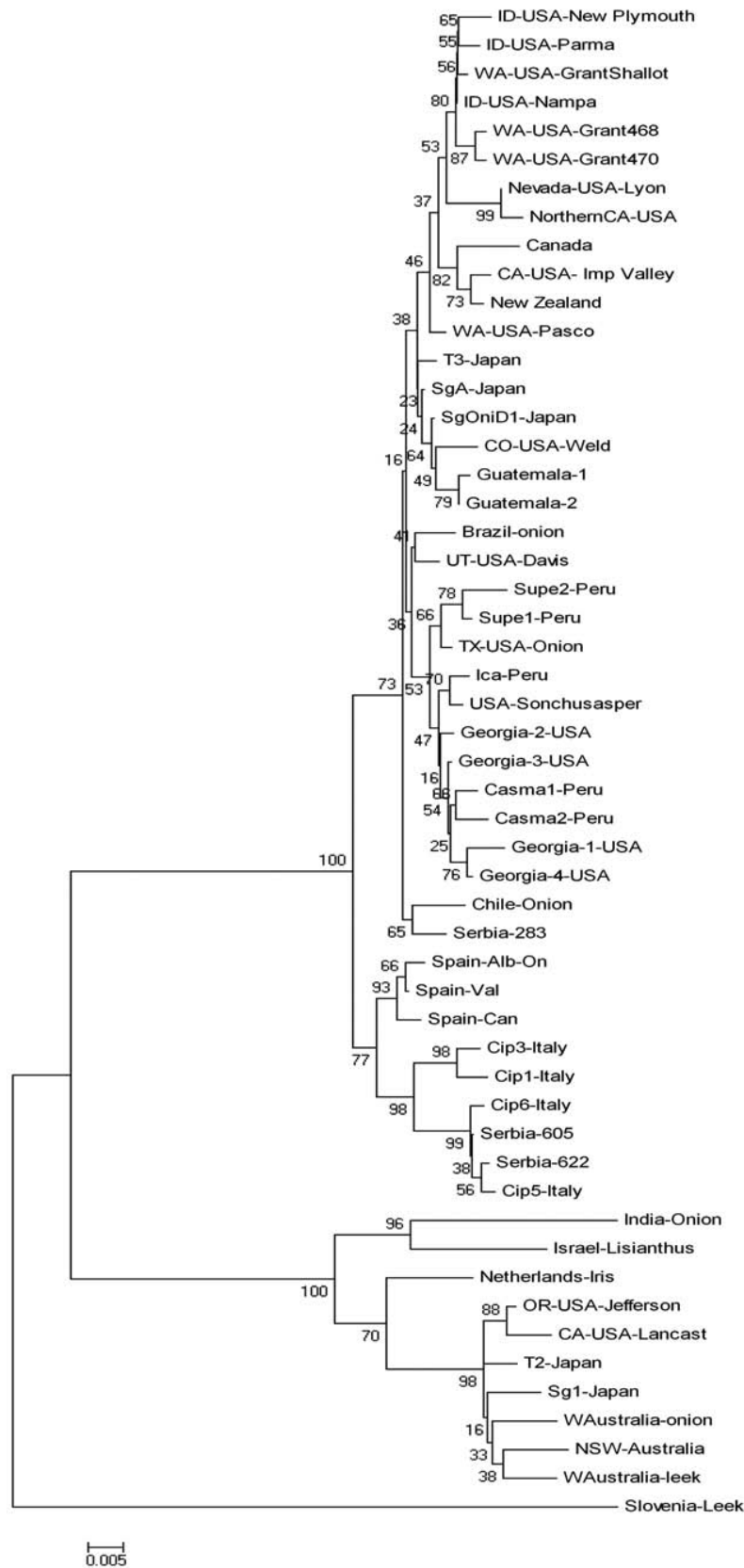


Fig. 3. Phylogenetic tree of nucleotide sequences in the nucleocapsid-protein coding region of 49 Iris yellow spot virus (IYSV) isolates from different origins and 4 Italian IYSV isolates. The accession numbers corresponding to each isolate are given in Table 2. The Minimal-Evolution tree was constructed with MEGA4 using the Maximum Composite Likelihood method and 1000 bootstrap replicates, as detailed in the text. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Table 2. Virus isolates used for the phylogenetic analysis shown in Fig. 3 and their accession number.

GenBank accession No.	Isolate name	Reference	GenBank accession No.	Isolate name	Reference
AB121026	T3-Japan	Doi <i>et al.</i> , 2003	DQ838587	Casma1-Peru	Nischwitz <i>et al.</i> , 2007
AB121025	T2-Japan	Doi <i>et al.</i> , 2003	DQ838588	Casma2-Peru	Nischwitz <i>et al.</i> , 2007
AB180918	Sg1-Japan	Unpublished data	DQ838589	Guatemala1	Nischwitz <i>et al.</i> , 2007
AB180919	SgOniD1-Japan	Unpublished data	DQ838590	Guatemala2	Nischwitz <i>et al.</i> , 2007
AB180921	SgA-Japan	Unpublished data	DQ838591	Georgia 1-USA	Nischwitz <i>et al.</i> , 2007
AF001387	Netherlands-Iris	Cortés <i>et al.</i> , 1998	DQ838592	Georgia 2-USA	Nischwitz <i>et al.</i> , 2007
AF067070	Brazil-Onion	Pozzer <i>et al.</i> , 1999	DQ838593	Georgia 3-USA	Nischwitz <i>et al.</i> , 2007
AF271219	Israel-Lisianthus	Gera <i>et al.</i> , 1998	DQ838594	Georgia 4-USA	Nischwitz <i>et al.</i> , 2007
AY345226	NSW-Australia	Coutts <i>et al.</i> , 2003	EF419888	Spain-Alb-On	Cordoba-Selles <i>et al.</i> 2005
EU078327	USA-Sonchus asper	Unpublished	EF427447	Spain-Val	Cordoba-Selles <i>et al.</i> 2005
AY538778	WAustralia-leek	Smith <i>et al.</i> , 2006	EF427448	Spain-Can	Cordoba-Selles <i>et al.</i> 2005
AY556424	WAustralia-onion	Smith <i>et al.</i> , 2006	EU727180	Serbia-283	Bulajic <i>et al.</i> , 2008
DQ150107	Chile-onion	Rosales <i>et al.</i> , 2005	EU586203	Serbia-605	Bulajic <i>et al.</i> , 2008
DQ270004	India-onion	Ravi <i>et al.</i> , 2006	EU750697	Serbia-622	Bulajic <i>et al.</i> , 2008
DQ233468	WA-USA-Grant468	Pappu <i>et al.</i> , 2006b	AY377428	Slovenia-leek	Mavric and Ravnikar, 2001
DQ233469	WA-USA-Pasco	Pappu <i>et al.</i> , 2006b	EU477515	New Zeland	Ward <i>et al.</i> , 2009
DQ233470	WA-USA-Grant 470	Pappu <i>et al.</i> , 2006b	EU287943	Canada	Hoepting <i>et al.</i> , 2008
DQ233471	WA-USA-Grant-Shallot	Pappu <i>et al.</i> , 2006b	FJ713699	Nevada-USA-Lyon	Unpublished
DQ233472	ID-USA-Nampa	Pappu <i>et al.</i> , 2006b	FJ713700	NorthernCA-USA	Unpublished
DQ233473	ID-USA-New Plymouth	Pappu <i>et al.</i> , 2006b	FJ185142	Cip3-Italy	This Study
DQ233474	ID-USA-Parma	Pappu <i>et al.</i> , 2006b	FJ842093	Cip1-Italy	This Study
DQ233475	CA-USA-Imp. Valley	Pappu <i>et al.</i> , 2006b	FJ842094	Cip5-Italy	This Study
DQ233476	CA-USA-Lancaster	Pappu <i>et al.</i> , 2006b	FJ842095	Cip6-Italy	This Study
DQ233477	CO-USA-Weld	Pappu <i>et al.</i> , 2006b	DQ838585	Supel-Peru	Nischwitz <i>et al.</i> , 2007
DQ233478	UT-USA-Davis	Pappu <i>et al.</i> , 2006b	DQ838586	Supel2-Peru	Nischwitz <i>et al.</i> , 2007
DQ233479	OR-USA-Jefferson	Pappu <i>et al.</i> , 2006b			
DQ658242	TX-USA-onion	Miller <i>et al.</i> , 2006			
DQ838584	Ica-Peru	Nischwitz <i>et al.</i> , 2007			

ent relative intensity, which may indicate a differential reactivity due to a diversity in the antigenic properties of the viral isolates from field samples compared to the isolate from The Netherlands used as positive control.

To characterize IYSV at the molecular level, RNA was extracted from sample Cip3 using the “Plant RNA

purification reagent” (Invitrogen, USA) following the manufacturer’s protocol and RT-PCR was performed using the degenerate primers (Euro-Tospo-90F-5’GAY-GAYTGGACNTTYMGMNG-3’ and Euro-Tospo-180AA R-5’TTYTTNACRTTYTGRAARTANGC-3’) (Turina *et al.*, 2006). These primers can equally amplify

the target sequences of the three tospoviruses [IYSV, TYRFV and Polygonum ringspot virus (PolRSV)] which were shown to be related in sequence and serologically (Ciuffo *et al.*, 2008). Direct sequence analysis of amplified PCR product showed that Cip3 isolate shared 99% similarity at the amino acid level with IYSV isolates from Spain and Serbia.

The primer pair IYSV-N-F – 5'ATGTCTACTG-CAAGGGT-3' and IYSV-N-R- 5'CTATGAGTATCT-GTCTTCTT-3' was used to amplify the nucleocapsid (N) gene of the virus. Amplified PCR product was sequenced using the oligonucleotides IYSV-300F- AGT-GCATATGGTTTGAAACC and IYSV-500R-GAA-GAGCAGCTGCAGCAAAG. Further characterization of the rest of the ELISA-positive samples was carried out by processing different leaf portions in the senior author's laboratory (Rome). Total RNAs were extracted using the RNeasy Mini Kit (Qiagen, Germany) and one-step RT-PCR was performed (Tomassoli *et al.*, 2007) using primers IYSV- Nc5 – AAATCGGGGAA ATTCACATTC and IYSV- Nc3 – CTTTCTTG-GAGGGATTCTTGG, respectively.

PCR products of all samples were sequenced as previously reported and, after sequence analysis, those of three isolates (Cip1, Cip5 and Cip6) were deposited in GenBank under accession numbers FJ842093, FJ842094 and FJ842095. Analysis of Cip3 confirmed 100% identity with the Cip3 previously deposited in GenBank (accession No. FJ185142).

Phylogenetic analyses of the cDNA sequences were conducted using MEGA4 (Tamura *et al.*, 2007). Evolutionary history was inferred using the Minimal Evolution method (Rzhetsky and Nei, 1992). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The Minimal Evolution tree was searched using the Close-Neighbor-Interchange algorithm at a search level of 1 (Nei and Kumar, 2000). The Neighbor-Joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 659 positions in the final dataset. The same program was used to derive phylogenetic trees using UPGMA and Neighbor-Joining methods (not shown). A number of clades were well supported by all the different methods used (Fig. 3 and not shown). The Slovenian leek isolate constitutes one clade, another clade includes isolates from the Netherlands, Israel, Japan and Australia; a third clade includes mostly isolates from the western United States, Chile, Guatemala, one isolate from Serbia and one from Brazil; a fourth clade includes isolates from Peru and Georgia, USA; a fifth statistically well supported

clade includes isolates from Serbia, Spain and Italy, with the Italian isolates dividing into two different subclades, one comprising of Serbian and Italian isolates, the other comprising of the remaining Italian isolates. Overall our data support a certain amount of diversification of the Italian isolates, which could be explained by the co-existence of two different founder effects in a rather narrow area (the fields are all located in a 70 km radius).

Others have pointed out the diversity among various IYSV isolates, and even though some authors did not include the data for European isolates that are now present in the databases, their results can be considered equivalent to ours (Pappu *et al.*, 2006; Smith *et al.*, 2006; Nischwitz *et al.*, 2007).

Overall, IYSV as a viral species seems to display wide diversity, also suggested by our serological analysis. In fact, although partial and not quantitative, our analysis points to the presence of serologically distinct isolates of IYSV, and this complicates diagnosis of this virus through serology. In particular, some isolates from southern Europe are not optimally recognized by some of the serological tools available (our own kit and the DSMZ-based one), which are based on the original isolate intercepted in the Netherlands. Furthermore, although our analysis was not strictly quantitative, we noted a different behavior in DAS-ELISA of isolates Cip1 and Cip3, from isolates Cip5 and Cip6 (compare LOEWE kit results vs other kits) and this difference was confirmed by the phylogenetic analysis.

Given the importance of onion bulb and seed crops in Italy, it is of utmost importance to establish a more extensive survey of onion-producing areas, with particular attention to possible alternative hosts and the presence of viruliferous thrips. The possibility of this virus spreading to ornamental species should also be taken into account, given its wide host range.

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