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Emergence of tomato leaf curl New Delhi virus in Italy: estimation of incidence and genetic diversity

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Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus (family *Geminiviridae*) identified for the first time in 1995 in Asia, from where it spread into several countries of the Mediterranean basin. ToLCNDV was found in Spain in 2012, and subsequently in Tunisia and Italy. The first outbreak in Italy occurred at the end of 2015 in Trapani province (Sicily) on zucchini squashes. Then in 2016, ToLCNDV was found in infected zucchini plants in Campania, Lazio and Sardinia regions, and in 2017 in Calabria. This study addressed the dispersion and genetic diversity of ToLCNDV isolates in Italy. A total of 1400 plants were analysed. Phylogenetic analysis showed low variability among the Italian isolates, probably as a consequence of the recent introduction and rapid spread of this virus in Italy. Two statistically significant clusters were reported: one grouping only Italian isolates and the other grouping Italian, Spanish, Tunisian and Moroccan isolates. Furthermore, the highest incidence of ToLCNDV was observed in Sicily, although the disease also appears to be critical in other Italian regions. In this work, a high efficiency of ToLCNDV mechanical transmission into *Cucurbita pepo*, *Cucumis melo inodorus* and *Cucumis melo cantalupensis* has been demonstrated. The rapid spread of ToLCNDV in the Mediterranean basin represents a threat for horticultural production, thus it is very important to develop suitable crop management practices, applying genetic resistance strategies and more restrictive phytosanitary measures.

Keywords: begomovirus, epidemiology

Introduction

Zucchini squash (*Cucurbita pepo*) is an important horticultural crop in Italy that accounts for more than 18 800 ha in greenhouse and open-field, with production of about 550 000 tonnes in 2017 (Agri ISTAT, 2017). This crop is cultivated throughout the country, with Apulia, Sicily, Lazio and Calabria regions representing about 70% of the total production in Italy (Agri ISTAT, 2017). In recent years, zucchini squash cultivation has increased in Italy (Agri ISTAT, 2017), probably because of strong interest by the food industry. Every year the presence of endemic diseases as well as the emergence, and likely establishment, of new pathogens undermine the cultivation of zucchini crops.

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Among the most common and dangerous pathogens, viral diseases cause considerable economical losses worldwide (Hanssen *et al.*, 2010). Some aspects of epidemiology of these pathogens are well understood but the control of viral diseases is often difficult. This is mainly due to the great ability of viruses to evolve rapidly through mutation and genetic recombination, which enables them to quickly adapt to changing environmental conditions and to overcome plant genetic resistances (Elena *et al.*, 2014). Therefore, it is extremely important to understand the dispersion of these pathogens and their evolutionary tendency, especially in anthropized agroecological contexts such as intensive cultivation, in order to plan appropriate intervention measures.

In recent years, some begomoviruses (genus *Begomovirus*, family *Geminiviridae*) have spread to temperate regions, becoming a serious threat to a number of economically important crops, as they are responsible for severe production losses (Navas-Castillo *et al.*, 2011). For example, *Tomato leaf curl New Delhi virus* (ToLCNDV), originating from India, was recently

Plant Pathology

detected in Murcia (southeastern Spain) (Juárez *et al.*, 2014), where it has been responsible for severe epidemic outbreaks in zucchini squash and for significant economic losses to cucurbit crops (López *et al.*, 2015). Furthermore, ToLCNDV was reported in Tunisia (Mnari-Hattab *et al.*, 2015) and in Italy (Panno *et al.*, 2016). In this scenario, the presence and dispersal of ToLCNDV in the Mediterranean basin represents a threat for zucchini squash production, and other economically important crops such as tomato and pepper, as well as other Solanaceae and Cucurbitaceae crops. In fact, the number of new hosts of ToLCNDV increases every year, as reported by the European and Mediterranean Plant Protection Organization (EPPO, 2018).

ToLCNDV is a typical bipartite begomovirus whose genome consists of two circular ssDNA components, named DNA-A and DNA-B (Padidam *et al.*, 1995) of 2.7 and 2.6 kb, respectively. DNA-A encodes all information for viral encapsidation and replication, produces virions, and can replicate autonomously (Rogers *et al.*, 1986).

DNA-B cannot replicate in the absence of DNA-A, but it is required for symptom expression, systemic movement, nuclear localization and systemic infection (Sanderfoot & Lazarowitz, 1996). An intergenic region (IR) is present in both DNA-A and DNA-B, and contains a common region (CR). The main topological feature of the CR is a hairpin structure with a conserved nonanucleotide sequence (TAATATT↓AC) that spans the virion strand origin of replication (*ORI*, indicated by ↓) (Padidam *et al.*, 1995).

ToLCNDV symptoms may range from foliar yellowing or spotting, necrosis or mosaic on leaves, leaf area reduction and stunting. In zucchini squash, ToLCNDV causes severe leaf curling, swelling of veins of young leaves, shortening of internodes, yellow mosaic in young leaves, roughness of the skin of fruit and reduced fruit size (Panno *et al.*, 2016), reducing the marketability of the fruits. The virus is transmitted by the whitefly *Bemisia tabaci*. This vector exists in tropical and subtropical regions and the Mediterranean basin, and is a major threat to agronomically important plants (Sharma & Prasad, 2017). *Bemisia tabaci* transmits begomoviruses in a circulative persistent manner, which are mostly restricted to the phloem of infected plants (Rosen *et al.*, 2015).

The aim of this study was to evaluate the dispersion and genetic diversity of ToLCNDV in Italy, following outbreaks occurring in different regions in 2015 and 2016 (Luigi *et al.*, 2016; Panno *et al.*, 2016; Bertin *et al.*, 2018). Further, the present work reports a genetic analysis of this begomovirus with the aim of better understanding its evolutionary dynamics and field epidemiology.

Materials and methods

Isolate collection

A total of 1400 zucchini squash plants were collected, regardless of whether the plants showed symptoms or not, from different areas in the following Italian regions: Sicily, Campania, Lazio, Sardinia and Calabria (Table 1). The surveys began in Sicily in 2015 and were extended to Campania, Lazio and Sardinia in 2016, and to Calabria at the end of 2017. The sampling areas selected in each region were marked by GPS using the PLANTHOLOGY mobile application (Davino *et al.*, 2017), and inspected every year.

For each area, 50 samples were collected from two different fields, according to the following scheme: out of 100 rows of plants, 10 were selected (one every 10 rows). Then, on each selected row, a sample was taken every 10 plants, for a total of five samples per row.

Screening of ToLCNDV using loop-mediated isothermal amplification (LAMP) assay

In order to assess the presence/absence of ToLCNDV, the collected samples were analysed using a LAMP kit (Enbiotech srl). LAMP experiments were carried out using unprocessed sap extracts, taking advantage of the low sensitivity of *Bst* enzyme to the inhibitors (Francois *et al.*, 2011).

Each sample was placed in an extraction plastic bag (BIOREBA) containing 1 mL of extraction buffer (50 mM Tris pH 9.0, 150 mM LiCl, 5 mM EDTA), and homogenized with a HOMEX 6 homogenizer (BIOREBA). Healthy zucchini plants grown in a phytotron with a 16/8 h light/dark photoperiod, 27 °C and 70–90% relative humidity, were used as negative controls.

LAMP reaction was performed by adding 3 μL of sap extract in a 0.2 mL tube containing the reaction mixture, following the manufacturer's instructions. LAMP was carried out in an

Table 1 Incidence of *Tomato leaf curl New Delhi virus* (ToLCNDV)-infected plants detected by LAMP, for every year of survey in each inspected area, and number of samples used for molecular analysis for each Italian region investigated.

		Year					
		Incidence (%)[a]			No. of samples used for molecular analysis		
Region	Area or city	2015	2016	2017	2015	2016	2017
Sicily	Agrigento	0	78	84	0	3	3
	Catania	0	14	54	0	3	3
	Ragusa	0	38	60	0	3	3
	Siracusa	0	28	44	0	3	3
	Trapani	54	78	84	3	3	3
	Subtotal	11	47	65	3	15	15
Campania	Napoli	N.A.	36	38	0	8	3
	Caserta	N.A.	40	40	0	4	3
	Subtotal	N.A.	38	39	0	12	6
Lazio	Terracina	N.A.	26	26	0	7	0
	Fondi	N.A.	14	14	0	3	0
	Sabaudia	N.A.	10	10	0	2	0
	Subtotal	N.A.	17	17	0	12	0
Sardinia	Cagliari	N.A.	42	34	0	2	0
	Subtotal	N.A.	42	34	0	2	0
Calabria	Catanzaro	N.A.	N.A.	24	0	0	2
	Subtotal	N.A.	N.A.	24	0	0	2
ITALY	TOTAL	—	—	43	3	41	23

N.A., no available data.

[a]50 samples were collected every year from each area.

ICGENE mini-machine supplied by Enbiotech srl, incubating the mixture at 65 °C for 40 min. The results were reported in a tablet, running a specific program showing the trend of the curve in real time, connected to the ICGENE mini via Bluetooth.

Mechanical transmission and preliminary host range characterization

Three ToLCNDV isolates, one from Sicily (SIC17RG), one from Campania (CAMP16NA) and one from Lazio (LAZ16RO) were mechanically inoculated in *Cucurbita pepo*, *Cucumis melo inodorus*, *C. melo cantalupensis*, *Cucumis sativus*, *Solanum lycopersicum*, *Solanum melongena* and *Capsicum annuum*. Sap extracts of each isolate were mechanically inoculated into three plants per host. Plants were grown in sterilized soil in an insect-proof glasshouse, with a photoperiod of 14 h light and a target air temperature set at 28/20 °C day/night. Symptoms were recorded weekly for the host range characterization, and the presence of ToLCNDV was evaluated in all plants by PCR.

Polymerase chain reaction and sequencing

From samples with positive LAMP results, 67 (Table 1) were retrieved for subsequent molecular analysis. Total DNA was extracted following the method described previously by Noris *et al.* (1994). The purified DNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The PCR assay was carried out using primers ToLCNDV-CP1 5′-CTCCAAGAGATTGAGAAGTCC-3′ (nucleotides 191–212; GenBank acc. no. KF891468) and ToLCNDV-CP2 5′-TCTGGACGGGCTTACGCCCT-3′ (nucleotides 1220–1240; GenBank acc. no. KF891468), specially designed for this work, which encompassed the AV1 (coat protein) gene. PCR was performed in a 25 μL final reaction volume, containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 μM each primer, 0.08 mM KB extender buffer and 2 U Platinum *Taq* DNA polymerase (Thermo Fisher Scientific). A final concentration of 10 ng μL^{-1} DNA was used as template. PCR was carried out in a MultiGene OptiMax thermal cycler (Labnet International Inc.) according to the following conditions: 94 °C for 3 min; 40 cycles of 94 °C for 50 s, 52 °C for 1 min, and 72 °C for 2 min; and a final elongation at 72 °C for 10 min. PCR products were sequenced in both directions using an ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems).

Sequence data analysis

Phylogenetic analyses were carried out using the obtained nucleotide sequences as well as the CP sequences of 38 ToLCNDV isolates from different countries, retrieved from GenBank. The CLUSTALW algorithm (Larkin *et al.*, 2007) was used to construct a multiple nucleotide sequence alignment. A mathematical model, considering nucleotide frequencies and instantaneous rate change, was used to estimate the number of nucleotide substitutions. The model that fitted best was the Tamura–Nei model TN93 (Tamura, 1992), modelled by using a discrete gamma distribution (+G) = 0.56 with five rate categories and by assuming that a certain fraction of sites is evolutionarily invariable (+I). Phylogenetic relationships were inferred by the maximum-likelihood method (ML) (Nei & Kumar, 2000) with 1000 bootstrap replicates to estimate the statistical significance of each node (Efron *et al.*, 1996). Initial trees for the heuristic search were obtained automatically by applying neighbour-joining and BIONJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All analyses were performed using MEGA v. 7 (Kumar *et al.*, 2016).

To investigate the presence of recombination events among the nucleotide sequences obtained from Italian isolates, the algorithms GENECONV, BOOTSCAN, MAXCHI, SISCAN, 3SEQ, LARD and RDP within the RDP 3 program suite (Martin *et al.*, 2010) were used. Only concordant results among different algorithms were considered as a positive result.

Nucleotide sequence diversity of the ToLCNDV-AV1 gene was estimated within and between different countries (for this experiment 114 sequences retrieved from GenBank were used), which were considered as geographic populations, using the Jukes–Cantor model (Jukes & Cantor, 1969). The genetic differentiation between populations was assessed by three permutation-based statistical tests: K_S*, Z* and S_{nn} (Hudson, 2000). At the same time the rate of gene flow adapted for the

Table 2 Host range of the three different isolates of *Tomato leaf curl New Delhi virus* (ToLCNDV) on different plants.

	Host plant (infected/inoculated)							
Isolate	*Cucurbita pepo*	*Cucumis melo inodorus*	*C. melo cantalupensis*	*Cucumis sativus*	*Solanum lycopersicum*	*Solanum melongena*	*Capsicum annuum*	WPI[a]
SIC17RG	0/3	0/3	0/3	0/3	0/3	0/3	0/3	I
	0/3	0/3	0/3	0/3	0/3	0/3	0/3	II
	3/3	2/3	3/3	0/3	0/3	0/3	0/3	III
	3/3	2/3	3/3	0/3	0/3	0/3	0/3	IV
CAMP16NA	0/3	0/3	0/3	0/3	0/3	0/3	0/3	I
	0/3	0/3	0/3	0/3	0/3	0/3	0/3	II
	3/3	2/3	3/3	0/3	0/3	0/3	0/3	III
	3/3	2/3	3/3	0/3	0/3	0/3	0/3	IV
LAZ16RO	0/3	0/3	0/3	0/3	0/3	0/3	0/3	I
	0/3	0/3	0/3	0/3	0/3	0/3	0/3	II
	3/3	1/3	3/3	0/3	0/3	0/3	0/3	III
	3/3	1/3	3/3	0/3	0/3	0/3	0/3	IV

[a]WPI, weeks post-inoculation.

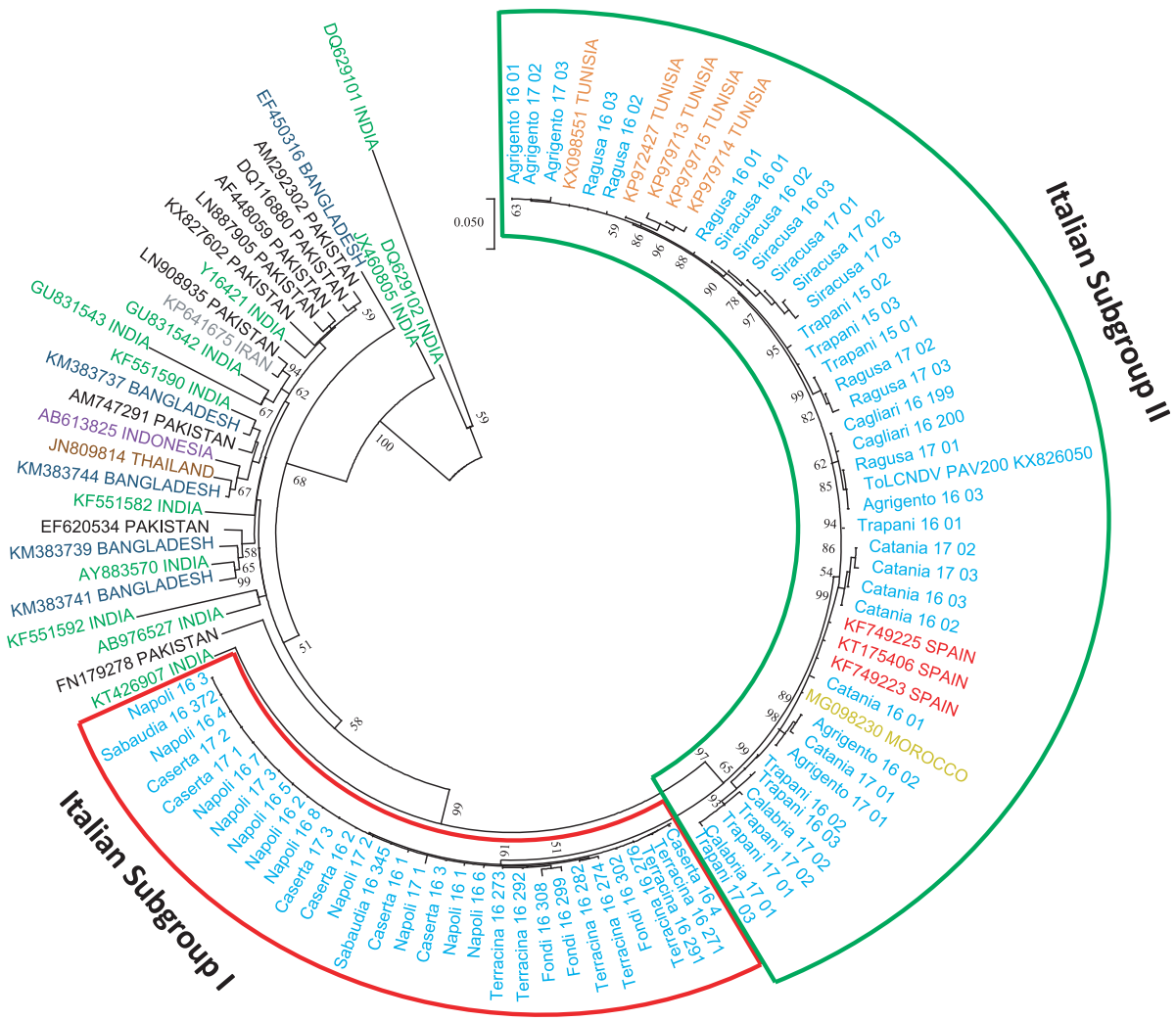

Figure 1 Phylogenetic relationships between coat protein genes (AV1) of Italian tomato leaf curl New Delhi virus (ToLCNDV) isolates and isolates from other countries inferred by maximum-likelihood method (ML) based on the Tamura 3-parameter model with bootstraps of 1000 replications, conducted with MEGA 7. Only bootstrap values >50% are indicated in the nodes. Colours (online) represent different countries: Italy, blue; Tunisia, orange; Spain, red; Morocco, gold; India, green; Pakistan, black; Bangladesh, dark-blue; Iran, grey; Thailand, brown; Indonesia, purple. [Colour figure can be viewed at wileyonlinelibrary.com].

virus population, here referred to as the 'migration rate', was estimated applying the statistic F_{ST} index (Weir & Cockerham, 1984). It is important to note that viruses are haploid and, for this reason, in order to calculate the allelic frequency, each sequence was coded as doubled haploid. All these tests were carried out within the DNASP v. 5.0 program (Librado & Rozas, 2009).

The role of natural selection in the Italian isolates at the molecular level was studied by evaluating the rate of synonymous substitutions per synonymous site (dS) and the rate of nonsynonymous substitutions per nonsynonymous site (dN), separately. These values were estimated by the Pamilo–Bianchi–Li method (Pamilo & Bianchi, 1993) within the MEGA v. 7 program. Selection at individual codon sites was statistically tested using the fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) methods available from the Datamonkey server (http://www.datamonkey.org) (Kosakovsky Pond & Frost, 2005). Only concordant results obtained through both methods were considered.

Results

Incidence of ToLCNDV infection

A total of 34.7% of zucchini squash samples (485 out of 1400) collected in different Italian regions from 2015 to 2017 and analysed by LAMP assay were infected by ToLCNDV (Table 1). In Sicily, the incidence showed an upward trend from 11% in 2015, to 47% and 65% observed in 2016 and 2017, respectively. In Sardinia, from 2016 to 2017 the disease moved from Cagliari province to the northern province of Oristano, mainly affecting melon crops (data not shown); in Lazio the rate of incidence by visual inspection according to typical symptom presence appeared constant, in 2016 to 2017. In Campania, the incidence showed a moderate but

Table 3 Nucleotide diversity[a] and gene flow (F_{ST}) of *Tomato leaf curl New Delhi virus* (ToLCNDV) in different geographical populations.

Population	*n*[b]	Bangladesh	India	Iran	Pakistan	Thailand	Spain	Tunisia	Italy
Bangladesh	7	**0.047 ± 0.004**	0.062 ± 0.004	0.047 ± 0.005	0.049 ± 0.004	0.048 ± 0.005	0.080 ± 0.007	0.086 ± 0.007	0.085 ± 0.007
India	73	0.041	**0.071 ± 0.005**	0.055 ± 0.004	0.058 ± 0.004	0.068 ± 0.006	0.090 ± 0.007	0.096 ± 0.007	0.095 ± 0.007
Iran	3	0.449	0.300	**0.004 ± 0.001**	0.042 ± 0.005	0.058 ± 0.007	0.084 ± 0.009	0.090 ± 0.009	0.090 ± 0.008
Pakistan	17	0.127	0.051	0.498	**0.003 ± 0.003**	0.053 ± 0.006	0.078 ± 0.008	0.084 ± 0.008	0.083 ± 0.008
Thailand	4	0.302	0.337	0.801	0.476	**0.018 ± 0.003**	0.888	0.090 ± 0.008	0.089 ± 0.008
Spain	3	0.701	0.599	0.973	0.759	0.083 ± 0.008	**0 ± ∞**	0.012 ± 0.003	0.009 ± 0.002
Tunisia	5	0.659	0.569	0.914	0.713	0.835	0.533	**0.011 ± 0.002**	0.020 ± 0.003
Italy	68	0.628	0.541	0.899	0.682	0.809	0.180	0.336	**0.015 ± 0.001**

Nucleotide diversity within a group is reported in bold in the diagonal, while nucleotide diversity between groups are reported above the diagonal. Gene flow is shown below the diagonal.

[a]Nucleotide diversity was measured by the Jukes–Cantor method.

[b]Number of isolates for each population.

constant increase over time, while in Calabria the end of 2017 represents the first important outbreak in this region.

Preliminary host range

As reported in Table 2, mechanical inoculation successfully transmitted SIC17RG, CAMP16NA and LAZ16RO to *C. pepo*, *C. melo inodorus* and *C. melo cantalupensis*. All the isolates induced symptoms related to ToLCNDV disease, such as curling and interveinal yellowing in young leaves. None of the tested ToLCNDV isolates was mechanically transmitted to *C. sativus*, *S. lycopersicum*, *S. melongena* and *C. annuum*.

Polymerase chain reaction and sequencing

All the samples analysed by PCR yielded the expected fragment of 1050 bp, comprising the entire AV1 gene. The obtained sequences were trimmed to remove the external nucleotides, leaving only the 771 nt coding for the AV1 gene. The sequences were deposited in GenBank under the accession numbers MH475370–MH475436.

Sequence data analysis

Molecular analysis was carried out on the AV1 genes of 68 ToLCNDV Italian isolates (67 from this work and one deposited in GenBank in 2016 (Panno *et al.*, 2016)), and isolates retrieved from GenBank from different countries. Regarding the sequences of the Italian ToLCNDV isolates, no recombination events were detected from the analysis carried out using the RDP 3 program (data not shown), so all of them were used in phylogenetic analysis.

Evolutionary relationships among ToLCNDV sequences used in the present study, represented by the phylogenetic tree in Figure 1, showed that isolates of ToLCNDV were separated into two statistically significant clusters: the first one composed only of isolates from the Mediterranean basin, showing low variability, and the second one composed of isolates from the Asian continent, which were more variable. The Italian isolates were grouped into two clades: one clade, with a bootstrap confidence of 91% (subgroup I) including isolates from Lazio and Campania regions, and one clade, with a bootstrap confidence of 54% (subgroup II) consisting of isolates from Sicily, Calabria and Sardinia together with isolates from Spain, Tunisia and Morocco.

Analysis of nucleotide diversity between the different Mediterranean countries showed very low differentiation between Italy and groups from Spain and Tunisia, while for Morocco it was impossible to calculate the nucleotide diversity because only one sequence was available. Interestingly, this unique sequence, from a phylogenetic point of view, is not divergent from the other Mediterranean populations of ToLCNDV.

A greater difference was observed between Mediterranean basin isolates and the other groups from Asia

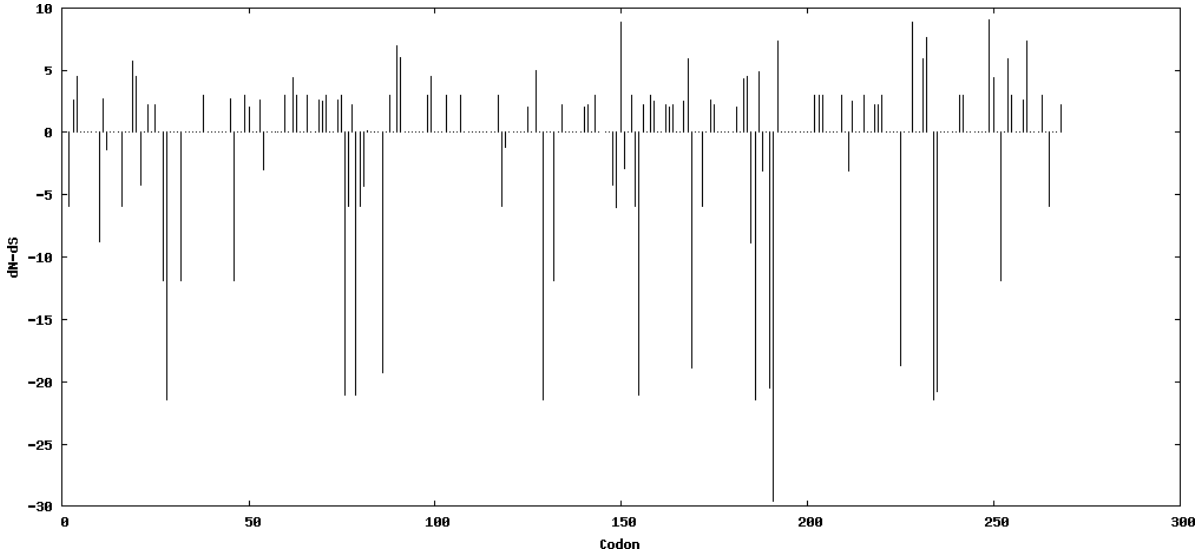

Figure 2 Amino acid sites of the coat protein gene of *Tomato leaf curl New Delhi virus* (ToLCNDV) under positive or negative selection. The *x*-axis represents amino acid coordinates in the coat protein while the *y*-axis represents normalized [dN − dS] values.

(Table 3). The intragroup analysis reported very low differentiation in the Italian, Spanish and Tunisian groups, suggesting a common origin, while a certain level of differentiation seems to have developed in the countries of the Asian continent. The genetic differentiation between Mediterranean basin groups and Asian groups was significant ($P \leq 0.05$) by means of all K_S*, Z* and S_{nn} tests (data not shown). To evaluate the gene flow level between different populations, the statistical F_{ST} value was calculated; this parameter was used, generally, to evaluate the 'migration rate' at genetic level between different populations. When two populations have a value close to zero it means that they have a similar distribution of sequence variants, while when the value is proximal to one, it indicates total separation and no 'migration' is underway between the two populations. In this sense, the results clearly indicated a high gene flow between Spain and Italy and Tunisia (and probably Morocco; however, no statistical tests support this hypothesis because only one sequence is available from this country), and separation between these populations and the Asiatic populations (Table 3). Analysing the sequences, a very low degree of genetic variability was observed in the Spanish population, which would also explain the stability of the Italian population, apparently derived from the Spanish one. This hypothesis is also supported by the phylogenetic analysis (Fig. 1).

The AV1 gene of the Italian ToLCNDV isolates showed dN and dS values of 0.012 ± 0.001 and 0.039 ± 0.010, respectively, with a dN/dS ratio of 0.307. These values confirm the hypothesis of negative selection. Finally, analysis of selection at individual codons carried out only for the Italian sequences with Datamonkey tools identified some positively selected sites (Fig. 2), that could indicate the occurrence of adaptive processes.

Discussion

Tomato leaf curl New Delhi virus has been recently introduced in Europe, first in Spain (Juárez *et al.*, 2014) in 2012 and in subsequent years in several countries of the Mediterranean basin, such as Tunisia and Italy (Mnari-Hattab *et al.*, 2015; Panno *et al.*, 2016). It was demonstrated that the isolates emerging in the western Mediterranean basin constitute a group of closely related isolates, according to the criteria currently accepted by the International Committee on Taxonomy of Viruses, i.e. that ≥91% and ≥94% nucleotide sequence identity account for begomovirus species and strain demarcation, respectively (Brown *et al.*, 2015). Therefore, these isolates constitute a new strain of this virus, for which the name ES (ToLCNDV-ES) was proposed (Moriones *et al.*, 2017). Evolutionary relationships among ToLCNDV sequences used in this study showed that all the Italian isolates are closely related to those from Spain, Tunisia and Morocco and thus a common origin can be assumed for these isolates, identifiable in ToLCNDV-ES. As ToLCNDV rapidly spread in a few years within the Mediterranean area, the low level of variability found among isolates supports the hypothesis of the founder effect associated with a population bottleneck during the spread to this new area (Moriones *et al.*, 2017). As reported by different authors (Juárez *et al.*, 2014; Mnari-Hattab *et al.*, 2015; Luigi *et al.*, 2016; Panno *et al.*, 2016; Moriones *et al.*, 2017), ToLCNDV in the Mediterranean basin found zucchini squash as its elected host. In these countries, this cucurbit is intensively cultivated during the entire year in the open field (summer crops) as well as in the greenhouse (autumn-spring crops). The prolonged presence of this host, as well as the mild climate favouring overlapping cycles of the vector, have facilitated the rapid spread of the virus.

It has been demonstrated that ToLCNDV-ES is probably the result of genetic recombination between a ToLCNDV-type isolate and other begomoviruses (Moriones *et al.*, 2017). Recombination exchanges at the intraspecies and interspecies level, due to mixed infection in the same host, are frequent in begomoviruses and considered more significant than mutation as a driving force in the evolution of begomoviruses, by increasing genetic variability and allowing adaptation to new agroecological contexts, including novel hosts (Davino *et al.*, 2009, 2012). For these reasons, the appearance of new ToLCNDV variants in Italy cannot be ruled out as a result of recombination events with endemic begomoviruses. In this regard, new hosts of significant economic value such as tomato, eggplant and pepper could suffer significant losses in the future.

To date, very low genetic diversity has been found within Italian isolates (0.015 ± 0.001) and between Italian and Spanish isolates (0.009 ± 0.002). Interestingly, genetic diversity between Italian and Tunisian and between Spanish and Tunisian isolates was higher (0.020 ± 0.003 and 0.012 ± 0.003, respectively) compared to that found between Italian and Spanish isolates, strengthening the hypothesis of the Spanish origin of Italian isolates. A low level of variability was observed among the Italian isolates, probably as a consequence of the recent introduction and fast spread of ToLCNDV in Italy, so few mutations have accumulated. Furthermore, the most likely hypothesis seems to be that the virus was introduced in Sicily through viruliferous whiteflies onto plants of strawberry (*Fragaria* spp.) imported from Spain. Nevertheless, considering the low level of gene flow, it is not clear if another accession of ToLCNDV arrived in Sicily from Tunisia, or the population structure of Sicilian and Tunisian isolates are closely related due to having a common origin from Spain.

The results obtained here show that Sicily has the highest number of infected plants among the Italian regions, due to the fact that the first outbreak of ToLCNDV in Italy was in Sicily, and the disease moved to the other regions a year later. However, between 2016 and 2017, the incidence of ToLCNDV in the other Italian regions also appears to be critical, and as a result ToLCNDV must be continuously monitored in order to understand the epidemiology and prevent any host change. In fact, it is of fundamental importance to prevent the virus from also infecting tomato crops that, today, represent one of the most economically important crops of southern Italy.

Sicily is one of the most important horticultural regions in the Mediterranean basin and represents the principal access point of plant material from the countries of the European Union, increasing the risk of introducing new pathogens. The trade could be adversely affected by these new introductions and Italian horticulture would be particularly at risk.

As reported by López *et al.* (2015), ToLCNDV can be transmitted in *C. melo*. The present study confirmed the mechanical transmission of the virus to *C. pepo*, *C. melo inodorus* and *C. melo cantalupensis*.

To avoid the spread of this virus and the introduction of new pathogens or pests to Sicily and other Italian regions, it is very important to carry out correct crop management, to use new rapid molecular methods (Panno *et al.*, 2014) to identify pathogens for early warning and surveillance and, of substantial importance, develop genetic resistance strategies and more restrictive phytosanitary measures at international borders.

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