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RESEARCH PAPERS

Simultaneous detection of mixed ‘*Candidatus Phytoplasma asteris*’ and ‘*Ca. Liberibacter solanacearum*’ infection in carrot

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Summary. Symptoms of shoot and root malformation were observed in carrot plants in fields located in the North of Gran Canaria Island (Spain), during surveys carried out in spring 2015 and 2016. Total DNA extraction from the leaves of symptomatic plants was performed and PCR assays were carried out to detect the agents possibly associated to the observed symptoms. While ‘*Candidatus Liberibacter solanacearum*’ was detected in the majority of tested symptomatic samples, phytoplasmas belonging to the ribosomal group 16SrI were detected only in two samples. Phylogenetic analyses and sequencing, together with virtual RFLP, confirmed that ‘*Ca. L. solanacearum*’ strains detected in carrot samples belong to haplotype D.

Keywords: ‘*Candidatus Liberibacter solanacearum*’, ‘*Candidatus Phytoplasma*, haplotypes, nested PCR, RFLP.

Introduction

‘*Candidatus Liberibacter*’ and ‘*Candidatus Phytoplasma*’ are phloem limited and insect-transmitted bacteria associated with economically important plant diseases. During surveys on carrot fields (*Daucus carota* L.) carried out in the municipality of Guía, on the North Atlantic island of Gran Canaria (Spain), symptoms of leaf curling with yellow, bronze, and purple discolouration, twisting of petioles, stunted growth of shoots and roots, and proliferation of secondary roots were observed. Nine fields showing symptoms possibly related to ‘*Candidatus Liberibacter*’ and ‘*Candidatus Phytoplasma*’ presence were observed. The percentage of symptomatic plants ranged from approx. 5 to 35% per field and no psyllids were detected in any of the fields at the time of the survey.

The symptom observed (Figure 1) resembled those reported by Duduk *et al.*, (2009) and Munyaneza (2010a), associated with the presence of phloem-inhabiting bacteria, in particular ‘*Candidatus Liberibacter*’ and ‘*Candidatus Phytoplasma*’ species. The first group are prokaryotes related to the α -*Proteobacteria*, which are Gram negative bacteria with a thin cell walls (Munyaneza, 2012), and are associated with economically important diseases such as citrus huanglongbing (HLB) (Bové, 2006) and zebra chip (ZC) of potato (*Solanum tuberosum*) in America (Secor *et al.*, 2009; Wen *et al.*, 2009; Crosslin *et al.*, 2010). Yellows in tomato (*Solanum lycopersicum*) associated with the presence of potato psyllids (Munyaneza *et al.*, 2010b; EPPO, 2013) and vegetative disorders in celery (*Apium graveolens*) evidenced by abnormal numbers of shoots, curling of stems, and yellowing (Teresani *et al.*, 2014), were also linked with the presence of these prokaryotes and in particular of ‘*Candidatus Liberibacter solanacearum*’ haplotypes. Phytoplasmas are

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Figure 1. Two fields showing disease symptoms in carrot (variety Cordoba), in Guía, Gran Canaria Island (Spain).

also phloem limited and insect-transmitted bacteria that lack cell walls and are associated with severe diseases in many important crops worldwide (Bertaccini *et al.*, 2014). Aster yellows (16SrI-B) phytoplasmas have previously been detected in carrots in mainland Spain, while “stolbur” (16SrXII-A) phytoplasmas were detected in the Canary Islands, Spain (Font *et al.*, 1999). We examined a number of samples in order to verify the presence of ‘*Candidatus*’ species in symptomatic carrots.

Materials and methods

Field sampling

Samples from carrot (variety Cordoba) collected in 2015 and 2016 from, respectively, 26 and eight symp-

tomatic plants, were randomly selected in two fields located in the North of Gran Canaria Island. Samples were also collected from two asymptomatic plants in each year and each field as negative controls.

Total DNA extraction

Total DNA was extracted from 1 g of leaf tissue samples ground in mortars with liquid nitrogen, using a reported method based on cetyl-trimethylammonium-bromide (CTAB) (Angelini *et al.*, 2001).

Amplification protocols

The extracted DNA samples were tested by PCR using the three primer pairs reported in Table 1 to

Table 1. The table describes the PCR systems used to detect '*Ca. L. solanacearum*' presence in carrot DNAs using primer pairs specific for its 16S rRNA (Clipo-F /O12c and OA2/O12c) and ribosomal protein *rplI/rplL* (CL514F/CL514R). To detect '*Ca. Phytoplasma*' spp. The phytoplasma universal (P1/P7, R16F2n/R16R2 and M1/M2) and group 16SrI- specific primers (R16(I)F1/R16(I)R1) were employed.

Name	Sequence 5'-3'	Literature	Amplicon length (bp)	Gene(s)
Reported target ' <i>Ca. L. solanacearum</i> '				
Clipo-F (f)	TACGCCCTGAGAAGGGGAAAGATT	Secor <i>et al.</i> , 2009	1,070	16S rRNA
O12c (r)	GCCTCGCGACTTCGCAACCCAT	Jagoueix <i>et al.</i> , 1996		
OA2 (f)	GCGCTTATTTTTAATAGGAGCGGCA	Liefting <i>et al.</i> , 2009	1,168	16S rRNA
O12c (r)	GCCTCGCGACTTCGCAACCCAT	Jagoueix <i>et al.</i> , 1996		
CL514F	CTCTAAGATTTCGGTTGGTT			
CL514R	TATATCTATCGTTGCACCAG	Munyaneza <i>et al.</i> , 2009	669	ribosomal protein <i>rplI/rplL</i>
Reported target ' <i>Ca. Phytoplasma</i> ' spp.				
P1	AAGAGTTTGATCCTGGCTCAGGATT	Deng & Hiruki, 1991		
P7	CGTCCTTCATCGGCTCTT	Schneider <i>et al.</i> , 1995	1,784	16S rRNA + spacer + beginning of 23S rRNA
R16F2n	GAAACGACTGCTAAGACTGG	Gundersen & Lee, 1996		
R16R2	TGACGGGCGGTGTGTACAAACCCCG	Lee <i>et al.</i> , 1993	1,248	16S rRNA
R16(I)F1 R16(I)R1	TAA AAG ACC TAG CAA TAG G CAA TCC GAA CTA AGA CTC T	Lee <i>et al.</i> , 1994	1,095	16S rRNA
16R758f (=M1)	GTCTTTACTGACGCTGAGGC	Gibb <i>et al.</i> , 1995		
16S1232r (=M2)	CTTCAGCTACCCTTTGTAAC		509	16S rRNA

verify the presence of '*Ca. L. solanacearum*' and '*Ca. Phytoplasma*' spp. DNA samples from phytoplasma strains maintained in collection (Bertaccini, 2015) were also employed as positive controls for phytoplasma detection: in particular, ash yellows (ASHY, 16SrVII-A); European stone fruit yellows (ESFY, 16SrX-B); "stolbur" (STOL, 16SrXII-A); aster yellows (AY, 16SrI-B); faba bean phyllody (FBPSA, 16SrII-C); peach X disease (CX, 16SrIII-A) and *Picris echioides* yellows (PEY, 16SrIX-C) were used. DNA samples from asymptomatic carrots and samples containing sterile distilled water as template were used as negative controls. A 25 µL reaction was prepared by mixing 5 µL PCR 5× Buffer with MgCl₂ (Promega), 2 µL of 10 mM dNTPs, 0.5 µL (20 µM) forward and reverse primer, 0.5 µL (20 µM), 0.16 µL (1.25 units) Promega GoTaq G2 DNA polymerase (Promega), and 15.4 µL nuclease-free molecular biology water (Sigma). For

the primers employed to detect '*Ca. Liberibacter*' 1 µL of DNA template was used, while 1 µL from a 1:30 template dilution was employed for the primers to detect the presence of phytoplasma. PCRs were performed in a Thermal Cycler BIOMETRA (Germany) with the following parameters: i) for primer pair ClipoF/O12c: 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min, and a final extension cycle of 72°C for 10 min; ii) for primer pair OA2/O12c: 94°C 3min, 35 cycles of 94°C 45 s, 66°C 45 s, 72°C for 1 min and 10 min at 72°C; and iii) for primer pair CL514F/R: 94°C 3 min, 35 cycle of 94°C 45 s, 54°C 45 s, 72° 1 min and 10 min at 72°C. To detect phytoplasmas, the direct PCRs were carried out with primer pair P1/P7 (Table 1) and the first nested amplification was performed in two systems: system I, with the primer pair R16F2n/R16R2 (Table 1); and system II, with primer pair R16(I)F1/R16(I)R1 (Table 1). A sec-

ond nested PCR was carried out with the primer pair 16R758f/16S1232r (= M1/M2) in both systems (Table 1). The template for nested PCR reactions was 1 µL of the 1:30 dilution of previous amplicon and the cycling parameters for both systems were as described by Schaff *et al.*, (1992). PCR products were visualized in 1% agarose gel stained with ethidium bromide, and were documented with a benchtop UV transilluminator at 312 nm. Identification of detected phytoplasmas was performed using RFLP analyses with *TruI* (Fermentas) restriction enzymes in a 6.7% polyacrylamide gel, stained with ethidium bromide and visualised under the UV transilluminator. Virtual RFLP analyses were also carried out on 967 bp of the 16S ribosomal gene from the obtained '*Ca. L. solanacearum*' sequences and reference sequences for the reported haplotypes (A, B, C, D and E) available in GenBank, using pDRAW32 (<http://www.acaclone.com/>).

Phylogenetic analyses

Amplified products of selected DNA samples obtained with all the primer pairs employed were purified and directly sequenced in both directions with the primers used in amplification by Macrogen (the Netherlands). The sequences were aligned using ChromasPro 2.4 software. They were then compared with nucleotide sequences in the GenBank database using BLAST at the National Center for Biotechnology Information (NCBI) website (<http://ncbi.nlm.nih.gov/BLAST>).

Phylogenetic analyses were carried out using the obtained 16S rDNA sequences, sequences from ribosomal protein gene *rplJ/rplL* and sequences of '*Ca. L. solanacearum*' haplotypes described in the literature. '*Ca. P. asteris*', carrot 2 sequence and '*Ca. L. asiaticus*' were used as outgroups according with the sequence employed. The evolutionary history in both cases was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and were in the units of the number of base substitutions per site. The ME trees were searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial trees. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

Results

Positive results were obtained in PCR and nested PCR with all the primer pairs and primer combinations used. The negative controls as well as four of the symptomatic samples resulted negative with all the primers and systems employed. In particular, in 2015 and 2016 respectively, the results were: 16 of 26 (61.5%) and four of eight (50%) positive samples with primer pair Clipo-F/O12c (998 bp), 22 of 26 (84.6%) and eight of eight (100%) with primers OA2/O12c (1,168 bp), and 22 (84.6) and six (75%) with primers CL514F/R (669 bp). All the amplicons were of the expected length (data not shown). Twenty-six positive samples were obtained with primer pairs M1/M2 in nested amplification: 16 (61.5%) in 2015 and six (75%) in 2016 were obtained by system I, and four (15.4%) in 2015 by system II (Table 2).

RFLP analyses carried out on the M1/M2 amplicons using *TruI* restriction enzyme showed the presence of diverse profiles. In particular, 22 positive samples, amplified with nested PCR system I, showed RFLP profiles identical to each other that matched with the virtual profile of the same amplicon from carrot 1 (Figure 2a). The remaining four amplicons obtained with nested PCR system II showed profiles referable to those of phytoplasmas belonging to the ribosomal group 16SrI (Figure 2b).

The CL514F/R sequenced amplicons showed 100% identity to each other and to the homologous sequence of a '*Ca. L. solanacearum*' strain from Morocco [GenBank accession number (AC) KJ754507]; the 526 bp sequence of sample carrot 1 was deposited in GenBank under AC KX181862. The direct sequencing and alignment of selected ClipoF/O12c and OA2/O12c amplicons showed 100% of sequence homology to each other and to the '*Ca. L. solanacearum*' strain found in carrots in Morocco (AC KJ740160). The aligned sequence of 967 bp from OA2/O12c amplicon of the same carrot 1 strain was deposited in GenBank under AC KX163276.

The sequences obtained from M1/M2 amplicons in nested PCR system I (510 bp) were identical to each other and showed 99% of identity to '*Ca. L. solanacearum*' strain detected in carrots in Finland (AC GU373048); in particular they showed one single nucleotide polymorphism (SNP) at position 116 where a T was substituted with a C. One or two further SNPs were also present to other '*Ca. L. solanacearum*' strains detected in *Bactericera cockerelli*, in pepper, potato and tomato in the USA, Mexico and

Table 2. Results of '*Ca. L. solanacearum*' and '*Ca. P. asteris*' detection in carrot with the different primer combinations. '*Ca. L. solanacearum*' was detected with the primers ClipoF/O12c, OA2/O12c and CL514 F/R but also with the system I (P1/P7 + R16F2n/R2 + M1/M2). '*Ca. P. asteris*' was detected with the system II (P1/P7 + R16(I)F1/R1 + M1/M2).

Primers and primer combinations	2015		2016
	' <i>Ca. P. asteris</i> '	' <i>Ca. L. solanacearum</i> '	' <i>Ca. L. solanacearum</i> '
<i>16S rRNA</i> gene primer combinations			
ClipoF/O12c	-	61.5%	50.0%
OA2/O12c	-	84.6%	100%
System I (P1/P7 + R16F2n/R2 + M1/M2)	-	61.5%	75.0%
System II (P1/P7 + R16(I)F1/R1 + M1/M2)	15.4%*	-	-
Ribosomal protein gene <i>rplJ/rplL</i> primer			
CL514 F/R	-	84.6%	75.0%

* Mixed infection with '*Ca. L. solanacearum*'; -, not tested.

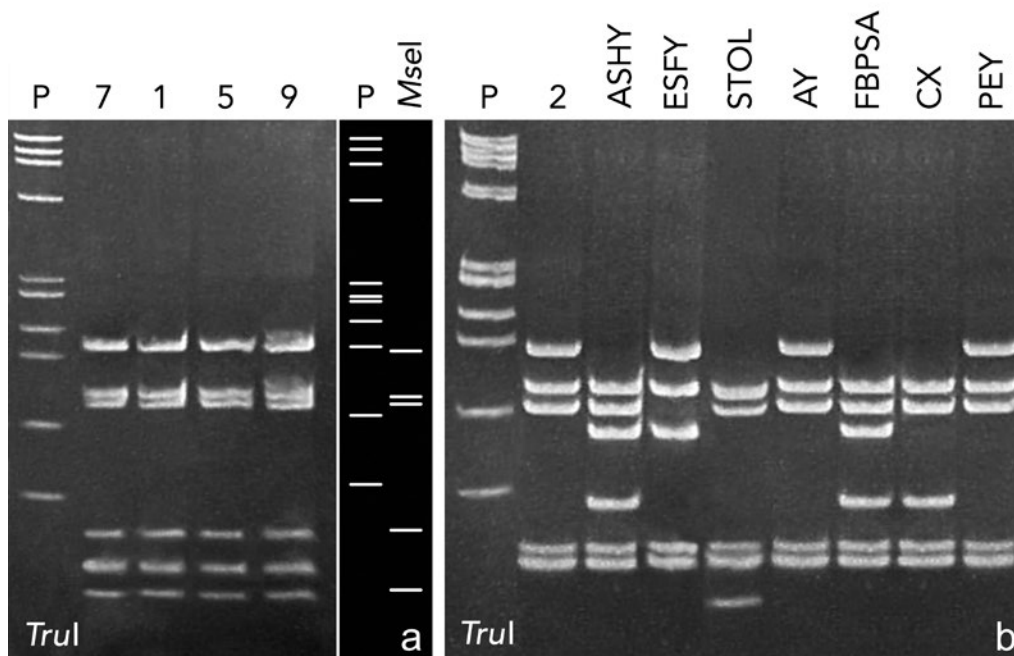


Figure 2. RFLP analyses in polyacrylamide gels of samples amplified in nested PCR with primers M1/M2 and digested with *TruI* (= *TruII*). In a), carrot samples 7, 1, 5, 9 amplified with system I and the virtual RFLP profile of the strain carrot 1 (AC KX163276) sequence cut at the same length as the M1/M2 amplicons and digested with the same enzyme (*MseI* and *TruII* are isoschizomers). The middle bands present in the real RFLP (on the left) represent non-specific amplification (primer dimers). In b) carrot sample 2 amplified with system II and reference phytoplasma strains: ash yellows (ASHY, 16SrVII-A); European stone fruit yellows (ESFY, 16SrX-B); "stolbur" (STOL, 16SrXII-A); aster yellows (AY, 16SrI-B); faba bean phylloxy (FBPSA, 16SrII-C); CX, peach X disease (16SrIII-A); *Picris echioides* yellows (PEY, 16SrIX-C). P, marker phiX174 *HaeIII* digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

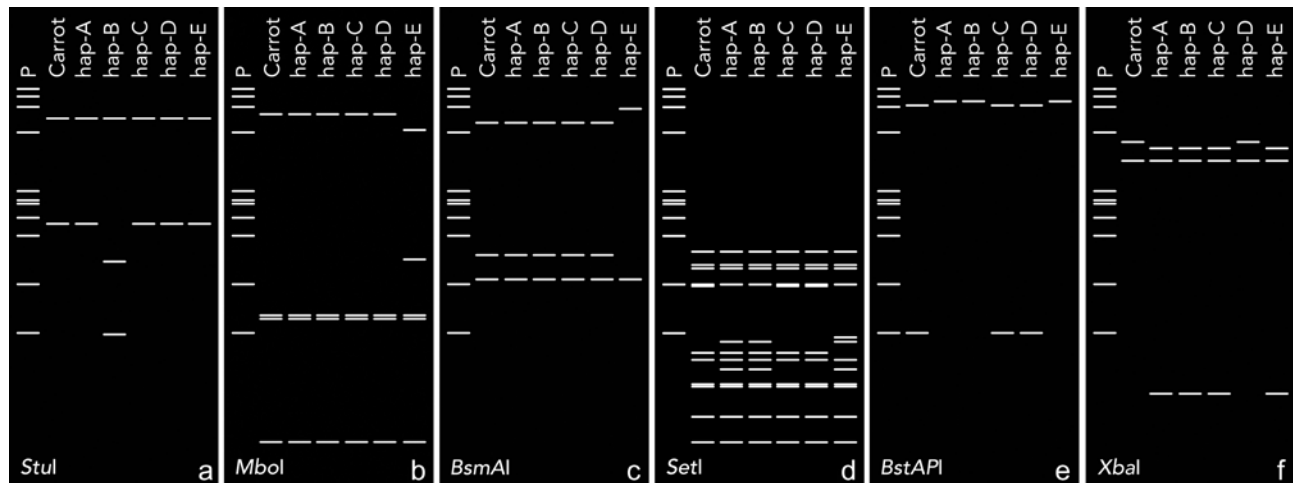


Figure 3. Virtual RFLP gel with selected restriction enzymes showing the polymorphisms among ‘*Ca. L. solanacearum*’ detected in carrot and the diverse haplotypes reported on full length OA2/O12c sequences, obtained in this work (Carrot) and retrieved from Genbank. Enzymes used are reported at the bottom of each gel. P, marker phiX174 *Hae*III digested with fragment sizes in base pairs from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

Thailand (AC KF776424, KF776423, KF776422 and KC771216). The carrot sequence from sample 1 was deposited in Genbank under AC KX163277. The sequenced strains amplified with nested PCR system II were 100% identical to each other, and the 498 bp sequence of strain carrot 2 was deposited in GenBank (AC KX163275). These sequences showed 99% identity to aster yellows phytoplasma strains available in GenBank, with one SNP at position 269 where a C substitution of a T was present.

These sequencing results verify that the profile obtained in RFLP analyses in all the samples amplified in nested PCR with system I was referable to the ‘*Ca. L. solanacearum*’ profile: (Figure 2a). On the other hand, the four positive samples obtained in amplification system II showed RFLP profiles referable to phytoplasmas (Figure 2b), in particular to aster yellows (16SrI) as confirmed by the sequencing results. Phytoplasma presence was therefore detected in only four samples and in mixed infection with ‘*Ca. L. solanacearum*’. The primer pair M1/M2, known as universal for phytoplasmas, detected ‘*Ca. L. solanacearum*’ when used in nested PCR system I and aster yellows phytoplasmas in nested PCR system II (Table 2).

The detected SNPs in the 16S rRNA, 16S/23S ISR and rplJ/rplL ribosomal protein sequences of ‘*Ca. L. solanacearum*’ strains from Gran Canaria agreed

with those present in the haplotype D (Nelson *et al.*, 2012). The same results were obtained using the virtual RFLP analyses on OA2/O12c amplicon sequences. These further confirm the assignment of the studied ‘*Ca. L. solanacearum*’ strains to the haplotype D, and allow differentiation of all the reported haplotypes (Figure 3). In particular, *StuI* (Figure 3a) differentiates haplotype B from all the others; *MboI*, *BsmAI*, *SetI* (Figures 3b, c, d) and *BsaI* (data not shown) differentiate haplotype E from all the others; *SetI* (Figure 3d) differentiates haplotypes C and D from haplotypes A and B, *BstAPI* (Figure 3e) and *MwoI* (data not shown) differentiate haplotypes C and D from all the others; while *XbaI* (Figure 3f) differentiates the haplotype C from all the others.

Phylogenetic analyses on CL514F/R sequences confirmed the clustering of the obtained sequences with those of ‘*Ca. L. solanacearum*’, in particular with haplotype D sequences (GenBank AC HQ454302). The CL514F/R sequences showed 100% identity with ‘*Ca. L. solanacearum*’ strains haplotype D (Figure 4).

Discussion

The results obtained in this study indicate the presence of ‘*Ca. L. solanacearum*’ and phytoplasmas belonging to the ribosomal group 16SrI in mixed infections in samples of carrots from Gran Canaria

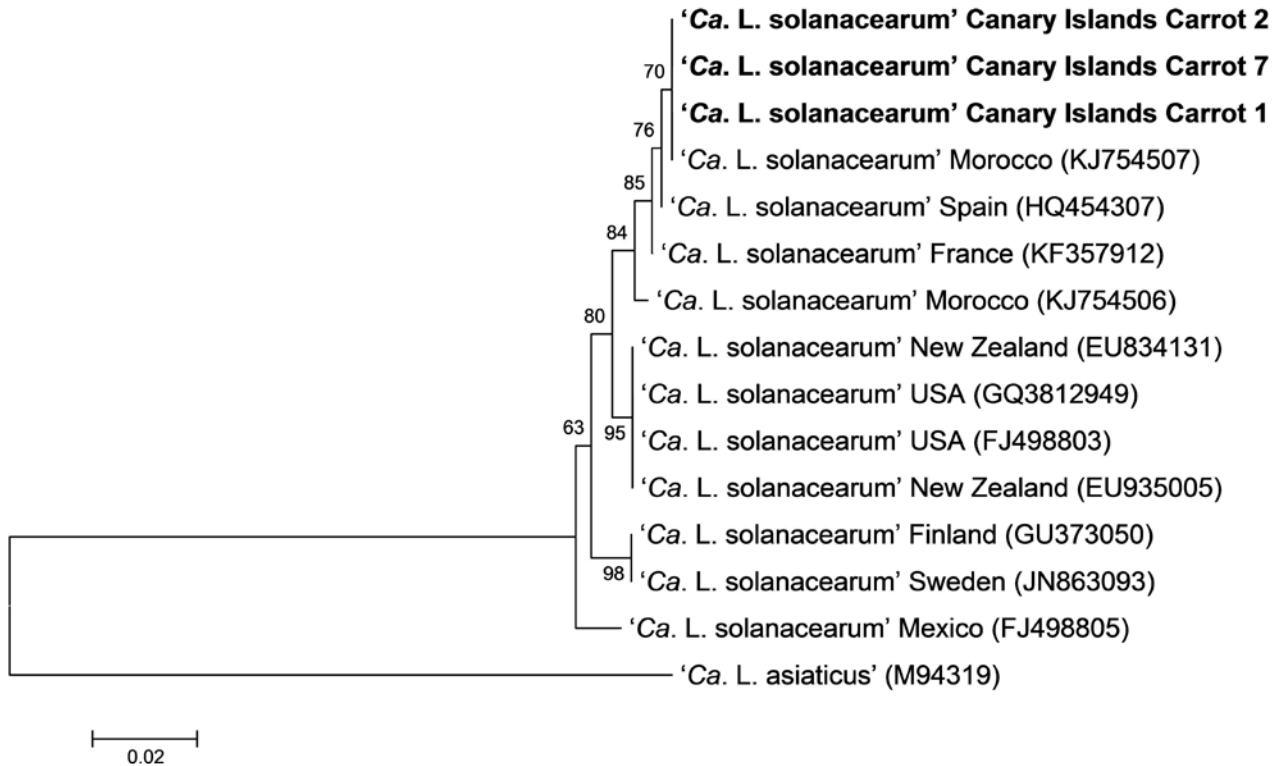


Figure 4. Phylogenetic tree showing the evolutionary history using the Minimum Evolution method enclosing ribosomal protein gene *rplJ/rplL* sequences from '*Ca. L. solanacearum*' from different geographic areas; '*Ca. L. asiaticus*' was used as outgroup. In bold sequences of strains obtained from carrots 1, 2 & 7 from Guia, Gran Canaria, Canary Islands, Spain; on the right geographic distribution and in parenthesis GenBank accession numbers. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 524 positions in the final dataset.

Island (Spain). Virtual RFLP analyses was applicable for '*Ca. L. solanacearum*' as an alternative tool for haplotype discrimination. It could be easier than SNPs detection on two genes as required now since only one gene and no sequencing can provide the same result. The nested PCR system(s) reported also allowed detection and RFLP differentiation of the two detected prokaryotes, as confirmed by the sequencing results.

'*Ca. L. solanacearum*' was described in 2008 (Hansen *et al.*, 2008; Liefing *et al.*, 2008) and it was shown to be associated with zebra chip disease of potato tubers, an economically important pathogen for solanaceous crops in New Zealand and the USA. In Europe, '*Ca. L. solanacearum*' was only been previously detected in carrot and celery (Hansen *et al.*, 2008; Liefing *et al.*, 2009; Secor *et al.*, 2009; EPPO, 2012; Munyaneza *et al.*,

2010a, 2010b; Buchman *et al.*, 2011; Alfaro-Fernández *et al.*, 2012a; Munyaneza, 2012). In addition, the bacterium causes serious damage in pepper (*Capsicum annuum*) in Mexico, aubergine (*Solanum melongena*) and tobacco (*Nicotiana tabacum*) in Honduras, tamarillo (*Solanum betaceum*) and tomatillo (*Physalis peruviana*) in New Zealand. It also infects weeds in the family Solanaceae (Munyaneza, 2012; EPPO 2013). Five haplotypes of '*Ca. L. solanacearum*' have been described that are discriminated by the presence of specific single SNPs in the 16S rRNA, 16S/23S ISR and 50S rplJ and rplL ribosomal protein genes (Nelson *et al.*, 2011). Two haplotypes (LsoA and LsoB) are transmitted by the psyllid *Bactericera cockerelli*. A third (LsoC) was detected in carrots in Finland, Sweden and Norway, and is transmitted by the carrot psyllid *Trioza apicalis* (Nissinen *et al.*, 2007; Nelson *et al.*, 2011). Haplotypes D and E were as-

sociated with carrots and celery in Spain and in the Canary Islands and are vectored by the psyllid *Bactericera trigonica* (Alfaro-Fernández *et al.*, 2012b; Teresani *et al.*, 2014). The same psyllid was previously demonstrated to vector the disease from carrot to carrot, when only phytoplasmas were reported to be associated with the disease (Font *et al.*, 1999).

Further research is in progress to confirm the transmission of both prokaryotes by seeds (Calari *et al.*, 2011; Bertolini *et al.*, 2015) and/or by insect vectors, considering that psyllids were not detected in the present survey and that the disease is present in Gran Canaria at epidemic levels similar to those reported in other affected areas.

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