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# Typing of tomato yellow leaf curl viruses in Europe

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**Abstract**

Tomato yellow leaf curl disease is spreading in southern Europe, where it has quickly become a serious problem. In recent years, several virus isolates have been characterised. Although with some genetic variability, all isolates found in Europe belong to one of two species *Tomato yellow leaf curl-Sardinia* (TYLCV-Sar) or *Tomato yellow leaf curl-Israel* (TYLCV-Is). Several methods were tested to identify and type TYLCV isolates from field samples: (1) RFLP of a DNA fragment amplified from the coat protein gene; (2) PAGE of a fragment amplified from the C2 gene; (3) dot-blot hybridisation. All methods enabled the detection of the TYLCVs and provided good indications for attributing them to one species or the other. However, for typing purposes, the RFLP method was the most reliable, due to the easily recognisable pattern produced by the two virus species present in Europe. Dot-blot hybridisation is less expensive for identifying TYLCVs in large numbers of samples, particularly when a mixture of two probes is used. PAGE of the C2 fragment is the fastest of the methods tested.

## Introduction

Severe crop losses in tomato (*Lycopersicon esculentum*) are attributed to diseases caused by geminiviruses in tropical and sub-tropical areas worldwide. Among these viruses, several have been named tomato yellow leaf curl virus (TYLCV) (for a review, see Czosnek and Laterrot, 1997). In Europe, TYLCV was reported in the late eighties and early nineties in some Mediterranean areas of Italy (Credi et al., 1989; Luisoni et al., 1989), Spain (Moriones et al., 1993), and more recently in Portugal (Louro et al., 1996). In 1997, outbreaks of a different TYLCV were described in Spain (Navas-Castillo et al., 1997). As a result of the presence of its insect vector, the whitefly *Bemisia tabaci*, in protected crops in most European countries, further spread is feared.

Some European isolates of TYLCV have been characterised, and a western Mediterranean cluster was proposed by Noris et al. (1994a). However, the TYLCV isolated in Portugal and the new one from Spain appear to be more closely related to eastern Mediterranean isolates. Following accumulation of molecular data on TYLCV and other geminiviruses, the International Committee on Taxonomy of Viruses (ICTV; Fauquet and Mayo, 1999) decided to divide TYLCV into different species: among them *Tomato yellow leaf curl-Sardinia* (TYLCV-Sar, first isolated in Sardinia, Italy) and *Tomato yellow leaf curl-Israel* (TYLCV-Is, first isolated in Israel), that would approximately coincide with the western and eastern Mediterranean clusters, respectively. TYLCVs have so far been detected and identified using DNA probes, labelled either radioactively (Navot et al., 1989) or non-radioactively (Crespi et al., 1991; Noris et al., 1994b). Due to occurrence of both TYLCV-Sar and TYLCV-Is in some parts of Europe, convenient methods to distinguish these two viruses need to be developed.

In this work, three methods for detecting and typing European isolates of TYLCVs are reported, namely: (i) RFLP of a PCR fragment amplified from the coat protein (CP) gene; (ii) polyacrylamide gel electrophoresis (PAGE) of a PCR fragment amplified from the C2 gene; (iii) dot-blot hybridisation.

## Materials and methods

### *Sample collection and preparation*

Tomato leaves from plants showing TYLCV-like symptoms were collected in fields from Italy, Spain and Portugal, from 1991 to 1998 (Table 1). Samples from other Mediterranean countries were also tested. In order to evaluate the different protocols using the same samples, one procedure was adopted for the extraction of the plant material. About 0.15 g of leaf tissue was extracted as described by Noris et al. (1994b), and finally dissolved in 500 µl of TE buffer. This is referred to below as the sample.

Extracts from plants infected with well-characterised isolates of the two viruses were included as standards in every assay. These were a TYLCV-Sar isolate collected in 1992 in Spain (sample 13 in Table 1; Noris et al., 1994a), and a TYLCV-Is isolate collected in 1995 in Portugal (sample 17; Louro et al., 1996). From each isolate, infectious clones were sequenced (Noris et al., 1994a; J. Navas-Castillo and G.P. Accotto, unpubl.).

### *RFLP of a CP gene fragment*

Primers amplifying a DNA fragment of about 580 bp in the gene encoding the CP were designed by analysing sequences in the EMBL/GenBank databases. The primers were: TY1(+): 5′-GCCCATGTA(T/C)-CG(A/G)AAGCC-3′ and TY2(−): 5′-GG(A/G)TTA-GA(A/G)GCATG(A/C)GTAC-3′. Their 5′-ends are located at nucleotide (nt) 447 and 1024, respectively, on the sequence of TYLCV-Sar, Acc. No. X61153. These primers are similar to those devised by Wyatt and Brown (1996) for detecting whitefly-transmissible geminiviruses, but are shorter and less degenerate, and were carefully studied to avoid mismatches in the 3′-end.

PCR conditions were: 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, followed by a final extension step of 7 min at 72 °C. Final concentrations of reaction components were: 200 µM of each dNTP, 0.4 µM of each primer, 2 mM $MgCl_2$ and 0.04 U $\mu l^{-1}$ AmpliTaq DNA polymerase (Perkin-Elmer). Small PCR reactions (25 µl) with 1 µl of sample were initially performed to test the ability of the primers to amplify the expected DNA fragment from all samples available. Aliquots (5 µl) of reaction products were analysed on 1% agarose or 3% NuSieve agarose (FMC) gels.

Larger PCR reactions (100 µl) were performed, followed by a quick purification of the products either on minicolumns (High Pure PCR Product Purification Kit, Boehringer Mannheim) or by phenol/chloroform extraction and ethanol precipitation. The purified PCR products were digested with the restriction enzyme *Ava* II, which, on the available sequences, was predicted to cut the TYLCV-Sar differently from TYLCV-Is. Digested DNA was finally analysed on 3% NuSieve agarose (FMC) gels.

### *PAGE of a C2 gene fragment*

Two primers, able to amplify a 348 bp fragment corresponding to 85% of the C2 open reading frame were designed (Navas-Castillo et al., 1998) based on the sequence of a TYLCV-Sar isolate from Spain (GenBank Acc. No. Z25751): MA17 (5′-GAAAACATTTGTGAATCC-3′, nt 1285–1302) and MA13 (5′-AATGCAATCTTCGTCACC-3′, nt 1632–1615). The C2 gene was selected to design wide range primers for PCR because it contains regions well conserved among monopartite TYLCV species. Fifty µl PCR reactions were performed, containing 1 µl of sample, 200 µM of each dNTP, 0.3 µM of each primer, 1 mM $MgCl_2$, and 1.5 U AmpliTaq DNA polymerase (Perkin-Elmer). Cycling conditions were: 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by a final extension step of 6 min at 72 °C. Aliquots (5 µl) of the amplification reactions were analysed under non-denaturing conditions by 1.5% agarose gel electrophoresis in TAE buffer, pH 8, or 8% PAGE in TBE buffer, pH 8, at room temperature. Gels were stained with ethidium bromide.

### *Dot-blot hybridisation*

Samples were loaded at three five-fold dilutions (corresponding to 1, 0.2 and 0.04 µl of sample) on positively-charged nylon membranes (Boehringer Mannheim) using a Minifold apparatus (Schleicher and Schuell). Nucleic acids were fixed to membranes with UV light, then membranes were prehybridised, hybridised

*Table 1*. List of the tomato samples used, with origin and collection year, followed by results of the RFLP analysis of the CP gene fragment, the PAGE analysis of the C2 gene, and the dot-blot hybridisation assays with single and mixed full-length probes

| Sample number and origin | RFLP pattern | PAGE pattern[1] | Hybridisation with | | |
|---|---|---|---|---|---|
| | | | Mixed probe | TYLCV-Is | TYLCV-Sar |
| 1 (Sardinia, 1992) | Sar | s | + | − | + |
| 2 (Sardinia, 1997) | Sar | s | + | − | + |
| 3 (Sardinia, 1997) | Sar | NT[2] | + | − | + |
| 4 (Sardinia, 1997) | Sar | s | + | − | + |
| 5 (Sardinia, 1997) | Sar | NT | + | − | + |
| 6 (Sardinia, 1997) | Sar | NT | + | − | + |
| 7 (Sardinia, 1997) | Sar | NT | + | − | + |
| 8 (Sardinia, 1997) | Sar | NT | + | − | + |
| 9 (Sardinia, 1997) | Sar | NT | + | − | + |
| 10 (Sicily, 1991) | Sar | s | + | − | + |
| 11 (Sicily, 1994) | Sar | s | + | − | + |
| 12 (Sicily, 1994) | Sar | s | + | − | + |
| 13 (TYLCV−Sar) | Sar | s | + | − | + |
| 14 (Spain, 1992) | Sar | s | + | − | + |
| 15 (Spain, 1992) | Sar | s | + | − | + |
| 16 Healthy control | — | — | − | − | − |
| 17 (TYLCV−Is) | Is | f | + | + | + |
| 18 (Portugal, 1995) | Is | f | + | + | + |
| 19 (Portugal, 1997) | Is | f | + | + | − |
| 20 (Portugal, 1997) | Is | f | + | + | − |
| 21 (Portugal, 1998) | Is | f | + | + | + |
| 22 (Spain, 1995) | Sar | s | + | + | + |
| 23 (Spain, 1996) | Sar | s | + | − | + |
| 24 (Spain, 1997) | Sar | s | + | + | + |
| 25 (Spain, 1997) | Is | f | + | + | − |
| 26 (Spain, 1997) | Is | f | + | + | + |
| 27 (Spain, 1996) | Is | f | + | + | + |
| 28 (Spain, 1997) | Is | f | + | + | + |
| 29 Healthy control | — | — | − | − | − |
| 30 (Spain, 1997) | Sar and Is | s and f | + | + | + |
| 31 (Spain, 1996) | Sar and Is | s and f | + | + | + |
| 32 (Spain, 1997) | Sar and Is | s and f | + | + | + |

[1]s and f denote bands migrating slower and faster than the 344 bp size marker, respectively; [2]Not tested.

and developed according to the Boehringer Chemiluminescent Detection Kit (Accotto et al., 1998). Two full-length probes were used, either singly or together, both obtained by incorporating digoxigenin-dUTP by random priming: one probe specific for the entire genome of TYLCV-Is from Portugal (GenBank AF105975), the other for that of TYLCV-Sar from Sardinia (Kheyr-Pour et al., 1991). The probes were used at a final concentration of 1 ng ml$^{-1}$ of hybridisation buffer, and the washing steps following hybridisation were at high stringency (0.1× SSC, 0.1% SDS at 65 °C).

Because of the cross-reactivity of full-length probes, smaller probes were tested. Intergenic region (IR)-specific probes were chosen, because DNA sequence in this region of the genome is significantly variable between *Begomovirus* species. Two probes were prepared (Navas-Castillo et al., 1999), specific for the IR of a TYLCV-Is and a TYLCV-Sar isolate from Spain, and used on dot-blots with a selection of samples. Nylon membranes were prepared by spotting 1 µl of each sample, fixed and hybridised as described above. Following hybridisation, the blots were washed under high (0.1× SSC, 0.1% SDS at 65 °C) and

low (0.5× SSC, 0.1% SDS at 50 °C) stringency conditions.

## Results

The primers described by Wyatt and Brown (1996) for whitefly-transmitted geminiviruses failed to amplify TYLCV-Sar (Figure 1). On the contrary, using TY1(+) and TY2(−) primers a 580 bp fragment was amplified from TYLCV-Sar, TYLCV-Is and all samples tested (Figure 2, left), and in most cases no other significant amplification product was visible. From samples collected in Portugal, minor fragments of larger size were detected. This probably reflects the ability of one of the primers to anneal, although with lower affinity, to other regions of the viral genome. Following digestion with *Ava* II (Figure 2, right), the TYLCV-Sar and TYLCV-Is controls produced the patterns expected from sequence data, i.e. 360, 150 and 68 bp (Sar-pattern), and 302 and 277 bp (Is-pattern), respectively. The results are summarised in Table 1. All samples from Sardinia and Sicily, and samples from Spain collected before 1996 produced the Sar-pattern. All samples from Portugal, and some recent ones from Spain, produced the Is-pattern. In some recent samples from Spain (30–32) both patterns were present (see Figure 2, sample 30), indicating a double infection by the two TYLCV species. This was confirmed by hybridisations using species-specific probes (Navas-Castillo et al., 1999).

Using the C2-specific primers MA17 and MA13, a single DNA fragment of about 350 bp was detected in agarose gels from TYLCV-Sar and TYLCV-Is controls, as well as from all analysed samples (not shown). However, different mobilities of the amplicons were observed in PAGE (Figure 3 and Table 1). TYLCV-Sar and samples that produced the Sar-pattern in the RFLP method always migrated more slowly than TYLCV-Is and samples that produced the Is-pattern. In spite of some heterogeneity observed among samples classified in the same virus species according to the RFLP method, an ideal line, drawn at the level of the 344 bp marker (see Figure 3), could separate isolates of TYLCV-Sar from those of TYLCV-Is. The two bands detected in some samples from Spain (samples 30–32), therefore, indicated a double infection with both TYLCV-Is and TYLCV-Sar.

In dot-blot experiments with full-length probes the mixed probes hybridised with TYLCV-Sar and TYLCV-Is controls and all samples analysed (Figure 4

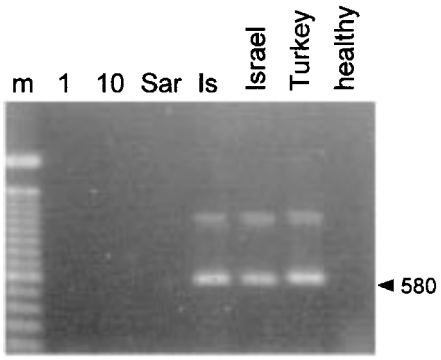



*Figure 1*. 1% agarose gel electrophoresis of DNA amplicons obtained from tomato samples using the primers described by Wyatt and Brown (1996) for the detection of whitefly-transmitted geminiviruses. Numbers correspond to samples in Table 1. Sar and Is are the TYLCV-Sar and TYLCV-Is controls (samples 13 and 17, respectively). One sample from Israel and one from Turkey are also included, along with a healthy control. m = 100 bp DNA ladder (Life Technologies).

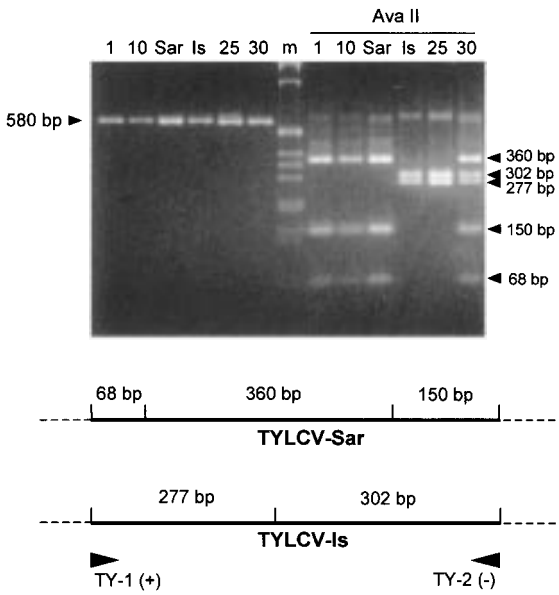



*Figure 2*. 3% NuSieve agarose gel electrophoresis of DNA amplicons obtained from tomato samples using primers TY1(+) and TY2(−). Numbers correspond to samples in Table 1. Lane m (1 Kb DNA ladder, Life Technologies) divides the undigested amplicons (left) from those digested with *Ava* II (right). Sar and Is are as in Figure 1. Below, a scheme of the amplified DNA fragment and the size of *Ava* II digestion products for TYLCV-Sar and TYLCV-Is, based on sequence data.

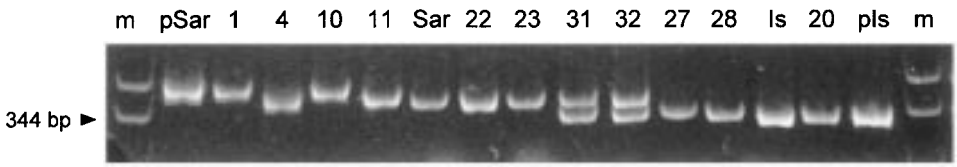



*Figure 3*. 8% PAGE analysis of C2 gene fragments amplified with primers MA17 and MA13 from tomato samples. Numbers correspond to samples in Table 1. m = 1 Kb DNA ladder (Life Technologies). pSar and pIs were from plasmids containing clones of TYLCV-Sar and TYLCV-Is, respectively. Sar and Is are as in Figure 1. The arrow indicate the DNA size marker which divides the fast from the slow migrating pattern.

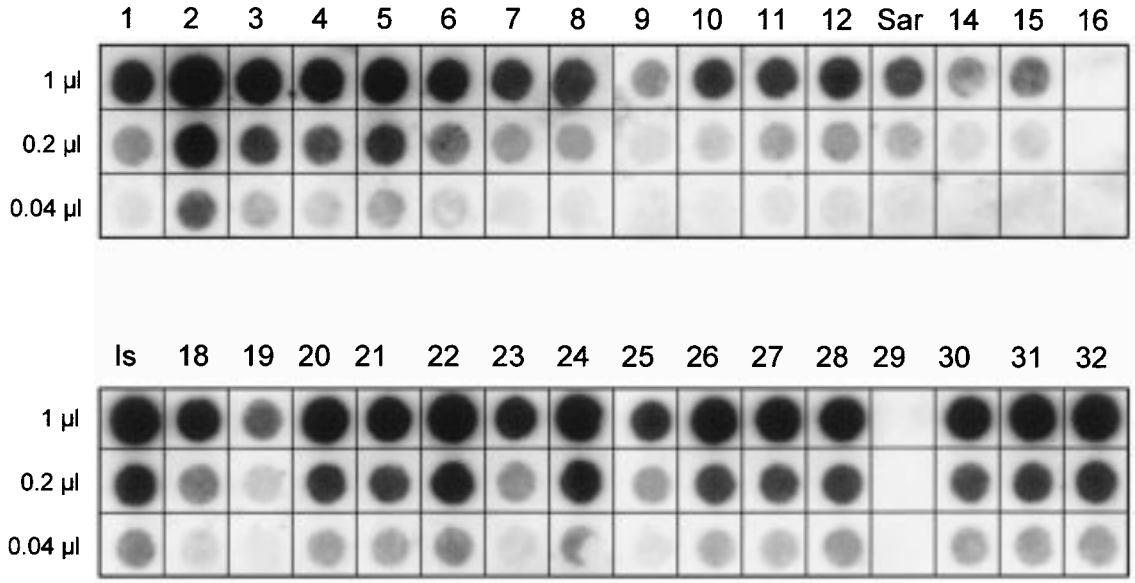


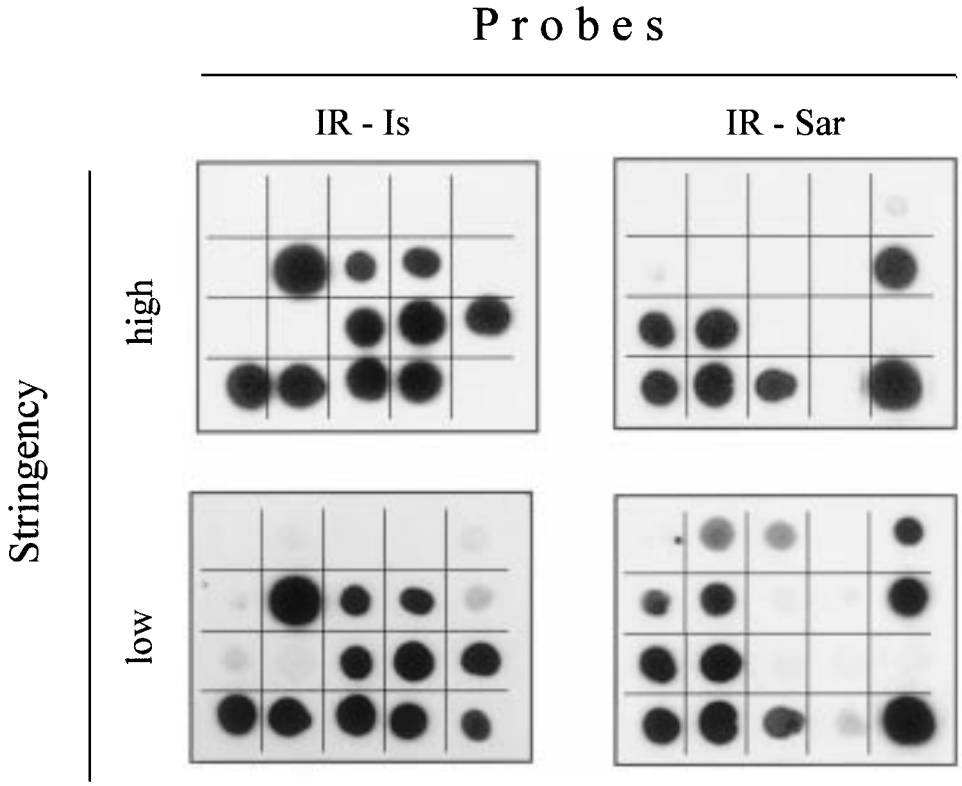

*Figure 4*. Dot-blot hybridisation of the tomato samples described in Table 1. Each dot in the column corresponds to 1, 0.2, and 0.04 µl of sample (or 300, 60, and 12 µg of leaf tissue), respectively. The membrane was hybridised with a mixture of full-length probes to TYLCV-Sar and TYLCV-Is.

and Table 1). In most cases, signal intensity was high at the second dilution tested, and good at the third dilution. In no case did healthy controls produce a signal. Table 1 also shows the results obtained with each single probe. The full-length TYLCV-Sar probe detected isolates collected in Italy and Spain, and the TYLCV-Is probe detected isolates from Portugal and Spain. Some samples (18, 21, 22, 24, 26–28 and 30–32) were detected by both probes, one probe generally producing a stronger signal than the other. Since only samples 30–32 are infected by both TYLCV species (Table 1), cross-reactivity between probes must have occurred in the other cases.

When the smaller probes, specific for IR, were used under high stringency, the IR-Is probe detected TYLCV-Is samples, but not TYLCV-Sar samples. However the IR-Sar probe detected only TYLCV-Sar samples from Spain (22–24 and 30–32), and not those from Sardinia (3 and 4, no signal) and Sicily (11 and 12, very weak signal) (Figure 5 and Table 1). By lowering the stringency, it was possible to detect all TYLCV-Sar samples with the IR-Sar probe, but cross-reaction with some TYLCV-Is-infected samples occurred (samples 17 and -Is). Under these conditions, the IR-Is probe produced a weak signal with the other TYLCV species (samples 11, 12 and 22–24).

## Discussion

The results of RFLP analysis of the CP fragment obtained with TY1(+) and TY2(−) primers showed




| | | | | |
|---|---|---|---|---|
| 16 | 3 | 4 | 29 | 11 |
| 12 | 17 | 20 | 21 | 22 |
| 23 | 24 | 27 | 28 | 26 |
| 31 | 32 | 30 | -Is | -Sar |

*Figure 5*. Dot-blots of selected samples (numbers as in Table 1), hybridised with probes specific for the IR of TYLCV-Is (IR-Is) or TYLCV-Sar (IR-Sar), and washed at high (0.1× SSC, 0.1% SDS at 65 °C) and low (0.5× SSC, 0.1% SDS at 50 °C) stringency. Sar and Is were tomato samples from plants artificially inoculated with cloned DNA of a TYLCV-Sar and a TYLCV-Is isolate from Spain.

that this method can discriminate between the two TYLCV species present in Europe, and at the same time can also detect double infections. This approach provides qualitative data, and is not influenced by the virus concentration in the sample, a problem that can be encountered using tissue- or dot-blot assays. Moreover, if new geminiviruses infect tomato crops, this approach will probably produce different digestion patterns, and identify the new isolates easily.

The inability of previously-described primers (Wyatt and Brown, 1996) to amplify TYLCV-Sar isolates is probably explained by the presence of mismatches near the 3′-end of both primers.

With PAGE of the C2 fragment it is possible to identify, at least preliminarily, a new isolate as belonging to

TYLCV-Sar or TYLCV-Is. This assay is the fastest of the three techniques tested, being completed within one day. It was successfully used for identifying TYLCV-Is and -Sar in large scale samplings conducted in Spain during 1997 and 1998.

The differences in PAGE mobility of the C2 fragment could be caused by differences in size or composition. However, this fragment was also amplified from cloned and sequenced DNAs of TYLCV-Sar and -Is (Figure 3, lanes pSar and pIs). These two DNAs are both 348 bp in length, but in the electrophoretic conditions used they migrated differently, TYLCV-Sar above and TYLCV-Is below the 344 bp size marker. Their different behaviour therefore depends on differences in base composition, and not in size, of the DNA. It is known that DNAs having the same length but different composition show variations in electrophoretic mobility up to 10% (Sambrook et al., 1989). The heterogeneity observed among samples classified in the same virus species, more easily observed in isolates of TYLCV-Sar, could also be due to limited differences in base composition of the amplicons.

Analysis of samples by dot-blot hybridisation with IR-specific probes under high stringency showed that the IR-Is probe can be considered species-specific, while the IR-Sar one can be considered strain-specific. The different behaviours of the two probes reflects the level of homology between each probe and the isolates tested. Based on known sequences (Kheyr-Pour et al., 1991; Noris et al., 1994; Crespi et al., 1995; J. Navas-Castillo and G.P. Accotto, unpubl.) homology between the IR-Sar probe used and TYLCV-Sar from Sardinia and Sicily is limited to 63% and 83% respectively, levels that can explain the complete lack of reaction with isolates from Sardinia, and the very low signal with those from Sicily, under conditions of high stringency. Conversely, homology of the IR-Is probe with TYLCV-Is from Portugal is very high (96%), allowing discrimination of European isolates of this species in hybridisation with high stringency. When low stringency conditions are used, the IR probes perform like the full-length probes: they cannot be considered species-specific, and therefore are not suitable for typing.

The mixture of the two full-length probes can be considered a good and inexpensive tool for diagnosing the tomato yellow leaf curl disease in Europe. However, isolates cannot be assigned to TYLCV-Sar or -Is solely on the basis of dot-blot hybridisations, because of some probe cross-reactivity. Furthermore, double infections are difficult to detect. The combination of full-length and IR probes hybridisation analyses gives valuable information for typing TYLCV isolates. Digoxigenin-labelled probes are very stable over time, can be reused several times, and have virtually no reaction with healthy tomato extracts.

In conclusion, the three methods tested in the present work allowed detection of TYLCVs in all samples tested, including field isolates collected over several years from areas where TYLCV infections had been reported in Europe. However, only RFLP analysis of the CP fragment could reliably discriminate between the two TYLCV species present in Europe, and therefore it is proposed as a tool for quickly and easily classifying new isolates, without the need of sequencing. It may also be useful in following the appearance and spread of new TYLCV types in areas where the disease is already present, as has recently happened in Spain.

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