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RESEARCH PAPERS

Molecular analysis of the 3′ terminal region of *Onion yellow dwarf virus* from onion in southern Italy

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Summary. *Onion yellow dwarf virus* (OYDV) is an economically important pathogen causing severe disease in garlic, onion and other *Allium* crops. Eleven isolates of OYDV, all from onion originating from Calabria, southern Italy, were genetically analyzed. An OYDV onion isolate from Sudan was also included in this study. The 3′ terminal region of about 2.5 kb of the twelve isolates were sequenced and the sequences comprising a part of the nuclear inclusion a (NIa-Pro), the complete nuclear inclusion b (NIb) and coat protein (CP) genes and the 3′ untranslated region (3′UTR), were compared to each other and to corresponding sequences of other OYDV isolates from different countries and *Allium* hosts. The within-population nucleotide identity of the Italian OYDV onion isolates was very high (more than 99.3%), whereas nucleotide identity between them and OYDV onion isolates from Germany was 94%, Argentina 92% and Sudan 87%. Recombination analysis among the complete 3′ terminal sequences showed putative recombination breakpoints in the NIb region of the Argentine isolate, with the minor parent related to the Sudanese isolate. Comparison between OYDV isolates from onion and isolates from garlic produced identities of 77-78% for the complete nucleotide region. When the 3′ terminal nucleotide sequence and the complete NIb protein were analyzed, the phylogenetic analysis generated rooted trees with high bootstrap values (100%), showing a genetic grouping into two well separated clades distinctive for onion and garlic isolates of OYDV. Phylogenetic analysis of CP protein and 3′UTR showed lower bootstrap separation values and no distinct sub-grouping of the OYDV isolates from the two major *Allium* species.

Key words: OYDV, *Allium cepa*, molecular characterization, NIb and CP proteins, 3′UTR region.

Introduction

Onion yellow dwarf virus (OYDV), is one of the most important viruses affecting *Allium* species (Van Dijk, 1993). The virus has spread worldwide and a high incidence has been found in many countries, including Greece, Argentina, The Czech Republic, Egypt, Brazil, India and Sudan (Dovas *et al*., 2001; Conci *et al*., 2003; Klukáčková *et al*., 2004; Abd El Wahab *et al*., 2009; Fayad-André *et al*., 2011; Katis *et al*., 2012; Kumar *et al*., 2012; Mohammed *et al*., 2013). The virus is reported to be transmitted in a non-persistent manner by more than 50 aphid species (Drake *et al*., 1933), including *Myzus persicae* (Sulzer), which is the most efficient vector followed by *Aphis craccivora* (Koch), and *A. gossypii* (Glover) (Abd El-Wahab, 2009; Kumar *et al*., 2011). OYDV was first identified in onion (*Allium cepa* L.) in Iowa, USA (Melhus *et al*., 1929) and has a limited host range restricted to the *Allium* genus (family *Amaryllidaceae*). It mainly infects onion and garlic (*Allium sativum* L.), but also shallot (*A. ascalonicum* L.), leek (*A. fistolosum*) and other

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Allium species from Asia. No wild species of *Allium* or other weeds have been reported as natural hosts. The main pathways for OYDV survival are therefore volunteer onion plants from previous crops, onion sets or vernalized bulbs for bulb-to-seed crops (Katis *et al*., 2012).

In onion, OYDV causes yellow striping or yellowing, curling, flattening and crinkling of leaves and dwarfing of plants. In seed crops, flower stems remain turgid and round but show yellowing, distortion and curling. In addition, they are shorter than normal, and produce smaller flowers with reduced numbers of seeds that are often of poor quality (Kumar *et al*., 2012). OYDV infection causes seed yield loss up to 50%, reductions in bulb weight and size up to 40%, and impairs seed storage (Conci *et al*., 2003; Elnagar *et al*., 2011; Kumar *et al*., 2012).

Numerous surveys and epidemiological studies regarding the garlic strain of OYDV, named OYDV-G (Van Dijk, 1993; Katis *et al*., 2012) have been carried out in many countries where the virus causes severe damage to garlic and other minor *Allium* spp. (shallot, leek and scallion), frequently found in mixed infections with other viral species of the genera *Potyvirus, Carlavirus* and *Allexivirus* (Fajardo *et al*., 2001; Fayad-André *et al*., 2011; Bagi *et al*., 2012; Katis *et al*., 2012; Mohammed *et al*., 2013). Studies on OYDV infecting onion crops, hereafter designated as OYDV-O, have also been reported, concerning occurrence, distribution and transmission (Hoa *et al*., 2003; Kumar *et al*., 2011; 2012; Elnagar *et al*., 2011; Katis *et al*. 2012; Velasquez-Valle *et al*., 2012; Sevik and Akcura, 2013).

OYDV is a 10,538 nucleotide long, single-stranded positive sense RNA virus (GenBank database, Reference accession No: NC005029) of the genus *Potyvirus* (family *Potyviridae*). The genome encodes a single large polyprotein yielding up to ten mature proteins between the terminal untranslated regions (Gibbs and Oshima, 2010), and an overlapping open reading frame (ORF), termed *pipo*, encoding for an eleventh protein (Chung *et al*., 2008). Information regarding the variability of populations of OYDV has been obtained through molecular techniques, and genetic studies have provided data mainly on garlic isolates, whereas less information is available on onion isolates (Arya *et al*., 2006; Celli *et al*., 2013).

In Italy, the presence of OYDV has long been assumed, based on symptomathology recorded in onion (Marani and Bertaccini, 1983). However, OYDV was first identified only in southern Italy (Dovas and Vovlas, 2003; Parrella *et al*., 2005), without providing published sequences and phylogenetic analysis of the onion isolates detected.

In the present study, we identified OYDV in southern Italy (Calabria) in the onion cultivar 'Rossa di Tropea' (protected by the IGP European trademark), and the 3′ terminal sequences of eleven OYDV isolates were determined, including part of the NIa-Pro gene, the complete nuclear inclusion b (NIb) and coat protein (CP) genes and 3′ untranslated region (3′UTR). Nucleotide and amino acid sequences were examined and compared with those of three OYDV-O isolates, one from Sudan (included in this study) and two from Celli *et al*. (2013). The sequences were also compared with other OYDV-G isolates reported in the GenBank database, to determine the similarities and differences between the two viral subgroups of different host origin. Recombination analysis was accomplished among the complete 3′ terminal sequences of OYDV-O isolates.

Materials and methods

Virus isolates

This study focused on the onion cultivar 'Rossa di Tropea' (Red of Tropea), grown in the Tropea area of Calabria (southern Italy), collecting two different biotypes of the cultivar characterized by round (indicated as 'T') or long (indicated as 'L') red bulbs. In the same location, different fields regarding both bulb (first year: seed-to-bulb) and seed (second year: bulb-to-seed) productions were surveyed for OYDV infections during 2012-2013. Samples were collected from plants showing severe symptoms of yellow striping and crinkling of leaves and distortion and dwarfing of flower stems (Figure 1). OYDV infection was confirmed by DAS-ELISA tests (Loewe Biochemica GmbH), and 11 isolates were selected for molecular characterization. An OYDV-O isolate (designated O.70) from Sudan, previously identified by H.S. Mohammed at Plant Pathology Research Centre, Rome (Italy), was also included in this study.

Viral RNA and RT-PCR assays

Total RNA was extracted from fresh host leaf tissue using Real Total RNA from Tissue and Cell kit (Durviz) according to the manufacturer's instruc-

Figure 1. Flower stems from an onion seed crop showing distortion and curling caused by OYDV.

tions. Primer sets (Table 1) were designed by multiple nucleotide sequence alignments of OYDV isolates from *Allium* spp. available in GenBank, choosing highly conserved nucleotide regions within the species. The annealing positions of the primers were arranged in order to obtain contiguous fragments with the ends overlapping between each one. For each primer set, single-step RT-PCR amplification was performed in a total volume of 25 μL, including 2 μL of Total RNA, GoTaq buffer 1× (Promega), 2.5 mM each dNTPS, 4 μM of sense and antisense primers, 1.2 U of AMV-RT (Promega), 20 U of RNase Out (Life Technologies) 0.75 U GoTaq Polymerase (Promega). The RT-PCR consisted of a reverse transcription at 46°C for 30 min, initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. A genus-specific primer pair (CPUP/P9502) amplifying the 3′ CP- 3′UTR region of potyviruses was also used to complete the 3′ terminal region of the isolates (Van der Vlugt *et al*., 1999) under the following conditions: RT at 46°C for 60 min, initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 2 min, 52°C for 2 min, 72°C for 2 min, and final extension at 72°C for 10 min.

Cloning and sequencing of PCR products

The PCR fragment of one Italian and the Sudanese OYDV-O isolates were purified using Amicon Microcon-PCR Centrifugal Filter Devices (Millipore Corporation), ligated into pGEM-T Easy Vector (Pro-

Table 1. Newly designed primers used for NIb and CP gene amplification and sequencing of OYDV isolates.

Primer	Sequence[a] (5′ to 3′)	Gene	Nucleotide position[b]	Amplicon size (bp)
OYDV-NIb F1	AACATTGCATGGGGWTCWCT	NIa	7825-7844	706
OYDV-NIb R1	CCTTMCCACCAAGYAACG	NIb	8530-8513	
OYDV-NIB F2	GGCATCTGGAACGGATCTT	NIb	8419-8437	754
OYDV-NIb R2	CTATACGTTCCATGTCCAATTTT	NIb	9172-9150	
OYDV-NIb/CP F1	CATCCAGATCACGAGGGAAT	NIb	9001-9020	984
OYDV-NIb/CP R1	TGTGGCATTTCGGTATTCAA	CP	9984-9965	
OYDV-CP F2	YGTYGAYRCTGGMACHACYG	CP	9558-9577	615
OYDV-CP R2	RTTACCATCMARGCCAAACA	CP	10172-10154	

[a] IUPAC Code for the mixed bases.
[b] The position of the primers are indicated according to the OYDV genome sequence (JX433020).

mega) and transformed into *Escherichia coli* strain JM109 High Efficiency Competent cells (Promega) according to the manufacturer's instructions. Plasmid DNA containing an insert of the expected size was extracted from three clones for each viral isolate using Quantum Prep Plasmid Miniprep Kit (Bio-Rad Laboratories). Inserts were sequenced in both directions using T7 and SP6 universal primers (Bio-Fab Research). The amplified products of the other Italian OYDV-O were directly sequenced in both directions.

The consensus sequence for both cloned and non-cloned products assembly of the 3′ terminal region of OYDV was compiled using ClustalW implemented in MEGA 5 (Tamura *et al*., 2011).

Sequence comparison, phylogenetic and recombination analysis

Amino acid (aa) sequences were predicted using Translate Expasy (http://www.expasy.org), and pairwise and multiple alignments of all nt and aa sequence data sets, according to each genomic coding and uncoding region and the complete 2637 nt sequences, were performed by both EMBOSS-Needle (http://www.ebi.ac.uk) and ClustalW2 (MEGA 5). Phylogenetic and evolutionary relationships among OYDV isolates were examined by generating phylogenetic rooted trees of both nt and aa sequences using the neighbour–joining (NJ) method and the Kimura two-parameter model implemented in MEGA 5 (Tamura *et al*., 2011). Evaluation of statistical confidence in nodes was based on 1000 bootstrap replicates. *Shallot yellow stripe virus* (SYSV) was chosen as the out-group (accession No. NC007433) as the closest species among the members of the genus *Potyvirus* (Gibbs and Ohshima, 2010). Occurrence of recombination events amongst OYDV isolates was examined using the Recombinant Detection Program (RDP4).

Results

Analysis of the complete 3′ terminal region

The 3′ terminal nucleotide region of all OYDV-O isolates was successfully amplified in one-step RT-PCR using the specific primer pairs (Table 1) designed from the 3′ terminus of the NIa gene to the 3′ terminus of CP gene and the CPUP/P9502 primer set for amplification of 3′UTR codon. The consensus sequences of the insert ligated into pGEM-T Easy Vector from the three clones considered for the Italian 152T and the Sudanese O.70 isolates, were identical. The 3′ terminal nucleotide sequences of all isolates were deposited in the GenBank database (Table 2).

The analysis of the obtained sequences revealed a single ORF of 2427 nt encoding a polyprotein which included the C-terminal of the NIa-Pro protein and the complete NIb and CP proteins. The ORF was followed by an UTR of 210 nucleotides excluding the poly (A) tail.

The pairwise sequence alignment of the Italian OYDV-O isolates revealed nucleotide identity of up

to 99.9% with each other, and up to 94.2% with the isolate from Germany (JX433020), 91.9% with the isolate from Argentina (JX433019) and 87.1% with the Sudanese isolate (KF623541). The nucleotide identity ranged from 76.7 to 78.3% when all OYDV-O isolates were compared with the corresponding sequence of OYDV-G isolates retrieved from the GenBank (Table 3).

Table 2. Identification code and GenBank accession numbers of the OYDV-O isolates from the Tropea area in Calabria (southern Italy) and the OYDV-O isolate from Sudan.

Isolate[a]	Accession No.
152T	KF623530
154T	KF623531
155T	KF623532
9L.Se	KF623533
11L.Se	KF623534
5L	KF623535
8T	KF623536
6L.Se	KF623537
13L.Se	KF623538
2T.Se	KF623539
27T.Se	KF623540
Sudan O.70	KF623541

[a] T, round bulb biotype; L, long bulb biotype; Se, samples collected from onion fields for seed production.

The partial NIa Pro-CP region of all the onion isolates produced a predicted polyprotein of 809 amino acids with an identity of more than 99.1% amongst Italian isolates, and average identity values between the Italian OYDV-O population and the Argentina isolate of 97.5%, the German isolate of 97.2% and the Sudanese isolate of 94.5%. The pairwise alignments of aa sequences of the Italian onion isolates revealed an average identity of 86.2% with the corresponding sequences of garlic isolates.

The Sudanese isolate showed an amino acid identity of 96.3% with the corresponding NIa Pro-CP of the Argentine onion isolate, 93.6% the German onion isolate and from 85.3% to 86.4%, with all OYDV garlic isolates retrieved from GenBank.

The polyprotein of Italian isolates, Sudanese isolate and of two already published OYDV-O isolates (Celli *et al*., 2013) showed conserved cleavage sites (Adams *et al*., 2005a) at the NIa-Pro/NIb (SFQ/SSE) and the NIb/CP (RYQ/AGP). When OYDV-O were compared with OYDV-G isolates, the NIa-Pro/NIb and the NIb/CP cleavage sites were different in P1 (T in garlic isolates) and in P3′ (E in garlic isolates), respectively. Concerning the analysis of the partial C terminal NIa-Pro protein, six residues (at positions 8, 11, 12, 16, 17 and 32) of the 34 aa were conserved

Table 3. Range of nucleotide and amino acid sequence identities (%) in the studied genomic regions within OYDV-O populations, OYDV-G populations, and between OYDV-O and OYDV-G. Comparisons were performed using all the Italian isolates and the Sudanese isolate from this study, and all available sequences for each compared region retrieved from the GenBank and reported in the phylogenetic trees.

Genomic region	Onion strain				Garlic strain		Between garlic and onion strains	
	Within Italian population		Between isolates		Between isolates			
	nt	aa	nt	aa	nt	aa	nt	aa
NIa Pro-3′UTR	99.3–99.9	-	86.5–94.2	-	84.75–100	-	76.7–78.3	-
NIa Pro-CP	-	99.1–100	-	93.6–97.6	-	92.9–100	-	85.3–86.6
NIb	99.0–100	99.0–100	85.0–93.3	93.0–97.1	83.1–100	91.5–100	75.2–77.2	83.5–85.5
CP	99.2–100	98.4–100	87.5–96.9	93.8–100	88.2–99.9	86.8–100	79.0–87.5	81.3–96.5
3′UTR	99.0–100	-	94.7–98.6	-	87.5–100		80.0–90.5	

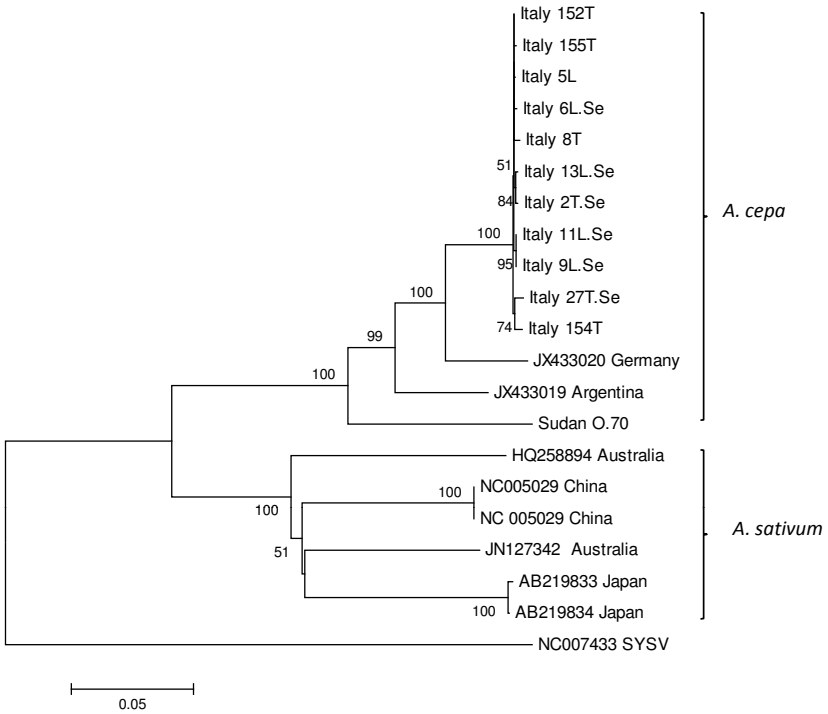

Figure 2. Neighbour-joining tree based on multiple alignment of partial nucleotide sequences, including partial NIa-Pro, complete NIb and CP genes and 3'UTR, of OYDV between Italian and Sudanese isolates from this study, German and Argentine isolates from onion and isolates from garlic available from the database. The sequence of SYSV (NC007433) was used as an out-group. Bootstrap percentages of clades, reported along the branches of the tree with value >50%, derived from bootstrap-resampled data sets (1000 replications).

in OYDV-O and different from OYDV-G. At position 10, the Sudanese and German isolates were different from the other onion isolates and produced the same residue as the garlic isolates.

Phylogenetic analysis of the obtained 3' terminal nucleotide region of the 12 onion isolates from this study, the Argentine and German OYDV-O and six available OYDV-G (NC005029, JN127342, HQ258894, AJ510223, AB219833 and AB219834) produced a rooted tree that differentiated OYDV according to the different host origins supported by 100% bootstrap value. Of the onion isolates, the Italian OYDV-O were grouped together and were phylogenetically closer to the German isolate than the Argentine or Sudanese isolates (Figure 2).

NIb protein: variability and analysis

The NIb gene sequences of the Italian OYDV-O isolates were 1554 nt long and encoded a protein 518 aa long, which contained all the conserved motifs CVDDFN, GNNSGQ, AMIEAWG, GQP-STVVD, FTAAPIE and DGSRFDS identified and used to detect members of the genus *Potyvirus* (Zheng *et al*., 2008, 2010). In addition, the putative active site of RNA-dependent RNA polymerase, $SG(X)_3T(X)_3NT(X)_{30}GDD$, was also conserved at the aa position 310–353 of the CP protein. When pairwise alignments of nt and aa sequences were performed, the Italian isolates showed up to 100% identity with each other, whereas they revealed an average identity of 93.2% and 88.4% (nt) and 96.9% and 96.3% (aa) with the German and Argentine isolates from *A. cepa*, respectively. The Sudanese OYDV isolate was more distant from the Italian and German isolates than the Argentine isolate, with respective nt identities of 85.2%, 85.3%, 88.9% and aa identities of 93.1%. 93.6%, and 96.3%.

Some residues differentiated the Italian OYDV-O isolates from the three foreign isolates, specifi-

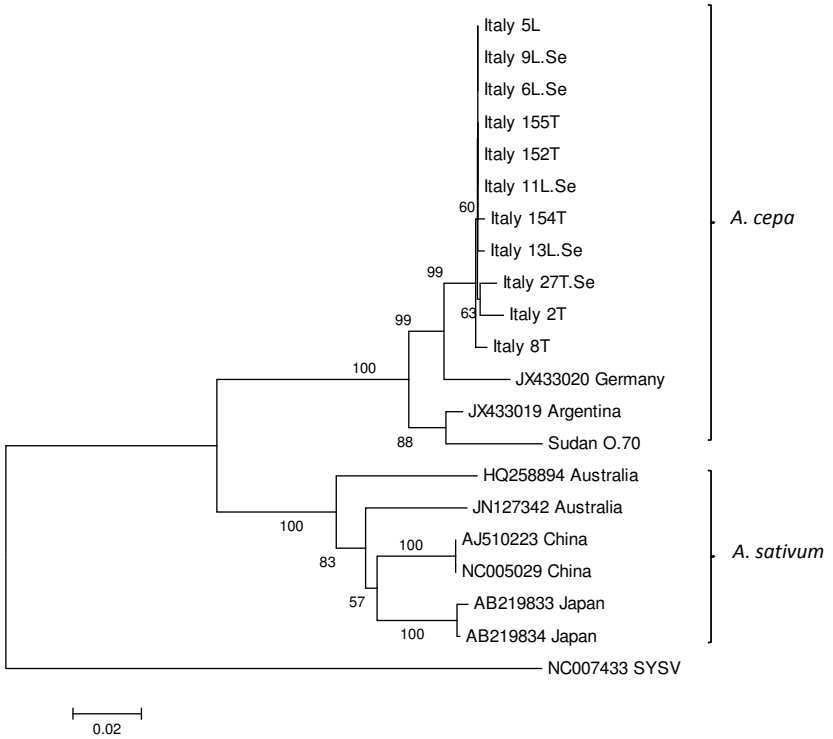

Figure 3. Neighbour-joining tree based on multiple alignment of complete NIb protein sequences of OYDV between Italian and Sudanese isolates from this study, German and Argentine isolates from onion and isolates from garlic available from the database. The sequence of SYSV (NC007433) was used as an out-group. Bootstrap percentages of clades, reported along the branches of the tree with value >50%, derived from bootstrap-resampled data sets (1000 replications).

cally at positions 79 (V instead of I), 279 (K instead of R/N/M), 338 (M instead of L/T/P), 343 (D instead of E) and 364 (E instead of G or K) of NIb gene. Moreover, at least 22 conserved residues of different polarities distinguished the onion isolates from the garlic isolates (data not shown) showing identity values from 75.2% to 77.2% at nucleotide levels and from 83.5% to 85.5% at amino acid levels. The highest variability was located in the C terminus just before the NIb/CP cleavage site. The phylogenetic analysis based on amino acid sequences (Figure 3), showed that onion isolates again grouped together in a separate cluster from the garlic isolates, maintaining a host grouping in the NIb coding region.

CP protein: variability and analysis

The CP coding region of Italian OYDV-O isolates was 771 nt long with a predicted protein of 257 aa. The CP coding region was highly conserved among the 15 isolates (11 from Italy and one from Sudan of this study and, three isolates from Argentina, Germany and The Netherland retrieved from the GenBank) sharing identity values with each other's from a minimum of 87.5% to 100% at the nucleotide level (Table 3). The lowest value of this range was related to the Sudanese OYDV-O when compared with Argentine, German and The Netherlands isolates, while this isolate showed an identity value of approx. 89% with Italian isolates. The predicted CP protein showed only a few differences at amino acid levels in a few OYDV-O isolates compared to the majority of the onion group (data not shown), where the Sudanese isolate was the most divergent from the other onion isolates with 95.3% of average aa identities due to 12 dissimilar residues, many of different polarities, mainly in the N-terminal region.

The putative CP protein of all OYDVs from onion contained the aphid transmissibility aspartic acid-alanine-glycine (DAG) triplet at the C-terminus of

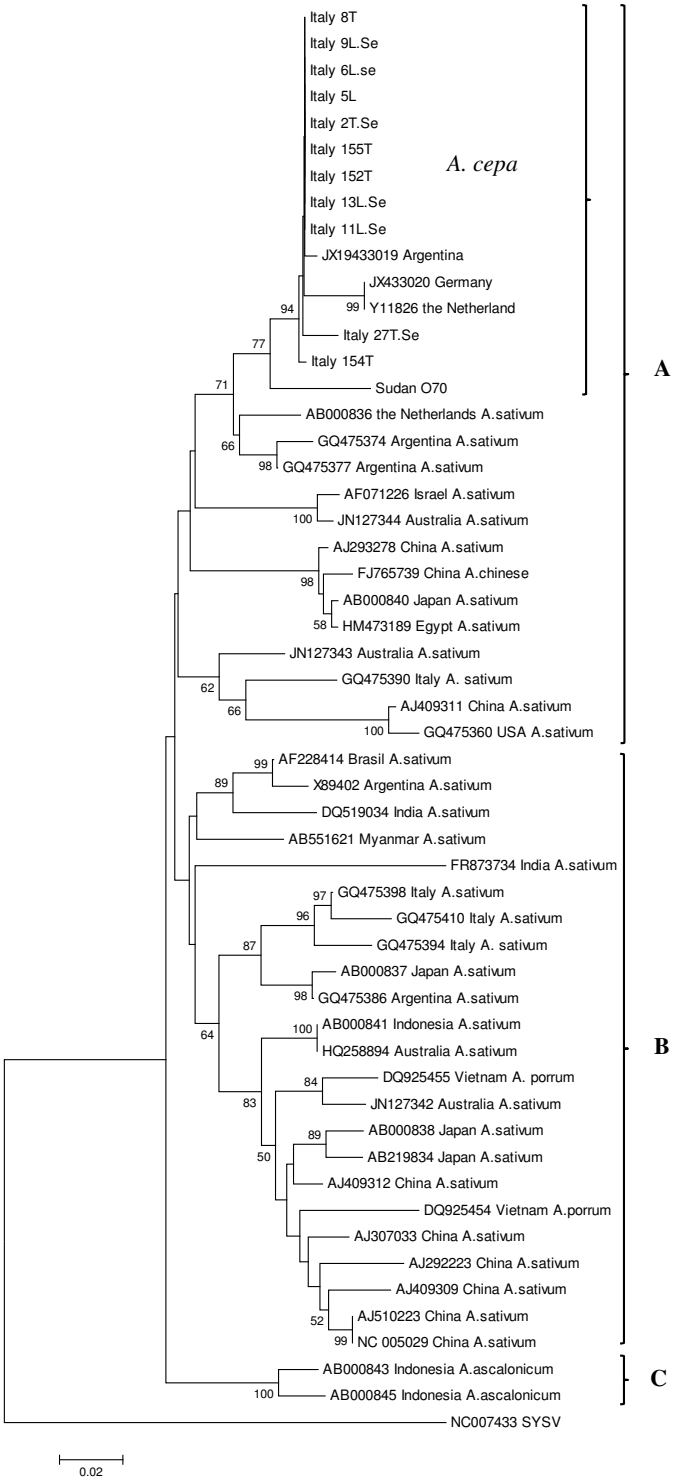

Figure 4. Neighbour-joining tree based on multiple alignment of complete CP protein sequences of OYDV between Italian and Sudanese isolates from this study, three onion isolates and 35 garlic isolates available from the database. The sequence of SYSV (NC007433) was used as an out-group. Bootstrap percentages of clades, reported along the branches of the tree with value >50%, derived from bootstrap-resampled data sets (1000 replications).

the protein. Sequence similarities between OYDV-O and OYDV-G were from 79% to 87.5% at the nt level and from 81.3% to 96.5% at the aa level (Table 3). The variability amongst the isolates was in the N-terminus of the CP region which also produced the greatest changes in aa residues in the predicted CP protein. Conversely, the residue at position 3 regarding the NIb/CP cleavage site, as mentioned above, was a neutral amino acid (P). This was conserved among the onion isolates but differed from almost all of the other 35 *Allium* isolates showing an acidic amino acid (D or E). In addition, at position 220 all onion isolates revealed an A instead of a T which is conserved in all *Allium* isolates.

Phylogenetic analysis based on the CP protein of 15 onion, 30 garlic and five from different *Allium* spp. isolates indicated that OYDV isolates, a part from two *A. ascalonicum* isolates, grouped into two main separate groups, in which the geographical origin was not a discrimination character (Figure 4). The onion isolates grouped together in group A but in a separate clade close to three OYDV-G isolates from

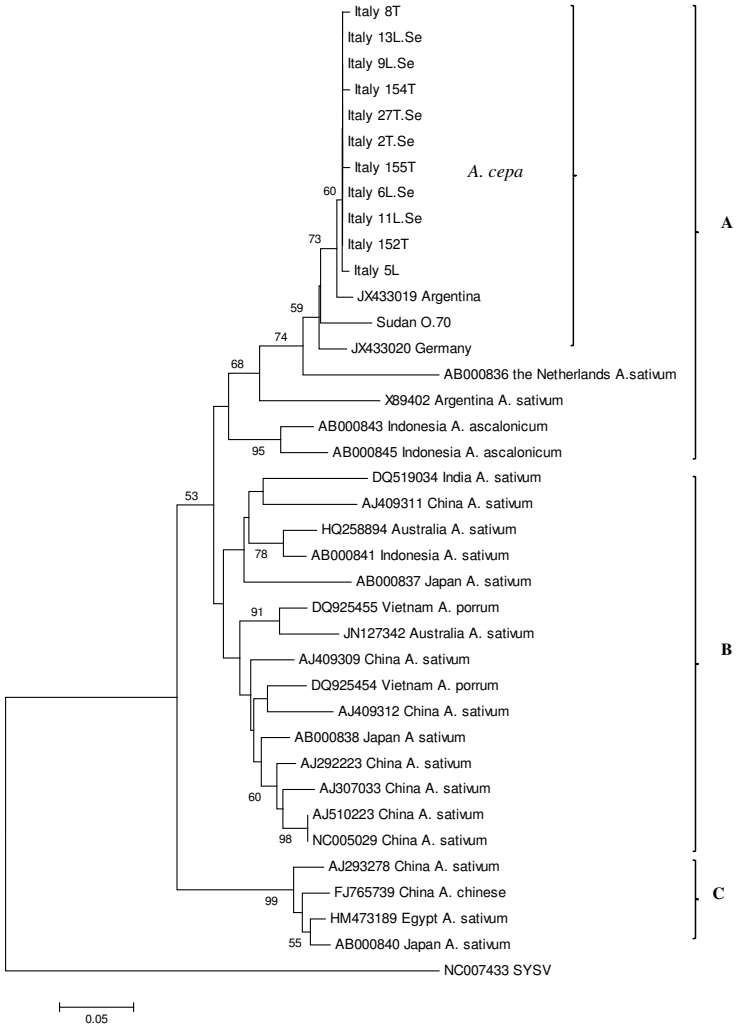

Figure 5. Neighbour-joining tree based on multiple alignment of 3'UTR sequences of OYDV between Italian and Sudanese isolates from this study, Argentine and German onion isolates and 23 *Allium* spp. isolates available from database. The sequence of SYSV (NC007433) was used as an out-group. Bootstrap percentages of clades, reported along the branches of the tree with value >50%, derived from bootstrap-resampled data sets (1000 replications).

non-Asian countries, which shared an aa homology of 95–96%. The Argentine OYDV-O showed the nearest genetic evolutionary relationship with Italian isolates. On the other hand, OYDV-O70 from Sudan was clearly distant from all the other onion isolates. The phylogenetic tree showed low bootstrap values (<50%) for the major branches. However, the analysis using the Maximum Parsimony method retained the sub-grouping as reported above.

3'UTR variability and analysis

The 3' UTR sequences of isolates from Italy were almost all identical, with the exception of two isolates which differed by only one nucleotide. In addition, they were more similar to the OYDV isolate from Argentina (98.6%) and slightly dissimilar (92.2%) from Sudanese and German isolates. The 3' UTR sequences of all OYDV isolates from onion contained 3 or 5 nt less among CP 37-38 residues than the corresponding sequences of the majority of OYDV-G isolates retrieved from the GenBank. Between OYDV-O and OYDV-G, nucleotide identity values in the 3'UTR region ranged from 80% to 90.48% (Table 3).

Phylogenetic analysis of the 3' UTR confirmed three major groups (Figure 5) with few differences from the CP phylogenetic tree. In A, all onion isolates were grouped on the evolution linkage of two OYDV-G from the Netherlands and Argentina.

Recombination analysis

Recombination detection analysis of the complete 2427 nucleotide sequences gave one significant event. This event shows recombination between the Italian genotype (in particular OYDV 152T with 95.3% similarity) as major parent and the Sudanese genotype (90.7% similarity) as the minor parent, which led to a recombinant isolate identified in the Argentine isolate. In this recombination event, a region (748-1626 nt of the analyzed nucleotide sequence) of the Argentine isolate was replaced with the core and 3' terminal NIb region of the Sudan isolate. A high degree of confidence was shown by six recombination detection methods implemented in RDP 4 program (i.e. average *P*-value of 5.035×10^{-7} by RDP; 3.673×10^{-6} by BOOTSCAN; 5.287×10^{-11} by Maximum ChiSquare; 5.354×10^{-12} by Chimera; 6.509×10^{-11} by Sister Scan; 1.983 × 10-^{10}by 3Seq).

Discussion

Although OYDV was firstly identified in onion (Melhus *et al*., 1929), studies on onion isolates have been mainly restricted to diagnosis and disease effects on the crops; moreover, only two complete (the Argentine and German isolates) and one partial (the Netherland isolate) sequences have been deposited in Genbank. On the contrary, many studies have been carried out on OYDV from infected garlic (OYDV-G) focusing on the characterization and phylogenetic analysis of partial or complete genome of the virus isolates mainly from Asia but also from Argentina, Africa and Europe including Italy (Shiboleth *et al*., 2001; Arya *et al*., 2006; Baghalian *et al*., 2010; Parrano *et al*., 2012; Soliman *et al*., 2012; Mohammed *et al*., 2013).

Our data extend existing knowledge since several OYDV-O isolates were partially sequenced and examined. Firstly, the comparison of the 3' terminal 2637 nt region of the genome, encompassing the partial NIa-Pro, the complete NIb and CP genes and 3'UTR, of the 11 OYDV-O Italian isolates revealed a high nucleotide and amino acid sequence identity each other (Table 3). This homogeneous population is undoubtedly due to the same geographic origin of the isolates (the same cultivar grown in the same IGP protected area during a limited period of survey). In the past, OYDV-O was detected in Italy but the isolates were not sequenced (Marani and Bertaccini, 1983; Dovas and Vovlas, 2003; Parrella *et al*., 2005) and during our previous studies no more Italian isolates were found in regions other than Calabria (data not shown). Consequently, molecular variability analysis amongst isolates from different Italian regions was not possible.

It was interesting to include in our study the geographically distant Sudanese isolate (OYDV-O.70) which showed significant differences in nucleotide and amino acid sequences when compared to the Italian onion isolates showing a nt and aa identities not more than 89% and 94.7%, respectively. When the phylogenetic relationship was extend to the other three available OYDV-O, the analysis of the 3' nt terminal region, the NIb and CP proteins revealed that the Sudanese isolate is an ancestor of the other onion isolates (Figures 2, 3 and 4) with a closer relationship to the Argentine isolate only in the NIb region (Figure 3).

The recombination analysis explained the different position of the Argentine isolate in the phyloge-

netic trees generated from different regions, showing that the recombination event with the Sudanese isolate as minor parent occurred in the NIb region. This first recombinant study of OYDV-O provides the first important context in the virus evolution, and motivates further research within the species.

In our study, the Italian and the Sudanese OYDV-O isolates were also compared with the corresponding published sequences of the isolates from different *Allium* spp. This analysis revealed that nucleotide identity in the NIb/CP/3'UTR region was greater in the maximum average value (85.0%) than 81.5% reported by Celli *et al*. (2013), who suggested that OYDV-O and OYDV-G might be different species. Equally, when NIb, CP and 3'UTR regions were separately analyzed for similarity to garlic isolates, the greater number of onion isolates determined an increase in the minimum and maximum identity values (Table 3).

Our data limited to the investigated region therefore indicate that onion and garlic isolates could still be considered strains of the same species, according to the proposed thresholds for species demarcation in the *Potyviridae* family (NIb: 75%; CP: 76%; 3'UTR: 76%) (Adams *et al*., 2005b).

When phylogenetic analysis of OYDV was performed, the separation of isolates into onion and garlic groups was only obtained when the 3' terminal nt region (partial NIa-Pro/NIb/CP/3'UTR) and NIb protein trees were constructed (Figures 2 and 3). On the contrary, the phylogenetic trees processed in the CP gene and 3'UTR (Figures 4 and 5) which included greater numbers of OYDV-G sequences from *A. sativum*, *A. chinense*, *A. porrum* and *A. ascalonicum*, showed that the onion isolates were not grouped separately from OYDV-G. Onion isolates were phylogenetically close to OYDV-G originating in non-Asian countries along the linkage of OYDV-G from the Netherlands (AB000836) and Argentina (GQ475374; GQ475377; X89402) sharing with them nt identity values from 83–87%. In the light of these results and according to the convention statement of the 9th ICTV Report on species demarcation criteria over whole genomes (< 76%) and in the CP protein (<80%) in the family *Potyviridae* (King *et al*., 2011), OYDV-O and OYDV-G might again be considered strains of the same virus and not two different virus species.

In accordance with this strain differentiation, substitutions of one residue at polyprotein cleavage sites, as observed at NIa-Pro/NIb and NIb/CP sites between OYDV-O and OYDV-G, are frequent in many potyvirus species (http://dpvweb.net/potycleavage/species.html). These do not always support species demarcation within the genus *Potyvirus* (Adams *et al*., 2005a).

Moreover, even if OYDV-O and OYDV-G are host-specific as onion isolates cannot infect garlic and leek whereas garlic isolates infects shallot, leek and scallion but not onion (Katis *et al*., 2012; Celli *et al*., 2013), this biological performance between strains of the same viral species is quite frequent in plant viruses (i.e., potyviruses). This is controlled by a few amino acid mutations in genes involved in virus-host interactions and long distance movement (Boevink and Oparca, 2005; Lopez-Moya *et al*., 2009).

There are not enough data (complete genomes) on OYDV-O from the Middle East and Central Asia, the probable regions of origin of *A. cepa* (Messiaen and Rouamba, 2004), to assume different ancestors for the two OYDV strains. In any case, the world exchange of vegetative materials and the cultivation in the same area of different *Allium* species has probably favoured mixed populations or recombinant isolates in many countries, since geographical origin is not a demarcation criterion in OYDV phylogenetic grouping (Figures 4 and 5).

In conclusion, this study and the recent genetic analysis of Celli *et al*. (2013) highlight the need for more extensive investigations to better clarify the taxonomic relationships between OYDV isolates originating from different *Allium* spp. hosts. It would be very worthwhile to examine more OYDV-O isolates from other countries, mainly from the geographical areas where the *A. cepa* is presumed to have originated as well as from countries where this crop is widely grown. Despite the failure in finding other Italian isolates from regions other than Calabria, where the typical 'Rossa di Tropea' onion is grown, the foreign isolate from Sudan enabled us to identify the recombination event amongst the OYDV-O and some molecular specificity of these isolates compared to those of garlic isolates (Parrano *et al*., 2012). Further research is now underway aimed at obtaining complete sequences of the Italian OYDV isolates and collecting more virus isolates.

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