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Additional Information

1 **Haplotypes of ‘*Candidatus Liberibacter solanacearum*’ identified in Umbeliferous**
2 **crops in Spain**

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10

11 **Abstract**

12 ‘*Candidatus Liberibacter solanacearum*’ is a phloem-limited Gram-negative bacterium
13 that causes serious damage to different crops of the botanical families *Solanaceae* and
14 *Apiaceae*. Five haplotypes have been described: LsoA and LsoB are present in
15 solanaceous crops in America and vectored by the tomato/potato psyllid *Bactericera*
16 *cockerelli*; LsoC affects carrots from Northern and Central Europe, and is transmitted by
17 the carrot psyllid *Trioza apicalis*; haplotypes LsoD and LsoE are present in Southern
18 Europe and Morocco in carrot and celery, and are associated with the psyllid *Bactericera*
19 *trigonica*. Thirty-four ‘*Ca. L. solanacearum*’ isolates were collected in six different
20 regions of Spain from distinct *Apiaceae* hosts (carrot, celery, parsley and parsnip) in eight
21 consecutive years and were analysed. Their haplotypes were determined by a sequence
22 analysis of 16S ribosomal RNA, the 16S-26S ribosomal RNA intergenic spacer, and the
23 23S ribosomal RNA and rplJ and rplL genes . Both haplotypes LsoD and LsoE were
24 found across Spain, and no host specificity appeared between these two haplotypes. This
25 is the first report of ‘*Ca. L. solanacearum*’ associated with parsley and parsnip.

26 **Keywords** carrot, celery, genetic variation, 16S ribosomal RNA

27

28 ‘*Candidatus Liberibacter solanacearum*’ is a Gram-negative bacterium that causes
29 serious damage to different crops of the botanical families *Solanaceae* (potato, tomato,

30 pepper, eggplant, tomatillo and tamarillo) and *Apiaceae* (carrot and celery) (EPPO 2013).
31 It has been described in several countries in Central and North America, New Zealand,
32 Northern and Central Europe, and also in the Mediterranean Region (Canary Islands,
33 Spain, France, Morocco) (EPPO 2012; Loiseau et al. 2014; Tahzima et al. 2014). This
34 bacterium is persistently transmitted by vegetative propagation by several psyllid species
35 (Munyaneza 2012), such as *Bactericera cockerelli* Sulc in tomato/potato (Munyaneza et
36 al. 2008) and *Trioza apicalis* Förster in carrots in Finland (Munyaneza et al, 2010a;
37 2010b).

38 Five different haplotypes have been described (LsoA, LsoB, LsoC, LsoD and LsoE) from
39 single nucleotide polymorphisms (SNPs) across three genome regions: partial sequences
40 of 16S, the 16-S/23S intergenic spacer region (ISR) and the rplJ and rplL genes.
41 Haplotypes LsoA and LsoB are present in solanaceous crops and are transmitted by vector
42 *B. cockerelli*. Both are present in North America and Mexico, but haplotype LsoA has
43 also been found in Central America and New Zealand (Nelson et al. 2011). The other
44 three haplotypes (LsoC, LsoD and LsoE) infect carrot, and haplotype LsoE has also been
45 identified in celery crops. All three have been detected in Europe: haplotype LsoC in
46 carrots from Northern Europe (Finland, Germany, Norway and Sweden) and haplotypes
47 LsoD and LsoE in Southern Europe (France and Spain) and Morocco (Nelson et al. 2011;
48 2013; Loiseau et al. 2014; Tahzima et al. 2014; Teresani et al. 2014; Munyaneza et al.
49 2015). *T. apicalis* has been described to transmit haplotype LsoC in Finland (Munyaneza
50 et al. 2010b; Nelson et al. 2011), while *B. trigonica* is associated with the transmission of
51 haplotype LsoD in carrot, and likely with LsoE in celery and carrot (Alfaro-Fernández et
52 al. 2012b; Nelson et al. 2013; Teresani et al. 2014). Two new *Bactericera* ssp. (*B.*
53 *tremblanyi* Wagner and *B. nigricornis* Förster), which carry ‘*Ca. L. solanacearum*’, could
54 be considered potential vectors of the bacterium (Teresani et al. 2015).

55 The objective of this study was to determine whether there was any geographic or
56 temporal pattern in the distribution of ‘*Ca. L. solanacearum*’ haplotypes in *Apiaceae*
57 crops in Spain.

58 Thirty-four isolates of ‘*Ca. L. solanacearum*’ were collected in different geographic areas
59 of Spain (Alicante, Albacete, La Rioja, Murcia, Segovia and Tenerife) in eight
60 consecutive years (2008-2015). The sampled *Apiaceae* crops were celery (*Apium*
61 *graveolens* L.), carrot (*Daucus carota* L.), parsnip (*Pastinaca sativa* L.) and parsley

62 (*Petroselinum crispum* (Mill.) Fuss.). Ten Spanish isolates from carrot and celery, whose
63 sequences were previously deposited in the GenBank (National Center of Biotechnology
64 Information, NCBI) database and were published (Nelson et al, 2013; Teresani et al.
65 2014), were also included in the assay. The isolate codes, accession number and
66 references of the publication of these previously published sequences are included in
67 Table 1.

68 The total DNA from 1 g of leaf tissue, including leaf petioles, was extracted with
69 cetyltrimethylammonium bromide (CTAB) buffer and the DNeasy Plant Mini Kit
70 (Qiagen, Valencia, CA. USA), as described by Green et al. (1999). Purified DNA was
71 stored at -20°C until use. Samples were analysed by PCR using three different primer
72 pairs: OA2/OI2c (1,168 bp), Lp Frag 4- 1611F/ LP Frag 4- 480R (918 bp) and
73 CL514F/CL514R (669 bp), which amplify sequences from 16S ribosomal RNA, the 16S-
74 26S ribosomal RNA intergenic spacer, and the 23S ribosomal RNA and rplJ and rplL
75 genes of '*Ca. L. solanacearum*', respectively (Hansen et al. 2008; Munyaneza et al. 2009).
76 Amplification was performed in 25- μ l reactions with final concentrations of 1x PCR
77 buffer (containing 2 mM MgCl₂), 10 pmol of each primer, 0.4 mM dNTPs, 1U of Biotools
78 DNA polymerase (Biotools B&M Labs S.A., Madrid, Spain) and 1 μ l of DNA extracts.
79 The PCR conditions were an initial denaturation cycle of 5 min at 94°C, followed by 39
80 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C
81 for 1 min. A final extension at 72°C for 10 min was introduced to finish the incomplete
82 PCR fragments.

83 The obtained PCR products were purified with the High Pure PCR Purification Kit
84 (Roche) and were directly sequenced. Sequences were submitted to the GenBank
85 database (NCBI). The obtained sequences and representative sequences of the previously
86 described haplotypes (Accession Nos. EU812559 and EU834131 for LsoA, FJ829813,
87 FJ830701 and FJ498807 for LsoB, GU373049, HM067883 and GU373051 for LsoC,
88 HQ454313, JX308304 and HQ454318 for LsoD and KF737348 for LsoE) were aligned
89 in gene regions using Clustal X (Larkin et al. 2007). SNPs were visually identified and
90 annotated as previously described (Nelson et al. 2011; 2013; Teresani et al. 2014).

91 The analyses performed of the 16S rRNA, 23S rRNA intergenic spacer and the rplJ and
92 rplL gene sequences identified haplotypes D and E, which were identified in the '*Ca. L.*
93 *solanacearum*' isolates collected from several *Apiaceae* crops across Spain (Table 1). The

94 haplotype described in America from *Solanaceae* crops, haplotypes LsoA and LsoB, and
95 LsoC from carrots identified in Northern Europe were not detected in the assayed Spanish
96 crops. Nine new SNPs of the ISR-23S gene were identified and completed (g.1963delAT,
97 g. 2034insT, g.2054A>T, g.2055C>T, g.2081G>A, 2218G>A, 2260C>T, 2405G>A)
98 from previous studies into haplotype E (Teresani et al. 2014). Seventeen samples of celery
99 (5), parsnip (2), carrot (9) and *B. trigonica* (1), collected between 2008 and 2010,
100 contained ‘*Ca. L. solanacearum*’ haplotype LsoD. These samples were collected from
101 Alicante in mainland Spain and Tenerife on the Canary Islands. Between 2011 and 2015,
102 27 samples were tested, including celery (5), carrot (17), parsnip (4) and parsley (1),
103 collected from Alicante, Albacete, Murcia Region, La Rioja and Segovia on mainland
104 Spain and from Tenerife on the Canary Islands. Samples contained a mix of ‘*Ca. L.*
105 *solanacearum*’ haplotypes LsoD and LsoE. The majority of the samples tested in this
106 study came from Alicante (SE Spain), whereby 24 celery, carrot and parsnip samples
107 were studied from 2008 to 2014, and the results showed that the haplotype LsoE levels
108 increased over time. It remains unclear whether the haplotype LsoE levels increased due
109 to the displacement of haplotype LsoD, or if the overall haplotype LsoE levels increased
110 and the sample size used herein was not large enough for this to be determined. No mixed
111 infection of both haplotypes was detected in a single field sample, nor did haplotype
112 specificity appear in the studied *Apiaceae* hosts because different samples of each host
113 plant were found to be infected with both bacterium haplotypes, except parsley, for which
114 only one isolate was studied.

115 Detection of ‘*Ca. L. solanacearum*’ in parsley and parsnip has not been previously
116 reported, and increases the knowledge of the host range of this organism in *Apiaceae*
117 species. Parsnip was collected in four of the study eight years, and the ‘*Ca. L.*
118 *solanacearum*’-infected samples showed yellowing and proliferation of leaves, as well
119 as stunting and proliferation of secondary roots with early root senescence. ‘*Ca. L.*
120 *solanacearum*’-infected parsley symptoms included yellowing, proliferation and
121 reddening of leaves. This finding of ‘*Ca. L. solanacearum*’ in two new *Apiaceae* species
122 is very important for carrot and celery growers in Spain as it increases the potential for
123 their crops to become infected with this bacterium.

124 Detection of haplotype LsoD and LsoE in carrot seeds from Agricola Villena Coop. V.
125 (Alicante, Spain), likely due to current commercial seed lots being composed of a mixture
126 of seeds from different origins and production years (Bertolini et al. 2015), could explain

127 the presence of both haplotypes in Spain. However, associating seed lot production and
128 haplotype was not possible. As reported in previous assays, the geographical distribution
129 of haplotypes LsoC and LsoD in Europe does not appear to result from introduction, but
130 are apparently native from their regions (Nelson et al. 2011). The results from the present
131 study might indicate that introduction or evolutionary divergence occurred with
132 haplotypes LsoD and LsoE. However, additional samples would need to be tested to
133 support this hypothesis. As haplotype LsoC, which was also identified in carrot, has been
134 never detected in either Spain or Southern Europe, it could indicate a relevant biological
135 difference among the three haplotypes described in *Apiaceae*, probably due to differences
136 in vector and climatic conditions.

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141

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Table 1. ‘*Ca. L. solanacearum*’ isolates collected in different geographic areas and years in Spain, including Genbank accession numbers and haplotype designation for each studied gene.

Isolate code	Collection year	Host	Geographic source	Genbank Accession no.			Haplotype
				16S	ISR-23S ^a	rplJ-rplL	
Ce-1071/08	2008	celery	Alicante	KU937831	KU937865	KU937901	LsoD
Ce-862/08		celery	Alicante	KU937832	KU937866	KU937902	LsoD
Pa-1054/08		parsnip	Alicante	KU937833	KU937867	KU937903	LsoD
Pa-1055/08		parsnip	Alicante	KU937834	KU937868	KU937904	LsoD
Ca-1057/08		carrot	Alicante	KU937835	KU937869	KU937905	LsoD
Ca-1065/08		carrot	Alicante	KU937836	KU937870	KU937906	LsoD
Ce-229/09	2009	celery	Alicante	KU937837	KU937871	KU937907	LsoD
Ce-253/09		celery	Alicante	KU937838	KU937872	KU937908	LsoD
140/09		carrot	Tenerife	HQ454312	np ^b	HQ454319	LsoD ^c
143/09		carrot	Tenerife	HQ454313	KU937873	HQ454320	LsoD
28/09		carrot	Alicante	HQ454303	np	HQ454306	LsoD ^c
Ce-447/10	2010	celery	Alicante	KU937839	KU937874	KU937909	LsoD
84/10		B. trigonica	Tenerife	HQ454316	np	HQ454321	LsoD ^c
31/10		carrot	Tenerife	HQ454314	np	HQ454317	LsoD ^c
33/10		carrot	Tenerife	HQ454315	KU937875	HQ454318	LsoD
4/10		carrot	Alicante	HQ454302	KU937876	HQ454305	LsoD
289/10		carrot	Alicante	HQ454304	np	HQ454307	LsoD ^c
Ce-961/11	2011	celery	Alicante	KU937840	np	KU937910	LsoD ^d
IVIA-1		celery	Alicante	KF737346	KF737347 ^e	KU937911	LsoE
Ca-879/11		carrot	Albacete	KU937841	KU937877	KU937912	LsoE
Ca-945/11		carrot	Alicante	KU937842	KU937878	KU937913	LsoE
Ca-946/11		carrot	Alicante	KU937843	KU937879	KU937914	LsoE
IVIA-V		carrot	Alicante	KF737348	KF737349 ^e	KU937915	LsoE
Ce-AP 1.1	2012	celery	Tenerife	KU937844	KU937880	KU937916	LsoD
Pa-46/12		parsnip	Alicante	KU937845	KU937881	KU937917	LsoE
Pe-P 1.1		parsley	Tenerife	KU937846	KU937882	KU937918	LsoE
Ca-670/12		carrot	Tenerife	KU937847	KU937883	KU937919	LsoD
Ca-821/12		carrot	Tenerife	KU937848	KU937884	KU937920	LsoE
Ca-21/12		carrot	Alicante	KU937849	KU937885	KU937921	LsoE
Ce-3065/13	2013	celery	Alicante	KU937850	KU937886	KU937922	LsoE
Pa-3042/13		parsnip	Alicante	KU937851	KU937887	KU937923	LsoE
Pa-3044/13		parsnip	Alicante	KU937852	KU937888	KU937924	LsoE
Ca-875/13		carrot	Murcia	KU937853	KU937889	KU937925	LsoE
Ca-872/13		carrot	Murcia	KU937854	KU937890	KU937926	LsoD
Ca-3053/13		carrot	La Rioja	KU937855	KU937891	KU937927	LsoE
Ca-1565/13		carrot	Murcia	KU937856	KU937892	KU937928	LsoE
Ca-3048/13		carrot	Alicante	KU937857	KU937893	KU937929	LsoE
Ce-461/14	2014	celery	Albacete	KU937858	KU937894	KU937930	LsoE
Pa-648/14		parsnip	Alicante	KU937859	KU937895	np	LsoE ^d
Ca-96/14		carrot	Murcia	KU937860	KU937896	KU937931	LsoD
Ca-97/14		carrot	Murcia	KU937861	KU937897	KU937932	LsoD
Ca-238/14		carrot	Murcia	KU937862	KU937898	KU937933	LsoE
Ca-630/14		carrot	Albacete	KU937863	KU937899	KU937934	LsoE
Ca-101/05	2015	carrot	Segovia	KU937864	KU937900	KU937935	LsoD

^a The first three SNPs of the ISR-23S fragment are missing in the sequences obtained herein, but nine new SNP are described at the end of the sequences of haplotype E, which are not detailed in Teresani et al. (2014).

^b np=not performed

^c Spanish ‘*Ca. L. solanacearum*’ isolates described in Nelson et al. (2013) that lack the ISR-23S gene sequence.

^d Spanish ‘*Ca. L. solanacearum*’ isolates from this study with missing sequences of one of the studied gene regions.

^e Spanish ‘*Ca. L. solanacearum*’ isolates described in Teresani et al. (2014) whose ISR-23S gene sequence was shorter than that studied and the 50S studied region was sequenced herein.

