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Founder effect, plant host, and recombination shape the emergent population of begomoviruses that cause the tomato yellow leaf curl disease in the Mediterranean basin

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Abstract

Tomato yellow leaf curl disease (TYLCD)-associated viruses present a highly structured population in the western Mediterranean basin, depending on host, geographical region and time. About 1,900 tomato and common bean samples were analyzed from which 111 isolates were characterized genetically based on a genome sequence that comprises coding and non-coding regions. Isolates of three distinct begomoviruses previously described were found (*Tomato yellow leaf curl virus*, TYLCV, *Tomato yellow leaf curl Sardinia virus,* TYLCSV, and *Tomato yellow leaf curl Málaga virus*, TYLCMalV), together with a novel recombinant virus. Mixed infections were detected in single plants, rationalizing the occurrence of recombinants. Except for TYLCV-type strain, single, undifferentiated subpopulations were present for each virus type, probably the result of founder effects. Limited genetic variation was observed in genomic regions, with selection against amino acid change in coding regions.

Keywords: Begomovirus; Common bean; Founder effect; Genetic diversity; Mixed infections; Population structure; Recombination; Tomato; Tomato yellow leaf curl disease

Introduction

Analysis of the genetic diversity and structure of plant virus populations is crucial for a better understanding of virus evolution and interaction with host plants. The forces that determine genetic variation and shape the genetic structures of natural plant virus populations (mutation, recombination, reassortment, selection, genetic drift, population bottlenecks caused by vectors, host plants and agricultural practices) have been reviewed (García-Arenal et al., 2001, García-Arenal et al., 2003; Gibbs et al., 1999; Roossinck, 1997). Detailed genetic analyses are available for plant RNA viruses, whose potential for genetic variation has been explained, in part, by the error-prone replication (Elena and Sanjuan, 2005; García-Arenal et al., 2001; Moya et al., 2004). However, reports on the genetic diversity and structure of natural populations of DNA plant viruses are scarcer and less detailed (Ooi and Yahara, 1999; Sánchez-Campos et al., 2002; Sanz et al., 1999; Stenger and McMahon, 1997). Based on available studies, either for RNA or DNA plant viruses the rule for natural populations seems to be high genetic conservation (reviewed in García-Arenal et al., 2001).

Viruses associated with the tomato yellow leaf curl disease (TYLCD) are single-stranded DNA viruses of the genus *Begomovirus* (family *Geminiviridae*) that severely constrain crop production and continue to emerge world-wide (Seal et al., 2006; Stanley et al., 2005; Varma and Malathi, 2003). Different virus species and strains of the same virus species have been recognized associated with TYLCD (Fauquet et al., 2003; Stanley et al., 2005). These begomoviruses pose a severe threat to tomato (*Solanum lycopersicum* L.) and common bean (*Phaseolus vulgaris* L.) production in many warm and temperate regions of the world (reviewed in Moriones and Navas-Castillo, 2000). Transmission in nature is through the whitefly *Bemisia tabaci* Gen. (*Hemiptera: Aleyrodidae*). Most begomoviruses have bipartite genomes comprising two DNA components (A and B). DNA A encodes a replication-associated protein (Rep), the coat protein (CP), and proteins such as a replication enhancer

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protein (REn), and a transcription activator protein (TrAP), which participate in the control of replication and gene expression. DNA B encodes virus-movement proteins. Open reading frames are organized bi-directionally in both genome components, separated by a non-coding intergenic region which contains key elements for replication and transcription (Gutierrez, 1999; Hanley-Bowdoin et al., 2000; Stanley, 1995). Several begomoviruses have a monopartite genome which resembles DNA A, among them most of the TYLCD-associated viruses (Stanley et al., 2005).

TYLCD epidemics occur in the Mediterranean basin since the late 1980's (Kheyr-Pour et al., 1991; Navot et al., 1991; Noris et al., 1994). In Italy, the type and Sic strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) have been present in Sicily and Sardinia since 1989 (Crespi et al., 1995; Kheyr-Pour et al., 1991), and the type strain of *Tomato yellow leaf curl virus* (TYLCV) was more recently reported in Sicily (Accotto et al., 2003). In Spain, the ES strain of TYLCSV (Noris et al., 1994) and the Mld and type strains of TYLCV (Morilla et al., 2003; Navas-Castillo et al., 1999) have been reported since 1992, 1997 and 2002, respectively. In addition, in this latter country the presence and spread in the population of a natural recombinant virus, *Tomato yellow leaf curl Málaga virus* (TYLCMalV), was reported in 1999, as the result of a genetic exchange between TYLCV-Mld and TYLCSV-ES (Monci et al., 2002). It should be noted that recombination ability in begomovirus populations (Padidam et al., 1999) provides a great variation potential and thus greater opportunity to evolve and rapidly adapt and emerge in changing environments (García-Arenal and McDonald, 2003; Seal et al., 2006).

For an evolutionary point of view, diversity of viruses should be studied at a population rather than at individual level (García-Arenal et al., 2001). Little is known about the genetic diversity and structure of populations of TYLCD-associated viruses, except for partial studies made in Spain and Italy (Davino et al., 2006; Sánchez-Campos et al., 2002). Populations studies in Spain concentrated on the diversity and evolution of a specific virus type, TYLCSV-ES, for which low genetic diversity and high genetic stability was found in the population during an 8-year period (Sánchez-Campos et al., 2002). In Italy, less detailed studies were done based on a 3-year survey which suggested that TYLCSV and TYLCV species could co-exist during epidemics (Davino et al., 2006), although the virus strains present were not examined. Following these preliminary studies, in this report we examine the population of viruses associated with TYLCD at a regional scale based on surveys performed in Italy and Spain between 1999 and 2003, to infer agro-ecological and diversification processes shaping the evolutionary pattern. It is of interest to understand factors driving evolution and structure of virus populations in order to design more durable control strategies. Results showed that distinct genotypes existed and that spatial, temporal, and host differences occur, probably associated with founder and agro-ecological effects. Moreover, mixed infections were found and a novel recombinant was detected which supported the role of recombination as a source of genetic diversity contributing to virus evolution. Interestingly genetic variation was restricted in genomic regions.

Results

TYLCD-associated virus population structure.

Table 1 summarizes the results of the analysis of about 1,900 samples collected during the survey conducted between 1999 and 2003 in Italy and Spain for samples of tomato and common bean plants infected with TYLCD-associated viruses. In Italy, only TYLCSV-like infections were detected in Sardinia, and in Sicily during 1999 and 2001. However, during 2002, a significant proportion of the samples collected in Sicily reacted with a probe to TYLCV. In Spain, samples that hybridized with TYLCV and/or TYLCSV probes were detected.

The nature of the begomoviruses involved in natural infections was determined more precisely by sequencing isolates from a number of positive samples randomly selected

Table 1
Analysis for infection with tomato yellow leaf curl disease (TYLCD)-associated viruses in samples from a survey conducted between 1999 and 2003 in commercial crops of tomato and common bean of the major growing areas of Italy and Spain [a]

Country	Zone	Crop	Year	No. of samples collected (no. of fields visited)	No. (%) of samples positive with probes to the IR of: [b]			Total no. (%) of TYLCD-infected samples [c]
					TYLCSV	TYLCV	TYLCSV+TYLCV	
Italy	Sicily	Tomato	1999	40 (4)	26 (100)	0 (0)	0 (0)	26 (65)
	Sicily	Tomato	2001	13 (3)	13 (100)	0 (0)	0 (0)	13 (100)
	Sicily	Tomato	2002	165 (4)	7 (5)	32 (23)	100 (71.9)	139 (84.2)
	Sardinia	Tomato	2003	135 (4)	117 (100)	0 (0)	0 (0)	117 (86.6)
Spain	Murcia	Tomato	2003	499 (9)	0 (0)	199 (99)	2 (1)	201 (40.3)
	Almería	Tomato	2003	470 (9)	0 (0)	169 (100)	0 (0)	169 (35.9)
	Málaga	Tomato	2003	316 (8)	8 (4.8)	150 (90.4)	8 (4.8)	166 (52.5)
	Almería	Bean	2003	94 (3)	0 (0)	5 (10.4)	43 (89.6)	48 (51.1)
	Málaga	Bean	2003	172 (5)	0 (0)	22 (28.6)	55 (71.4)	77 (44.8)

[a] Samples were collected during summer/autumn by systematic sampling regardless of presence of symptoms, following a W-shaped itinerary (Lin et al., 1979), and consisted of one terminal leaf per plant.

[b] Samples were tested by tissue blot hybridization on positively charged nylon membranes, using digoxigenin-labeled DNA probes specific to *Tomato yellow leaf curl Sardinia virus*(TYLCSV), or *Tomato yellow leaf curl virus*(TYLCV) based on the intergenic region (IR) (Navas-Castillo et al., 1999). Percentage was calculated over the total number of TYLCD-infected samples detected from each zone/crop/year.

[c] Total number of samples positive in hybridization analyses; percentage was calculated over the total number of samples collected from each zone/crop/year.

from the population. In Italy, 12 tomato samples were selected per year (for 1999 and 2002 in Sicily, and for 2003 in Sardinia), and in Spain, 15 samples were selected per region (Almería, Málaga, and Murcia provinces) and host species (tomato and common bean) from samples collected in 2003. Thus, 111 nucleotide sequences (referred here to as 'isolates') were derived (GenBank DQ317696 to DQ317806) from TYLCD-associated begomoviruses present in the analyzed samples, 99 from cloned molecules (for samples from Spain and Sicily), and 12 from direct sequencing of PCR amplified fragments (for samples from Sardinia) (cf. Materials and methods). Nucleotide sequences of about 780 nt (30% of the genome) were deduced from cloned molecules, encompassing coding (5′-proximal Rep and C4 ORFs, and 5′-proximal V2 and CP ORFs) and non coding (entire IR) regions, and about 590 nt (20% of the genome) from PCR fragments, encompassing the same regions except the V2 and CP.

As shown in Fig. 1, phylogenetic analyses revealed presence of isolates that grouped with three distinct TYLCD-associated begomovirus species previously described, and strains of the same virus species: the type, Sic and ES strains of TYLCSV, the type and Mld strains of TYLCV, and TYLCMalV. The twelve isolates from Sardinia, tightly grouped with the TYLCSV-Sic type virus (not shown).

Genetic structure studies revealed striking differences in the population depending on the host in Spain. Thus, 70% of the total variance of the population (F_{ST}=0.700, P<0.001) in this country was explained by the host origin of isolates. In tomato (red in Fig. 1), isolates of the type strain of TYLCV were the most frequently found, whereas in common bean (green in Fig. 1), TYLCMalV-like isolates were predominant. In addition to this host differentiation, spatial differentiation also was observed in the population. Thus, in tomato in Spain regional differences were revealed when comparing the subpopulation found in Málaga (red-italics text in Fig. 1) with that present in Murcia and Almería (red-boxed and red-normal text, respectively, Fig. 1). About 23% of the total variance (F_{ST}=0.229, P=0.001) was explained by the geographical origin of isolates. In Málaga, isolates of the type and Mld strains of TYLCV, TYLCSV-ES, and TYLCVMalV were detected whereas isolates of the type strain of TYLCV were almost the only found in Murcia and Almería. Spatial differences also were detected in Italy. In this case, the subpopulations from Sicily (isolates in blue in Fig. 1) or Sardinia (all isolates of the TYLCSV-Sic type, not shown) differed, and about 38% of the total variance (F_{ST}=0.378, P=0.001) was explained by the regional origin. Spatial differences in the population structures were also evident when comparing subpopulations from tomato present in Italy or Spain (isolates in blue and red, respectively, in Fig. 1), with about 52% of the total variance (F_{ST}=0.519, P<0.001) associated to country origin. In fact, Italian and Spanish subpopulations only shared isolates of the type strain of TYLCV, and even those grouped separately (Fig. 1). It should be noted that some temporal effect could not be excluded on spatial differences found between virus subpopulations from different regions of Italy or between subpopulations from Italy and Spain, because samples were not collected in the same year. In fact, differences in the population structure with time could occur, as revealed in Sicily when comparing 1999 and 2002 subpopulations (normal and italic blue text, respectively, Fig. 1). In this case, only TYLCSV-like isolates were detected in 1999, whereas in 2002 about 50% of the isolates grouped with the type strain of TYLCV.

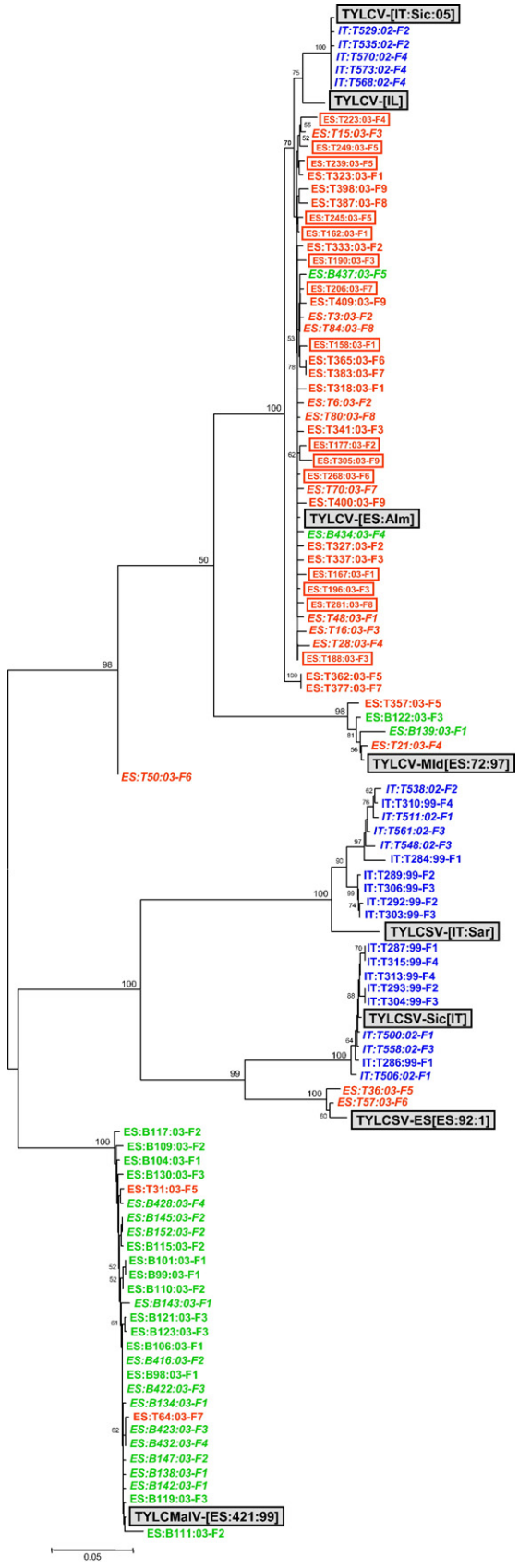
It was surprising the high frequency at which isolates of the type strain of TYLCV were detected in tomato in Spain, because this virus was recently reported in this country (Morilla et al., 2003). However, analyses of samples systematically collected in previous years (1997 to 2002) from commercial tomato crops (Monci et al., 2002; Sánchez-Campos et al., 1999, 2002) revealed that this virus has been present since 1998 (Fig. 2). Moreover, evolution of frequencies in each region suggested that a progressive spread of this virus type occurred first in Almería, then in Murcia, and finally in Málaga.

Genetic homogeneity is the norm in subpopulations of TYLCD-associated virus types

The genetic diversity within the subpopulations of begomoviruses well represented in the population was studied based on the nucleotide sequences derived from cloned molecules (Table 2). Either for the type or Sic strains of TYLCSV, for TYLCV, or for TYLCMalV subpopulations, low genetic diversity values were found in coding (Rep and V2) and non-coding (IR) regions (Table 2), suggesting genetic homogeneity, being the IR the most diverse region, except for TYLCMalV. Restriction to variation was suggested in coding regions based on the d_{NS}/d_S ratios lower than the unit (except for Rep in TYLCMalV). Moreover, variation in the V2 coding region seemed to be more constrained, as shown by the lower d_{NS}/d_S ratios (Table 2), except in TYLCSV-Sic.

As the IR region was the most variable, it was selected to further analyze differentiation between subpopulations of the different virus types, considering as a subpopulation the group of isolates from a given year or region. Temporal comparisons could be done for subpopulations of the type and Sic strains of TYLCSV collected in Sicily during 1999 and 2002. In either case no significant differences (P>0.05, t-test) were observed in values of between or within subpopulation comparisons (Table 3), suggesting no genetic differentiation with time. Interestingly, significantly higher within-population diversity values were observed for the type strain of TYLCSV than for TYLCSV-Sic (P<0.05) which suggests a more diverse subpopulation of the former virus.

Spatial comparisons could be done for subpopulations of TYLCMalV present in Málaga and Almería in Spain (14 isolates per zone). In this case, between-population diversity (0.00016±0.00011) was significantly smaller than within-population diversity (0.00454±0.00150 in Málaga, 0.00620±0.00196 in Almería) (P<0.005), suggesting no genetic differentiation related to geographic region. Similarly, for the type strain of TYLCV in Spain a single, undifferentiated population seems to be present as suggested by the smaller between-population nucleotide diversities than within-population diversities (P<0.001) (Table 4). In contrast, striking genetic



differences seemed to exist between subpopulations of the TYLCV type strain present in Spain and Italy, as suggested by the significantly larger diversity values found between-populations than within-populations ($P<0.001$) (Table 4). As mentioned above, this genetic differentiation also was presumed by the separate grouping of TYLCV type strain isolates from Spain and Italy observed in the phylogenetic tree shown in Fig. 1.

Mixed infections and emergence of recombinant viruses

Interestingly, in Italy and Spain numerous samples reacted with both TYLCSV and TYLCV probes (Table 1). This was especially evident in Sicily in 2002 and in Spain, in common bean, and suggested the possible existence of field samples with multiple infections (Table 1). However, as IR-based probes were used for detection, presence of viruses with recombinant IR could not be excluded. This might be the case of common bean in Spain, in which isolates of TYLCMalV, a virus with a recombinant IR (Monci et al., 2002), predominated (see above and Fig. 1). In contrast, in Italy phylogenetic relationships of isolates derived from samples of the same sampling year, indicated that different strains of the same virus species (e.g. type and Sic strain isolates of TYLCSV in Fields 1 and 3 in 2002, Fig. 1), or of different virus species (e.g. TYLCSV and TYLCV isolates in Field 2 in 2002. Fig. 1) could coexist in a single field. Therefore, existence of mixed infections in single plants was further studied. Restriction fragment length polymorphism (RFLP) analysis using *Bgl*II restriction endonuclease on clones obtained from single samples allowed to discriminate between virus species and strains (Fig. 3). Results for sample IT:T304:99-F3 suggested that isolates of the type and Sic strains of TYLSCV could be present co-infecting a sample (compare lane 3 with other lanes, Fig. 3A). Furthermore, mixed infections with isolates of TYLCV and of the two strains of TYLCSV also were suggested from results of sample IT:T570:02-F4 (compare e.g.

Fig. 1. Phylogenetic relationships for a sequence of about 780 nucleotides encompassing the non-coding intergenic region and 5′-proximal parts of Rep and V2 open reading frames derived for tomato yellow leaf curl disease (TYLCD)-associated begomovirus-like molecules cloned from tomato samples collected in Sicily (Italy) during 1999 and 2002 (normal and italics blue text, respectively) or from tomato and common bean (red and green text, respectively) samples collected in Spain during 2003 in Almería, Málaga, or Murcia provinces (normal, italics, and boxed text, respectively). Relationships were inferred by neighbor-joining analysis. Data were analyzed using MEGA 3.1 software (Kumar et al., 2004). Support for nodes in a bootstrap analysis with 1000 replications is shown for values over 50%. Horizontal branch lengths are drawn to scale with the bar indicating 0.05 nucleotide replacements per site. Vertical distances are arbitrary. GenBank accession numbers for sequences obtained in the present study are DQ317696 to DQ317784, and DQ317797 to DQ317806. Isolates are identified using a code that refers to country (ES, Spain; IT, Italy), host species origin (T, tomato; B, common bean), sample number:year, and field (F_i, field i). Representative isolates are included (boxed and shadowed text) of begomoviruses associated with TYLCD in the Mediterranean area: the type, Sic and ES strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV), type (isolates from Israel-IL-, Spain, and Italy) and Mld strains of *Tomato yellow leaf curl virus* (TYLCV), and *Tomato yellow leaf curl Málaga virus* (TYLCMalV) (GenBank accession numbers X61153, Z28390, Z25751, X15656, AJ489258, DQ144621, AF071228, and AF271234, respectively). Similar results were obtained with parsimony analyses.

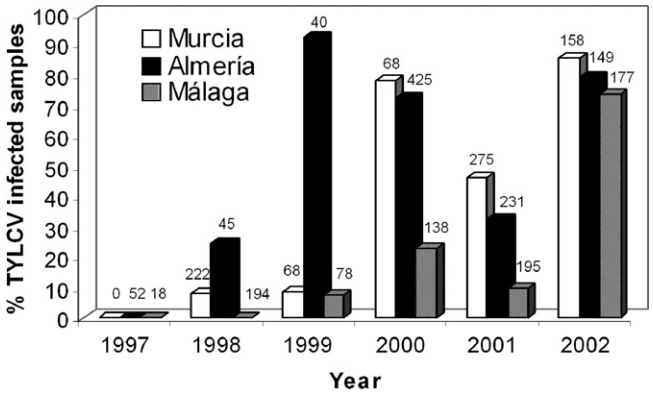


Fig. 2. Percentage of tomato samples that reacted with a probe specific to the type strain of *Tomato yellow leaf curl virus* (TYLCV) among samples infected with tomato yellow leaf curl disease (TYLCD)-associated viruses collected in Spain during random surveys of commercial crops conducted between 1997 and 2002 in Málaga, Almería, and Murcia. The numbers at the top of the bar indicate the total number of samples infected with TYLCD-associated viruses available in each case.

lanes 1–3 with lanes 7–8 and lanes 9–10, Fig. 3B). These results, confirmed by sequencing, indicated that mixed infections of isolates of different virus types occurred in single plants. Then, although not found, appearance of recombinants in the population seemed possible.

Interestingly, one isolate from Spain (ES:T50:03-F6, Fig. 1) did not group with any of the TYLCD-associated viruses reported so far. More detailed studies of its nucleotide sequence indicated contradictory phylogenetic relationships depending on the region (5′-half or 3′-half of the nucleotide sequence) compared (not shown), suggesting its recombinant nature. In fact, using the RDP program (Martin et al., 2005), a significantly high probability ($P = 1.0361^{-24}$) of recombination was found, with high affinities of the 5′-half with the equivalent region of the [ES:Alm] isolate of the type strain of TYLCV, and of the 3′-half with the equivalent region of the [ES:1:92] isolate of the ES strain of TYLCSV, both virus isolates reported from Spain (Morilla et al., 2005; Noris et al., 1994). This suggested the possible emergence of a novel recombinant virus genetic type in the population, evolved from extant viruses by genetic exchange.

Discussion

TYLCD-associated viruses are a model case of emerging plant viruses spreading worldwide causing severe damage to tomato and other crops. A better understanding of the genetic and biological composition of virus populations and factors affecting the rate and direction of their evolution will facilitate development of more effective and durable control strategies. Following local studies performed in Spain and Italy (Davino et al., 2006; Sánchez-Campos et al., 2002), this work presents the first complete population study of TYLCD-associated viruses at a regional scale. Information is provided here that can help to understand factors that drive their evolution.

A complex virus population was found associated with TYLCD epidemics in the western Mediterranean basin, with presence of three distinct but related TYLCD-associated begomovirus species including several previously described strains: the type, Sic and ES strains of TYLCSV, the type and Mld strains of TYLCV, and TYLCMalV (Accotto et al., 2000; Monci et al., 2002; Morilla et al., 2005; Navas-Castillo et al., 1999). This population structure is compatible with multiple introductions and spread of virus types in the region. The observed maintenance and spread in the population of TYLCMalV, a virus type of recent recombinant origin (Monci et al., 2002), was a confirmation that recombination can have profound effects driving evolution of this virus population. Moreover, we demonstrated here *de novo* creation of virus genetic differentiation as a result of genetic exchange between extant viruses. Thus, a novel virus genotype of recombinant nature was detected in Spain, that seemed to have resulted from

Table 2
Nucleotide diversities for different genome regions of isolates corresponding to the Sic and type strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV), the type strain of *Tomato yellow leaf curl virus* (TYLCV), and *Tomato yellow leaf curl Málaga virus* (TYLCMalV) present in Spain and Italy[a]

Genomic region	Nucleotide diversity	TYLCD-associated virus types			
		TYLCSV-Sic ($n=9$)	TYLCSV ($n=10$)	TYLCV ($n=45$)	TYLCMalV ($n=28$)
IR	d	0.00687 (0.00260)	0.02679 (0.00631)	0.02119 (0.00295)	0.00526 (0.00127)
Rep	d	0.00427 (0.00440)	0,00792 (0.00581)	0.01210 (0.00493)	0.00411 (0.00234)
	d_{NS}	0.00227 (0.00226)	0,00811 (0.00407)	0.00778 (0.00301)	0.00442 (0.00243)
	d_S	0.01384 (0.01721)	0,02920 (0.03238)	0.01595 (0.00686)	0.00296 (0.00225)
	d_{NS}/d_S	0.16	0.28	0.49	1.49
V2	d	0.00417 (0.00419)	0.01073 (0.00790)	0.00251 (0.00145)	0.00990 (0.00622)
	d_{NS}	0.00183 (0.00182)	0.00391 (0.00284)	0.00343 (0.00118)	0.00567 (0.00313)
	d_S	0.00471 (0.00510)	0.06829 (0.04530)	0.01226 (0.01182)	0.01961 (0.00830)
	d_{NS}/d_S	0.39	0.06	0.28	0.29

[a] Nucleotide sequences of about 780 nucleotides encompassing the non-coding intergenic region (IR) and 5′-proximal parts of Rep and V2 open reading frames derived for tomato yellow leaf curl disease (TYLCD)-associated begomovirus-like molecules cloned from samples collected in Italy and Spain during 1999 and 2002 (summarized in Fig. 1), were used. Nucleotide diversity (d), defined as the average number of nucleotide substitutions per site between pairs of sequences, was estimated according to Kimura's two-parameter method (Kimura, 1980). Nucleotide diversities were also computed separately for non-synonymous (d_{NS}) and synonymous (d_S) positions of Rep and V2 coding regions using the PBL method of the MEGA 3.1 software (Kumar et al., 2004). For this, nucleotides of Rep/C4 and V2/CP overlapping zones were not considered in the analysis. The number (n) of sequences compared is indicated in each case. Standard errors (in parenthesis) were calculated using the bootstrap method (Nei and Kumar, 2000) with 1000 replicates.

Table 3
Within- and between-population nucleotide diversity in the non-coding intergenic region for subpopulations of the type and Sic strains of *Tomato yellow leaf curl Sardinia virus* present in Sicily (Italy) by year[a]

Virus type	Year	1999	2002
TYLCSV-Sic	1999 (n=6)	0.00647 (0.00316)	0.00067 (0.00036)
	2002 (n=3)		0.00668 (0.00378)
TYLCSV	1999 (n=6)	0.02038 (0.00576)	0.01234 (0.00395)
	2002 (n=4)		0.01994 (0.00543)

[a] We considered a subpopulation as the set of isolates from a given growing season (1999 and 2002). Nucleotide diversity is defined as the average number of nucleotide substitutions per site between pairs of sequences and was estimated according to Kimura's two-parameter method (Kimura, 1980). Between-population diversity values correspond to net nucleotide substitutions as given by Nei (1987, p. 276). Standard errors (in parenthesis) were calculated using the bootstrap method (Nei and Kumar, 2000) with 1000 replicates. Nucleotide sequences derived from begomovirus genome clones obtained from infected samples were used. Number (n) of sequences available in each subpopulation is indicated.

a genetic exchange between TYLCV and TYLCSV-ES. This recombinant strongly recalls a new begomovirus recently characterized in this country from the wild reservoir *Solanum nigrum* (García-Andrés et al., 2006), thus suggesting its spread into cultivated hosts. Mixed infections of different virus types in single plants were identified here, rationalizing the occurrence of recombinants. It is worth noting that recombination might help evolution of these begomoviruses for rapid adaptation to the invaded area (García-Arenal and McDonald, 2003; Seal et al., 2006). Recombinant viruses might possess enhanced fitness, with unknown consequences for evolution of epidemics and virus population (Bürger, 1999; Moffat, 1999; Legg and Fauquet, 2004; Monci et al., 2002; Padidam et al., 1999).

Distribution of virus types in the population was not uniform. Striking differences were detected for example depending on the host species. A different population composition was found in tomato or in common bean in Spain, suggesting that host species can drive the structuration of the population, as observed in other pathosystems (Mastari et al., 1998; Moury et al., 2001). The type strain of TYLCV largely predominated in tomato, whereas TYLCMalV was the most frequently found in common bean. As tomato and common bean crops share growing regions and overlap in time, population differences should reflect differences in the host plant fitness of virus types. In fact, previous data also suggested that TYLCV is well adapted to tomato whereas TYLCMalV seems better adapted to common bean (Monci et al., 2002; Sánchez-Campos et al., 1999). However, we have not attempted to investigate the mechanisms involved in the differential host adaptation observed. This is an aspect that needs to be further studied because can shed light on viral pathogenesis mechanisms and thus provide tools to design new control strategies (Ohshima et al., 2002; Petty et al., 2000; Rojas et al., 2005; Sacristán et al., 2005).

There was indication of spatial structuration of the population. Differences were detected between regions in Italy and in Spain. Also, differences were observed between virus subpopulations present in these two countries, that only shared presence of the type strain of TYLCV (Fig. 1). It is worth noting that some temporal effect may account for the differences observed, because samples were not collected synchronously in all regions. In fact, temporal evolution of the population was suggested when comparing subpopulations present in Sicily in 1999 and 2002. In this case, differences probably associated with the introduction and spread of the type strain of TYLCV in Italy during that period (Accotto et al., 2003; Davino et al., 2006). Therefore, different histories of virus spread may determine spatial and temporal differences. Also, agro-ecological differences might influence. Thus, for example in Málaga, continuous cropping, overlapping begomovirus-susceptible plants species in open field and greenhouses, would favor the maintenance of the diverse virus types observed in that region. In contrast, in Almería and Murcia, a more pronounced disruption of growing cycle occurs during summer or winter, respectively, which might result in severe virus population bottlenecks that can accentuate founder effects in the following epidemics (Seal et al., 2006). Then, local extinctions and recolonizations from near or distant sources may determine the simpler genetic structures observed in the latter cases (Bebee and Rowe, 2004; Fraile et al., 1997; Novella et al., 1995; Sánchez-Campos et al., 2002). We cannot discard that differential adaptation to local *B. tabaci* populations of the begomoviruses detected could account for some of the differences observed.

The genetic structuration observed in the population of TYLCD-associated viruses present in the western Mediterranean basin is important because might condition the evolution of this population and therefore the success of the control programs implemented (García-Arenal et al., 2001; Gibbs et al.,

Table 4
Within- and between-population nucleotide diversities in the non-coding intergenic region for subpopulations of the type strain of *Tomato yellow leaf curl virus* by growing zone[a]

Zone	Almería (n=14)	Murcia (n=15)	Málaga (n=11)	Sicily (n=5)
Almería	0.01862 (0.00372)	0.00045 (0.00017)	0.00047 (0.00021)	0.03200 (0.00999)
Murcia		0.01691 (0.00316)	0.00037 (0.00017)	0.03278 (0.01027)
Málaga			0.01133 (0.00282)	0.03378 (0.01052)
Sicily				0.00130 (0.00128)

[a] We considered a subpopulation as the set of isolates from a given zone (Almería, Murcia, and Málaga in Spain, and Sicily in Italy). Nucleotide diversity is defined as the average number of nucleotide substitutions per site between pairs of sequences and was estimated according to Kimura's two-parameter method (Kimura, 1980). Between-population diversity values correspond to net nucleotide substitutions as given by Nei (1987, p. 276). Standard errors (in parenthesis) were calculated using the bootstrap method (Nei and Kumar, 2000) with 1000 replicates. Nucleotide sequences derived from begomovirus genome clones obtained from infected samples were used. The number (n) of sequences available from each subpopulation is indicated.

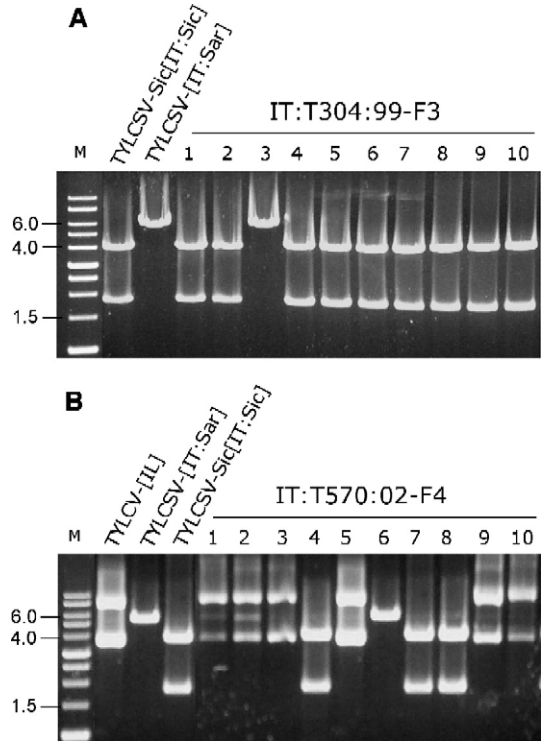


Fig. 3. Restriction fragment length polymorphism analyses with the restriction enzyme *Bgl*II on 10 almost full-length genome clones of tomato yellow leaf curl disease-associated begomoviruses derived from samples IT:T304:99-F3 (A) and IT:T507:02-F4 (B). Equivalent clones obtained from samples infected with known isolates of the type and Sic strains of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and of the type strain of *Tomato yellow leaf curl virus* (TYLCV) were used as controls. The size of DNA marker bands in lane M are indicated in kilobases.

1999; Lecoq et al., 2004). In this sense, it is worth noting that differences have been observed in the effectiveness of resistance genes depending on the TYLCD-associated virus that challenge resistant plants (de Castro et al., 2005).

Analyses of nucleotide sequences revealed a small genetic diversity within subpopulations of the different TYLCD-associated virus types analyzed here. Therefore, as suggested for natural populations of other plant viruses (García-Arenal et al., 2001), genetic stability also seems to be the rule for these viruses. Moreover, our data suggested that negative selection to variation exists in coding regions, similar to that reported for the begomovirus *Cotton leaf curl virus* (CLCuV) (Sanz et al., 1999). However, as shown in other plant viruses (Fargette et al., 2004; Rubio et al., 2001; Sanz et al., 1999; Schirmer et al., 2005; Tomimura et al., 2004), different restrictions to variation seemed to operate depending on the protein. Here we studied separately the Rep and V2 coding regions. The Rep region analyzed comprises the N-terminal part of the Rep, which is essential for specific recognitions during viral replication (Gutierrez, 1999; Hanley-Bowdoin et al., 2000; Heyraud-Nitschke et al., 1995; Orozco et al., 1997); the V2 region, comprises the 5′-end of V2 ORF, a protein that in TYLCSV plays a role in accumulation of single-stranded DNA and in successful infection of host plants (Wartig et al., 1997). Our data showed that except for TYLCV-Sic, the V2 was under tighter evolutionary constraints (lower d_{NS}/d_S ratios, Table 2), suggesting a more strict need to conserve features of this protein for virus functions, at least for the genome regions compared here. It is interesting to mention that data for the Rep coding region of TYLCMalV might suggest that positive selection is operating, based on the d_{NS}/d_S ratio above the unit deduced (Table 2). We can speculate that the recent emergence of this virus through genetic exchange between two TYLCD-associated virus species (Monci et al., 2002) put together genome fragments of diverse origin that might have determined less stable protein–protein or protein–DNA recognitions (Gutiérrez, 1999; Hanley-Bowdoin, 2000; Orozco et al., 1997). Therefore, evolution of the Rep could be needed for better fitness. However, the d_{NS}/d_S of TYLCMalV is larger than that of other viruses not because a d_{NS} value particularly higher, but because d_S is very low. Therefore, this is an aspect that needs to be further studied by examination of more TYLCMalV isolates.

Reports of very different detail are available on sequence variation in geminiviruses (Fauquet et al., 2005; Gilbertson et al., 1991; Hughes et al., 1992; Ndunguru et al., 2005; Pita et al., 2001; Stenger and McMahon, 1997). Genetic diversity studies within a single virus species are only available for few cases (e.g. Ooi et al., 1997; Sánchez-Campos et al., 2002; Sanz et al., 1999; Yahara et al., 1998). It is worth noting that nucleotide diversity values at synonymous positions of ORFs shown here for the TYLCD-associated viruses analyzed were much smaller than those reported for natural populations of the begomoviruses *Tobacco leaf curl virus* (Ooi et al., 1997) or CLCuV (Sanz et al., 1999). Therefore, less diverse natural begomovirus populations seemed to be present in the western Mediterranean basin. Also, comparison of subpopulations of the different begomovirus types suggested that except for the TYLCV-type strain, single, undifferentiated subpopulations were present. Founder effects could account for this highly homogeneous subpopulations, as suggested by Sánchez-Campos et al. (2002), because these viruses were not indigenous and spread recently into this area. For the type strain of TYLCV, data suggested that different virus types have founded the subpopulations of Italy and Spain. It should be noted that nucleotide sequence comparisons of isolates of the TYLCV-type strain reported so far suggested that at least four different phylogenetic types seem to be spreading worldwide (Fig. 1). Biological and epidemiological implications of such genetic differentiation are unknown. However, this is an aspect that merits to be further studied because TYLCVs seem to be very efficient invading new geographical areas (Davino et al., 2006; Polston et al., 1999).

Materials and methods

Field surveys

Fresh market tomato and common bean crops were surveyed for TYLCD-associated viruses. Surveys were conducted between 1999 and 2003 in affected regions of Italy and Spain. In Italy, tomato samples were collected in Sicily (1999, 2001 and 2002) and Sardinia (2003), the two major islands. In Spain, collections were made during 2003, in Málaga (southern Spain), Almería and Murcia (southeastern Spain) for tomato, and in

Málaga and Almería, for common bean. Samples were collected from plants by systematic sampling following a W-shaped itinerary (Lin et al., 1979), regardless the presence of TYLCD symptoms. Surveys were conducted during summer/autumn in affected commercial crops of the major growing areas. Each sample consisted of one terminal leaf per plant.

Sample analyses and isolate characterization

Freshly cross-sectioned petioles were squash-blotted on positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany). Tissue blots were analyzed by hybridization using digoxigenin (DIG)-labeled DNA probes able to detect all the TYLCD-associated viruses reported in the western Mediterranean basin (Accotto et al., 2000; Monci et al., 2002; Navas-Castillo et al., 1999). When needed, blots were also tested for specific detection of the type strain of TYLCV. For this, a polymerase chain reaction (PCR)-DNA probe was obtained using a DIG-labeling and detection Kit (Roche Diagnostics), based on TYLCV-[ES:Alm] (GenBank accession number AJ489258), with primers that amplify the 5′-half of the intergenic region (IR) (Navas-Castillo et al., 2000): MA250 (5′-GGTGTCCCTCAAAGCTCTATGGCAATCG-3′, corresponding to nt 2627 to 2654) and MA163 (5′-TTTGAATTTTGAATTTTGAATTGC-3′, complementary to nt 2753 to 2730). Total DNA was extracted from infected samples as described by Thompson et al. (2003). Fragments encompassing almost the full-length genome of the TYLCD-associated viruses present in infected samples were PCR-amplified from the extracted DNA. In order to minimize primer-oriented selection of sequence variants, amplification was performed with a primer pair designed on nucleotide sequences conserved among all the TYLCD-associated viruses reported from the western Mediterranean basin: MA241 (5′-GAATGGGCTTCCCATACTTTGTGTTGC–3′), corresponding to nt 1739 to 1765 of TYLCSV-ES [ES:1:92] (GenBank Z25751), and MA242 (5′-CACTATCTTCCTCTGCAATCCAGG-3′), complementary to nt 1719 to 1696 of this same virus. PCR was performed using the PCR Expand High Fidelity kit (Roche Diagnostics) in a GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA, USA) by first heating at 94 °C for 2 min, followed by 30 cycles at 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 2 min, adding 5 s of elongation at each cycle in the last 20 cycles, and then one cycle of elongation at 72 °C for 7 min. Amplified fragments were separated by electrophoresis in 0.8% agarose gels, excised from the gel and purified using the Ultrafree-DNA Kit (Millipore Corporation, Bedford, MA). The purified DNAs were cloned using the pGEM-T Easy System II Kit (Promega Corporation, Madison, USA), and plasmids were maintained in *Escherichia coli* DH5α. A fragment representing about one fifth of the viral genome was obtained from infected samples collected in Sardinia during 2003 by PCR amplification. In this case, a combination of three primers able to amplify any TYLCD-associated virus from the western Mediterranean basin was used: MA272 (5′-CTGAATGTTYGGATGGAAATGTGC-3′, corresponding to nt 2342–2365 on GenBank X61153), MA273 (5′-GGTTCGTAGGTTTCTTCAACTAG-3′, complementary to nt 225–247 on X61153), and MA274 (5′-GCTCGTAAGTTTCCTCAACGGAC-3′, complementary to nt 232–254 on GenBank X15656). PCR conditions were as described by Monci et al. (2002). Based on the almost full-length genome clones, a sequence of about 780 nt (approximately 30% of the genome) was derived from one randomly selected clone per sample using primer MA272, encompassing the 5′-proximal part of the Rep ORF (about 240 nt, including 88 nt overlapping with the 5′-end of the C4 ORF), the entire IR (about 310 nt) and the 5′-proximal part of the V2 ORF (about 230 nt, including 72 nt overlapping with the 5′-end of the CP ORF). By direct sequencing the partial genome PCR products obtained for samples from Sardinia with primers MA272 and MA273, a sequence of about 590 nt (approximately 20% of the genome) was derived, encompassing the same region as indicated above except for the V2 ORF. Sequences were obtained using an automatic sequencer (ABI Prism 3700 DNA analyser, Applied Biosystems).

Nucleotide sequences deduced for TYLCD-associated viruses present in the analyzed samples are referred here to as ‘isolates’. According to recommendations made by Fauquet and Stanley (2005) for isolate descriptors, isolates are identified here using a code that refers to country (ES, Spain; IT, Italy), host species origin (T, tomato; B, common bean), sample number: year, and field from which the sample was obtained (F_i, field i) (e.g. IT:T535:02-F2, means an isolate derived from sample 535 collected in Italy during 2002 from a tomato plant of Field 2). For hybridization or PCR, positive controls were obtained from tomato plants infected with known TYLCD-associated viruses, and negative controls, from healthy tomato and common bean plants.

Analysis of sequence data

Multiple nucleotide sequence alignments were done using CLUSTAL X (Thompson et al., 1997), with default parameters. The alignments were used to calculate pairwise genetic distances (see below). Phylogenetic analyses were done by the neighbor-joining (Saitou and Nei, 1987) and parsimony (Fitch, 1977) methods implemented with MEGA 3.1 software (Kumar et al., 2004). Robustness of inferred evolutionary relationships were assessed by 1000 bootstrap replicates and by comparing the trees obtained by neighbor-joining or parsimony methods.

Two main approaches were used to estimate genetic differentiation in the virus population. One involved analysis of the genetic structure using the frequencies of the different virus types. In this case, population differentiation was measured using the F_{ST} estimator, a fixation index describing genetic variation among subpopulations within a population (Nei and Kumar, 2000; Wright, 1965), calculated with Arlequin version 2.0 (Schneider et al., 2000). The second approach analyzed genetic diversity in the subpopulations of each virus type. For this, the nucleotide sequences of about 780 nt derived from the almost full-length genome clones (see above) were used. Multiple nucleotide sequence alignments were used to estimate genetic distance (d) between any two isolates by the Kimura’s two-parameter method (Kimura, 1980). Also, in coding regions

(Rep and V2), genetic distances for synonymous (d_S) and non-synonymous substitution (d_{NS}) were estimated separately as described by Pamilo and Bianchi (1993) and by Li (1993) (PBL method). In the latter case, Rep/C4 and V2/CP overlapping regions were discarded because multiple evolutionary constraints could exist in those regions (Sanz et al., 1999). Confidence estimates for d, d_S, and d_{NS} values were calculated using a bootstrap method (Nei and Kumar, 2000) with 1000 replicates. Contribution of selection to sequence variation was assessed by the ratio d_{NS}/d_S (Yang and Bielawski, 2000): a ratio of less than 1 indicates that selection against amino acid changes is uppermost, while a ratio greater than 1 evidences positive selection. Within-population and between-population nucleotide diversities were estimated from genetic distances as described by Nei (1987). Analyses were performed using the MEGA 3.1 software (Kumar et al., 2004). Equality of the nucleotide diversities was tested using the *t*-test (Sokal and Rolf, 1981).

Aligned sequences were checked for incongruent relationships, which might have resulted from recombination, using the Recombination Detection Program (RDP) version 2.0 (Martin et al., 2005). This program identifies possible recombinant and parental sequences (lineages likely to have contributed to the recombinant), estimates breakpoint positions and calculates probability scores using six different automated methods; a multiple comparison corrected *P*-value cutoff of 0.05 and default settings were used throughout and only events detectable with two or more different methods were retained.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this work can be found in the GenBank/EMBL/DDBJ/ databases under accession numbers DQ317696 to DQ317806.

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