New Insights into the Genetic Diversity of the Bacterial Plant Pathogen '*Candidatus* Liberibacter solanacearum' as Revealed by a New Multilocus Sequence Analysis Scheme

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Abstract

'*Candidatus* Liberibacter solanacearum' (Lso) has emerged as a serious threat on solanaceous and apiaceous crops worldwide. Five Lso haplotypes (LsoA, LsoB, LsoC, LsoD and LsoE) have been identified so far. To decipher genetic relationships between Lso strains, a MLSA study of seven housekeeping genes (*acnA*, *atpD*, *ftsZ*, *glnA*, *glyA*, *gnd* and *groEL*) was performed on a representative bacterial collection of 49 Lso strains. In all, 5415 bp spanning the seven loci were obtained from each of the 49 strains of our bacterial collection. Analysis of sequence data was consistent with a clonal population structure with no evidence of recombination. Phylogenies reconstructed from individual genes, and with concatenated data, were globally congruent with each other. In addition to the five highly supported and distinct genetic clusters, which correspond to the five established haplotypes, our phylogenetic data revealed the presence of a sixth haplotype, designated 'LsoG'. This new haplotype is currently represented by two strains from France which had distinct sequences in four out of the seven tested housekeeping genes. Altogether, the data presented here provide new information regarding the genetic structure of Lso and the evolutionary history of the haplotypes defined within this bacterial species.

Running title: '*Candidatus* Liberibacter solanacearum' genetic diversity

Keywords: '*Candidatus* Liberibacter solanacearum', genetic diversity, haplotype, MLSA, apiaceous and solanaceous crop

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Introduction

'*Candidatus* Liberibacter solanacearum' (Lso) is an unculturable phloem-limited bacterium that spreads from infected to healthy plants by psyllid vectors. This phytopathogenic bacterium includes strains responsible for diseases of several solanaceous and apiaceous crops (Haapalainen, 2014). Within this bacterial species, strains were classified into five haplotypes (LsoA, LsoB, LsoC, LsoD and LsoE). These haplotypes were defined based on genotyping of the 16S rRNA, 16S/23S ISR and 50S *rpIJ-rpIL* ribosomal gene loci, where any co-inherited single-nucleotide polymorphism (SNP) variant led to the classification in a new haplotype (Nelson et al., 2011, 2013; Teresani et al., 2014). LsoA and LsoB are associated with diseases of solanaceous crops, especially Zebra chip (ZC) disease of potato (*Solanum tuberosum*) in North America and New Zealand (Hansen et al., 2008; Liefting et al., 2008a; Lin el al., 2009), and are vectored to solanaceous plants by the potato/tomato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae) (Munyaneza et al., 2007; Secor et al., 2009). In addition to ZC disease, LsoA and LsoB cause diseases on other economically important solanaceous hosts, including tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), tomarillo (*Solanum betaceum*), tobacco (*Nicotiana tabacum)* and Cape gooseberry (*Physalis peruviana*) (Liefting et al., 2008b, Ling et al., 2011; Munyaneza et al., 2016). LsoC is vectored by the carrot psyllid *Trioza apicalis*, and is associated with carrot in northern Europe (Munyaneza et al., 2010a,b; 2012a,b; 2015; Nissinen et al., 2014). LsoD and LsoE are associated with the carrot psyllid *Bactericera trigonica*, and with vegetative disorders on several apiaceous crops in southwestern Europe and the Mediterranean area (Alfaro-Fernández et al., 2012a,b; Nelson et al., 2013; Loiseau et al., 2014; Tahzima et al., 2014, 2017; Teresani et al., 2014; Hajri et al., 2017; Mawassi et al., 2018). A best understanding of the relationships between Lso strains isolated from a set of various host plants in different geographical locations may help to better explain the increasing spread and extended host range of Lso.

Up to now, there has been no known biological mechanism explaining the different host range of the five haplotypes within Lso. The diversification of this species into different lineages could result from ecological adaptation, geographical structure or neutral processes (Haapalainen, 2014; Munyaneza, 2015). Genetic diversity investigations may provide an understanding of the population structure of a bacterial species and the evolutionary forces that led to its diversification (Puttamuk et al., 2014). For Lso, few studies have been attempted to investigate its population structure and genetic diversity, in particular due to its fastidious nature (Lin and Gudmestad, 2013). Sequencing of the 16S rRNA, 16S/23S ISR and 50S *rpIJ-rpIL* ribosomal gene loci has become the method of choice for the study of the genetic diversity of the bacterium (Lin et al., 2009; Wen et al., 2009; Nelson et al., 2011, 2013; Teresani et al., 2014; Alfaro-Fernández et al., 2017; Haapalainen et al., 2017; Hajri et al., 2017; Monger and Jeffries, 2018). However, genetic variation within these genes does not generally allow differentiation of closely related strains due to their high degree of conservation (Lin and Gudmestad, 2013). In addition to ribosomal genes, simple repeat sequences (SSR) and multilocus sequence typing (MLST) markers have been used to assess the genetic diversity of the bacterium (Lin et al., 2012; Glynn et al., 2012). However, these studies focused only on Lso solanaceous haplotypes (LsoA and LsoB), which is not sufficient to understand the extent of the genetic diversity of this bacterial species (Lin and Gudmestad, 2013; Haapalainen, 2014). More recently, genetic variation of LsoC was investigated by MLST (Haapalainen et al., 2018). To our knowledge, there is no information regarding the phylogenetic relationships between a large set of strains representing the five Lso haplotypes using multiple loci.

84   Multilocus sequence analysis (MLSA) has become the standard for phylogenetic analyses of
85   bacterial species and has been shown to be a powerful molecular method for microbial
86   population genetic studies. This method consists of the analysis of multiple (usually four to
87   eight) conserved housekeeping genes, which encode proteins that are essential for the survival
88   of the organism (Cooper and Feil, 2004; Gevers et al., 2005; Almeida et al., 2010). MLSA relies
89   on the concatenation of aligned DNA sequences from each gene. Mutations within
90   housekeeping genes are largely assumed to be selectively neutral, and therefore are more likely
91   to correctly reflect the phylogeny of the strains (Gevers et al., 2005; Hajri et al., 2012). In
92   addition, the concatenated sequence data could reduce the weight of horizontal gene transfer
93   'HGT' (Macheras et al., 2011) and/or recombination (Timilsina et al., 2015), which may
94   provide more reliable phylogenetic relationships among closely related strains (Glaeser and
95   Kämpfer, 2015). MLSA has been successfully used to describe the genetic structure of several
96   phytopathogenic bacteria (Castillo and Greenberg, 2007; Young et al., 2008; Almeida et al.,
97   2010; Trantas et al., 2013 ; Tancos et al., 2015; Constantin et al., 2016). Despite the availability
98   of several Lso genomic sequences (Lin et al., 2011; Thompson et al., 2015; Wu et al., 2015;
99   Wang et al., 2017), this strategy has never been applied to Lso.
100

101   In this paper, we studied the genetic structure of Lso and the phylogenetic relationships of
102   strains belonging to different haplotypes that have so far been defined in this species. For this
103   purpose, we designed a new MLSA scheme based on partial sequencing of seven housekeeping
104   genes (*acnA*, *atpD*, *ftsZ*, *glnA*, *glyA*, *gnd* and *groEL*). Using a representative bacterial collection
105   of 49 Lso strains, we evaluated its robustness with respect to defining the genetic structure of
106   the bacterium.
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108

109   **Materials and methods**
110

111   **Bacterial strains**

112   The 49 Lso strains used in this study are listed in Table 1. Strains were selected in order to
113   maximize the diversity in terms of geographical origin, host and year of isolation. Forty-two
114   strains have been isolated from several solanaceous and apiaceous crops and from different
115   psyllids (*B. cockerelli*, *B. trigonica* and *T. apicalis*). In addition, we included for comparative
116   purposes, the six published Lso genomes: strain ZC1 (Lin et al., 2011), strain RSTM (Wu et
117   al., 2015), strain NZ1 and strain HenneA (Thompson et al., 2015), and strains FIN111 and
118   FIN114 (Wang et al., 2017). We also included in our phylogenetic analysis the recently
119   deposited draft genome sequence of LsoD (GenBank accession number
120   NZ_PKRU00000000.1).
121

122   **DNA extraction**

123   In this study, genomic DNAs of the 42 strains were extracted using (i) a slightly modified
124   cetyltrimethylammonium bromide (CTAB) buffer extraction method (Murray and Thompson,
125   1980); and (ii) the magnetic-bead-based QuickPick™ SML Plant DNA Kit (Bio-Nobile)
126   according to the manufacturer's instructions. Each extraction series contained positive and
127   negative controls.

Selection of housekeeping genes

For the design of our MLSA scheme, several criteria were used in the selection of the potential genes. The genes included were (i) those encoding for putative housekeeping products with important biological functions (ii) evenly distributed across the genome as assessed from the Lso sequenced genomes, and (iii) present in one single copy in the Lso sequenced genomes. Using these criteria, we selected seven housekeeping genes: *acnA* (aconitate hydratase); *atpD* (ATP synthase subunit beta); *ftsZ* (cell division protein); *glnA* (glutamine synthetase I); *glyA* (serine hydroxymethyltransferase); *gnd* (6-phosphogluconate dehydrogenase) and *groEL* (molecular chaperone). Figure 1 shows the genomic location of the genes used in this study based on the Lso ZC1 genome.

Gene amplification and sequencing

Primers for partial sequencing of the seven housekeeping genes were designed (Table 2) based on the alignments of orthologous sequences collected from the six Lso genomes available in GenBank: strain ZC1, strain NZ1, strain HenneA, strain RSTM, strain FIN111 and strain FIN114. PCR amplification of each gene was performed in a 25-µL reaction mix using the Bio-X-Act short polymerase mix (Bioline, London, UK), 0.2 µM of forward and reverse primers, and 2 µL of DNA. The PCR conditions were an initial denaturation at 95°C for 5 min; followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 1 min of extension at 72°C and followed by 10 min of a final extension at 72°C. All PCR series included positive and negative controls. Amplified PCR products were separated on 1.5% agarose gels containing ethidium bromide for visualization. PCR products were directly sequenced with forward and reverse primers (Genewiz, Takeley, UK).

Sequence acquisition and alignment

Forward and reverse nucleotide sequences were assembled using the CAP3 contig assembly program (Huang and Madan, 1999) to obtain high-quality sequences. Multiple alignments were edited using the ClustalW tool of the BIOEDIT program version 7.2.6.1 (Hall, 1999). Sequences were concatenated following the alphabetic order of the genes, ending in a sequence of 5415 bp (bp 1 to 816 for *acnA*, 817 to 1575 for *atpD*, 1576 to 2349 for *ftsZ*, 2350 to 3132 for *glnA*, 3133 to 3879 for *glyA*, 3880 to 4623 for *gnd*, and 4624 to 5415 for *groEL*).

Sequence data analysis

Summary statistics for the sequences were computed using DnaSP version 5.10.01 (Librado and Rozas, 2009). The GC content, number of polymorphic sites (S), nucleotide diversities ($\theta\pi$ and θw) (Nei, 1987; Watterson, 1975), and neutrality test of Tajima's D (Tajima, 1989) were estimated for each of the seven genes and on concatenated sequences. DnaSP was also used to calculate the ratio of non-synonymous to synonymous substitutions (dN/dS) (Nei and Gojobori, 1986) in order to test the degree of selection on a locus. Values of dN/dS of 1, $dN/dS>1$ and $dN/dS <1$ indicate neutrality, diversifying selection and purifying selection, respectively. Occurrence of recombination was analyzed on each gene using the different recombination detection methods as implemented in RDP v4 (Martin et al., 2015). The analysis was performed with default settings for the different detection methods and the Bonferroni-corrected P-value cut-off was set at 0.05.

Phylogenetic analyses

Phylogenetic analyses were performed on individual gene sequences as well as on the dataset of concatenated sequences of the 49 strains of our bacterial collection. Strain psy62 of 'Ca. L. asiaticus' (Duan et al., 2009) was used to root trees. Maximum Likelihood trees were generated with the MEGA 7.0.14 software program (Kumar et al., 2016) using the Kimura two-parameter model (Kimura, 1980). Statistical support for tree nodes was evaluated by bootstrap analyses with 1000 replicates. To determine the haplotype affiliation of tested Lso strains, the sequenced genomes of strains from LsoA (strains NZ1, HenneA and RSTM), LsoB (strain ZC1), LsoC (strains FIN111 and FIN114) and LsoD (strain haplotype D1) were included in the analysis. For LsoE for which no published genome sequence is available, we used as reference, strain 14/235 that was previously determined as belonging to LsoE using the 16S rRNA gene and the 50S ribosomal protein *rpIJ-rpIL* gene region (Hajri et al., 2017).

Sequence accession numbers

Two hundred and ninety four sequences generated in this study were deposited in the GenBank database with accession numbers MH108648 - MH108689 for *acnA*, MH108690 - MH108731 for *atpD*, MH108732 - MH108773 for *ftsZ*, MH108774 - MH108815 for *glnA*, MH108816 - MH108857 for *glyA*, MH108858 - MH108899 for *gnd* and MH108900 - MH108941 for *groEL*.

Results

Sequence analysis and DNA polymorphism

In the present study, amplification was successful for all tested Lso strains with the seven housekeeping genes. To validate the choice of the seven loci as appropriate phylogenetic markers for Lso, descriptive statistics on nucleotide and allelic diversities were calculated for each locus and for the concatenated dataset. The 49 sequences of each locus were aligned; no gap and no insertion in the sequences of all tested Lso strains were detected. Analyzed sequence lengths ranged from 744 bp (*gnd*) to 816 bp (*acnA*), leading to a total of 5415 bp for the sequence of concatenated dataset (Table 3). The GC content for all loci ranged from 33.9% (*gnd*) to 43.2% (*ftsz*). All loci were polymorphic and the number of polymorphic nucleotide sites varied from 12 for the least polymorphic locus (*ftsZ*) to 27 for the most polymorphic locus (*glnA*) (Table 3). The value for the neutrality test of Tajima was positive for 4 loci (*ftsZ*, *glnA*, *glyA* and *groEL*), negative for 3 loci (*acnA*, *atpD* and *gnd*) and positive for the concatenated data but it was not significant for all tested loci and for concatenated data. The dN/dS ratios of all tested housekeeping genes were <1, indicating that these loci were subject to purifying selection. No recombination event was detected with the RDP program, indicating that the Lso population is highly clonal. For the concatenated dataset, the average GC content was 38.9%. Overall, 143/5415 nucleotide sites examined here are polymorphic, which indicates that Lso is genetically rather uniform. For neutrality tests, no significant departure from neutrality was observed on the overall dataset with the Tajima's D test (Table 3).

MLSA based on concatenated gene sequences

The phylogenetic tree generated from the concatenated sequences of the seven housekeeping genes (*acnA*, *atpD*, *ftsZ*, *glnA*, *glyA*, *gnd* and *groEL*) showed six well defined groups. High bootstrap values indicated that this clustering was well supported and that the phylogenetic tree was robust (Figure 2). The first group (LsoB) is composed of three strains isolated in the United States: one from *S. tuberosum*, one from *B. cockerelli* and the sequenced strain ZC1. The second

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group (LsoC) consists of one strain isolated in Finland from *T. apicalis* and the sequenced
strains FIN111 and FIN114. The third group (LsoA) was identified from one strain isolated
from *B. cockerelli* in the United States, one strain isolated from *S. lycopersicum* in New
Zealand, and the sequenced strains NZ1, RSTM and HenneA. The fourth group (LsoD)
included strain haplotype D1 from Israel, one strain isolated in Spain from carrot plants, one
strain isolated in Morocco from carrot plants, and 14 strains isolated in France: 12 from carrot
plants, one from *B. trigonica* and one from parsley. The fifth group (LsoE) included two strains
isolated from Spain: one from *B. trigonica* and one from carrot plants, and 17 strains isolated
from France (nine from carrot plants, one from *B. trigonica*, three from parsley, one from celery,
one from fennel, one from parsnip and one from chervil) (Table 1). In addition to these five
groups, which correspond to the five Lso haplotypes described so far, our phylogenetic tree
revealed the presence of a sixth haplotype, designated LsoG. This new haplotype clustered two
strains from France (17/0021-2c and 17/0021-2d). The bootstrap value (95) of the LsoG group
depicted the robustness of this phylogenetically novel population (Figure 2).

Regarding the phylogenetic relationships between the 49 tested Lso strains, a close relatedness
was observed between LsoD, LsoE and LsoG. These three haplotypes appeared to be more
closely related to LsoA than to LsoC. Strains of LsoB formed a cluster that was the most
genetically distant from the other five Lso haplotypes (Figure 2). Based on our MLSA analysis,
strains that are pathogenic on a same plant species or family were displayed in very divergent
groups. This was the case of LsoA and LsoB, both pathogenic on solanaceous crops, which did
not group together on the basis of our sequence data. For apiaceous haplotypes, LsoC form a
distinct phylogenetic group that was clearly separated from the three other apiaceous haplotypes
(LsoD, LsoE and LsoG) (Figure 2). These observations suggest that host specificity is not
correlated to Lso classification based on our phylogenetic data.

Individual gene phylogenies

Individual phylogenetic trees were constructed using the maximum likelihood method and are
shown in Figures S1 to S7. The tree topologies constructed from single genes presented roughly
congruent phylogenies. Some differences were observed in the topology of the individual
phylogenetic trees. For example, LsoC appears to be more closely related to the other apiaceous
haplotypes in the phylogenetic trees built with the *acnA*, *glnA* and *glyA* genes. This was not the
case for the trees built with the four other genes (*atpD*, *ftsZ*, *gnd* and *groEL*) since LsoC form
a cluster that was phylogenetically distant from the cluster formed by the other apiaceous
haplotypes.

All the individual phylogenetic trees confirmed the structuration of the tested Lso strains into
five groups (LsoA, LsoB, LsoC, LsoD and LsoE), with the formation of a sixth group (LsoG)
in the *glnA*, *glyA*, *groEL* and *ftsZ* phylogenetic trees (Figures S1 to S7). The gene sequences
from LsoG strains had distinct sequences in these four housekeeping genes: they exhibited one
new SNP in the *glnA* gene, one new SNP in the *glyA* gene and one new SNP in the *groEL* gene
(Table 4). In addition, the *ftsZ* gene sequences from these two strains varied from the *ftsZ* gene
sequences of the other LsoD strains by two nucleotide substitutions (Table 4). Regarding LsoE,
all tested strains have identical sequences except for the *atpD* gene (Figures S1 to S7), for which
two strains (14/427.3 from France and 15/150.5 from Spain) exhibited a different allele from

266   the other LsoE strains (presence of G instead of T at position 321 of the aligned sequence of
267   the *atpD* gene).
268

Discussion

270   Lso is an emerging phytopathogenic bacterium which is responsible for several economically
271   important diseases on solanaceous and apiaceous crops worldwide (Soliman et al., 2013;
272   Haapalainen, 2014). Previous sequence-based analyses of the genetic diversity of Lso have only
273   targeted genes of the ribosomal operon (Lin et al., 2009; Wen et al., 2009; Nelson et al., 2011).
274   These data, however, were obtained based on the analysis of a single locus, which may not
275   provide enough information for discrimination between different Lso strains (Lin and
276   Gudmestad, 2013). Since the publication of the first Lso genome sequence (Lin et al., 2011), a
277   number of loci including SSR and MLST markers were used to define the genetic relationships
278   among solanaceous Lso haplotypes (LsoA and LsoB) (Lin et al., 2012; Glynn et al., 2012). A
279   more recent study investigated the genetic variability of LsoC strains in Finland by MLST
280   (Haapalainen et al., 2018). These genetic diversity studies did not take into account LsoD and
281   LsoE variability. Thus, an updated phylogeny of Lso strains representing the five established
282   haplotypes is lacking. In recent years, the MLSA approach has shed new light on prokaryotic
283   phylogeny, and has been widely used in the classification and identification of diverse bacterial
284   species (Gevers et al., 2005; Almeida et al., 2010; Glaeser and Kämpfer, 2015). In this study,
285   we established a new MLSA scheme based on seven housekeeping genes (*acnA*, *atpD*, *ftsZ*,
286   *glnA*, *glyA*, *gnd* and *groEL*) for a representative collection of 49 Lso strains. The availability of
287   5415 bp of sequence data from each of the 49 strains gives an important resource to understand
288   the extent and nature of genetic diversity within this bacterial species.
289

290   Considering the seven housekeeping genes used in this study, 143 polymorphic sites in 5415
291   bp of sequence were identified. No insertions/deletions were found in the analyzed sequences.
292   The seven loci were informative, with *glnA* being the most variable gene. In addition to point
293   mutations, bacterial evolution may be driven by recombination which is considered as one of
294   the most important processes that increase genetic variability in bacterial genomes (Feil and
295   Spratt, 2001; Martin et al., 2011). Due to the association of LsoA and LsoB with the same
296   solanaceous crops and the same vector (*B. cockerelli*) (Munyaneza et al., 2007; Lin et al., 2012),
297   recombination between these two haplotypes could occur. Within apiaceous haplotypes, we
298   would expect recombination to occur between LsoD and LsoE since they are both associated
299   with *B. trigonica* (Alfaro-Fernández et al., 2012b; Teresani et al., 2014; Tahzima et al., 2017)
300   whereas LsoC is vectored by a different psyllid species (*T. apicalis*) (Munyaneza et al., 2010b).
301   Recombination events were estimated on each locus. No recombination events were detected
302   whatever the data analyzed. The absence of recombination for housekeeping genes was
303   previously reported for other phytopathogenic bacteria such as *Ralstonia solanacearum* and
304   *Xanthomonas* species (Castillo and Greenberg, 2007; Bui Thi Ngoc et al., 2010; Hajri et al.,
305   2012). Housekeeping genes are components of the 'core genome' and typically encode proteins
306   essential for the organism's survival. These genes are present in all strains of a bacterial species
307   and usually evolve slowly (Sarkar and Guttman, 2004). In the frame of this study, the levels of
308   selection acting on the tested housekeeping genes were evaluated with two population genetic
309   tests. Tajima's D statistic did not produce significant results. In addition, all dN/dS were less
310   than 1, indicating that the seven tested loci appeared to be under purifying selection, as expected
311   for genes chosen for MLSA studies. Taken together, our data revealed a high clonality for
312   housekeeping genes and support a predominant role of mutation in shaping the genetic diversity
313   of Lso.

314

315   Previous genetic studies based on the sequencing of three ribosomal gene loci (16S rRNA,
316   16S/23S ISR and 50S *rpIJ-rpIL*) led to consider Lso as a species complex constituted of five
317   haplotypes (LsoA, LsoB, LsoC, LsoD and LsoE) (Nelson et al., 2011, 2013; Teresani et al.,
318   2014). Our MLSA data showed that differences between the five haplotypes are not restricted
319   to the rRNA operon and are present within the core genome. The phylogenetic tree based on
320   concatenated sequences of the seven housekeeping genes confirmed the clustering of Lso
321   strains into five evolutionary lineages that correspond to the five previously described Lso
322   haplotypes, supporting the hypothesis of Nelson et al (2011) that Lso haplotypes represent
323   stable haplotypes. This clear correspondence between phylogenetic clustering and haplotype
324   classification was also supported by phylogenetic trees based on individual loci. The *atpD*, *ftsZ*,
325   *gnd* and *groEL* phylogenetic trees were found to be congruent with the tree generated from the
326   concatenated data, indicating a common evolutionary history for these four genes. However,
327   the *acnA*, *glnA* and *glyA* gene phylogenies did not accurately represent the same evolutionary
328   history as the other tested genes. The main incongruence concerned the position of LsoA, LsoB
329   and LsoC. A recent phylogenetic analysis based on 88 single-copy orthologs of the bacterial
330   core genome showed that LsoA is more closely related to LsoC than to LsoB (Wang et al.,
331   2017). This topology is in agreement with the *atpD*, *ftsZ*, *gnd* and *groEL* phylogenies, indicating
332   that these genes are more reliable to reflect the phylogenetic relationships among Lso
333   haplotypes. In addition, our phylogenetic data revealed a clear separation between the cluster
334   formed by LsoD and LsoE on the one hand and the cluster formed by LsoC on the other hand,
335   although displaying the same host range. Similarly, LsoA and LsoB which are both associated
336   with solanaceous crops, did not cluster together on the basis of our phylogenetic analysis. Thus,
337   no clear correspondence could be established between clustering of Lso strains and their host
338   specificities on the basis of our phylogenetic data. When considering the insect vector, our
339   phylogenetic analysis suggests that clustering of Lso strains associated with apiaceous crops
340   may be explained by differences in insect vector. Indeed, LsoD and LsoE, which appeared to
341   be very close phylogenetically, share the same insect vector (*B. trigonica*) (Alfaro-Fernández
342   et al., 2012b; Teresani et al., 2014; Tahzima et al., 2017), whereas LsoC which forms a more
343   distant cluster, is associated with a different psyllid species (*T. apicalis*) (Munyaneza et al.,
344   2010b). The genetic relatedness of LsoD and LsoE was reported previously based on sequence
345   analysis of the 50S *rpIJ-rpIL* gene (Hajri et al., 2017). For solanaceous haplotypes, this is not
346   true since LsoA and LsoB, which are both associated with *B. cockerelli*, did not cluster together
347   on the basis of our phylogenetic analysis. Altogether, the results presented here strongly suggest
348   that the current host plant range and the vector psyllid associations of the different Lso
349   haplotypes are not congruent with Lso phylogeny, as previously suggested by Haapalainen
350   (2014).

351

352   The present study not only clarifies the phylogenetic relationships among Lso haplotypes but
353   also reveals a part of the unknown genetic diversity of the bacterium. In addition to the five
354   previously described Lso haplotypes (LsoA, LsoB, LsoC, LsoD and LsoE), our phylogenetic
355   data revealed the presence of a sixth haplotype, designated LsoG, This new genetically distinct
356   group is represented by two strains (17/0021-2c and 17/0021-2d) from France and is associated
357   with asymptomatic carrots. Our sequence analysis revealed that the new haplotype G had
358   distinct sequences in four out of the seven tested housekeeping genes: one new SNP in the *glnA*
359   gene, one new SNP in the *groEL* gene, one new SNP in the *glyA* gene and one different allelic
360   form in the *ftsZ* gene. In the phylogenetic tree based on the concatenated gene sequences, strains
361   17/0021-2c and 170021-2d form a very tight and homogeneous cluster which is clearly separate
362   from the other Lso haplotypes. Because strains 17/0021-2c and 17/0021-2d were isolated from

363   the same geographical region, it is tempting to speculate that the newly described haplotype G
364   is a genetically structured population based on geographical area. To support this hypothesis,
365   further characterization of additional representative strains of LsoG is now necessary. Although
366   the haplotype nomenclature system has been useful in an agricultural context, its biological
367   justification is questionable since there are no biological distinctions that reliably differentiate
368   Lso haplotypes (Lin and Gudmestad, 2013). Thus, haplotype designation within Lso should
369   take into account both the genetic diversity and biological traits of the strains (Haapalainen,
370   2014; Munyaneza, 2015). More recently, two novel haplotypes (LsoU and LsoF) was identified
371   in Finland from the psyllid *Trioza urticae* and its host plant *Urtica dioica*, and in the USA in
372   potato tuber (Haapalainen et al., 2018; Swisher-Grimm and Garczynski, 2018). The
373   identification of three novel haplotypes (LsoU in Finland, LsoF in USA and LsoG in France)
374   indicates that genetic diversity of Lso is much higher than previously expected. In our study,
375   we focused on housekeeping genes which provide substantial evidence that strains 17/0021-2c
376   and 17/0021-2d represent a new Lso haplotype. However, we cannot exclude the possibility
377   that genetic variation among these strains is present elsewhere in the genome. Further work on
378   the biological traits (*i.e.* host range) of these two strains and further sequencing of more variable
379   genes like those involved in virulence are now necessary to confirm the status of this new
380   haplotype.
381

382   The interaction between Lso and its host plants is strongly influenced by the ecological niche,
383   the insect vector and the evolution of bacterial pathogenicity determinants (Lin and Gudmestad,
384   2013; Haapalainen, 2014). Currently, little is known about pathogenicity determinants that can
385   account for the differing host specificities between Lso haplotypes. Previous comparative
386   genomic studies focused only on LsoA, LsoB and LsoC and revealed relevant differences in
387   the genome organization between these haplotypes (Lin et al., 2011; Thompson et al., 2015;
388   Wang et al., 2017). However, these studies did not take into account haplotypes D and E since
389   no published genome sequence is available for LsoE and a draft genome sequence of LsoD was
390   made available only recently in Genbank. Notably, our MLSA data highlighted interesting
391   features for LsoD and LsoE: (i) they are very closely related, but phylogenetically distinct; (ii)
392   they form a cluster that is clearly separated from LsoC which is also pathogenic on apiaceous
393   crops. In addition, a recent study reveals that the newly described LsoU haplotype is more
394   closely related to LsoD and LsoA than to LsoC (Haapalainen et al., 2018). Altogether, these
395   observations raise interesting questions about the genomic basis of adaptation to the host of Lso
396   haplotypes. Sequencing of LsoE, LsoF, LsoU and LsoG genomes is now necessary to perform
397   a whole comparative genomic analysis at the species level which may enable the identification
398   of specific-haplotype genes. Such traits are excellent candidates to better understand the genetic
399   basis of the host range differences that exist between apiaceous and solanaceous haplotypes and
400   the factors involved in vector/plant host interactions. In this article, we have described the
401   genetic structure of Lso with special emphasis on housekeeping genes. However, the population
402   structure of a bacterium can also be tracked by focusing on other informative genetic markers
403   such as phage-related genes that could be linked to virulence. The potential contribution of
404   prophages and phage-related sequences to the genetic diversity of Liberibacter species has been
405   investigated more extensively in '*Ca.* L. asiaticus' (Zhou et al., 2013; Puttamuk et al., 2014;
406   Zheng et al., 2016, 2018). Furthermore, Thompson *et al.*(2015) highlighted that the most
407   important difference between the two genome sequences of LsoA is the location of their
408   prophage domains. Profiling the phage-related genes of Lso strains from different geographic
409   origin could help to better decipher the evolutionary forces responsible for the haplotype
410   diversification within this bacterial species and to identify the sources of disease outbreaks or
411   incursions.
412

Author contributions

AH: conceived and designed the experiments, analyzed and interpreted the data, wrote the paper; PCS: performed the experiments; PG: revised the manuscript; ML: analyzed the data with the maximum likelihood method and revised the manuscript.

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715   **Figures**

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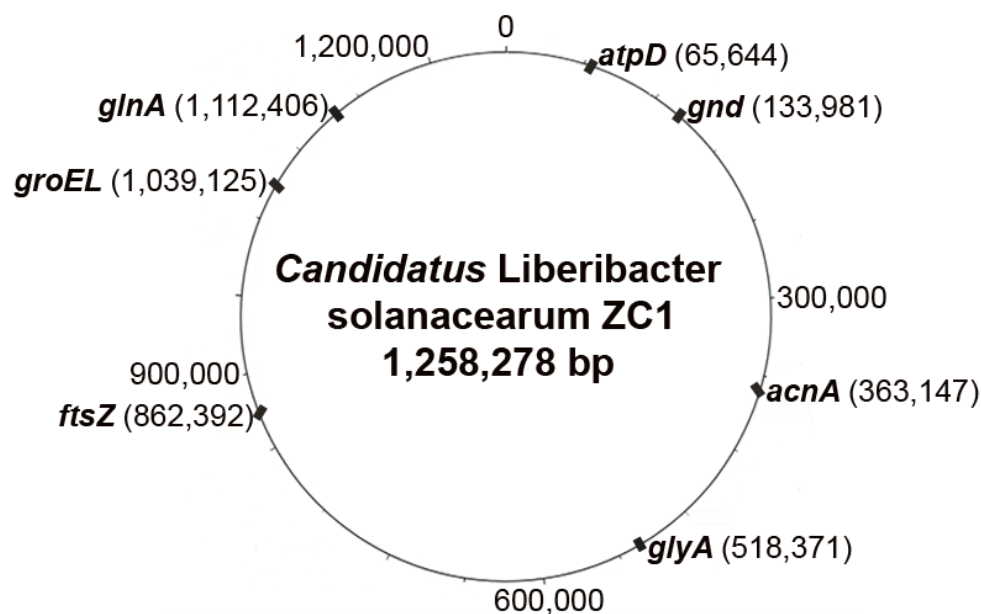
717
718   **Figure 1.** Schematic representation of the positions of the seven housekeeping genes used in
719   this study based on the sequenced Lso ZC1 genome (Genbank accession number CP002371).
720   The position of each locus (in base pairs) is given between brackets.
721
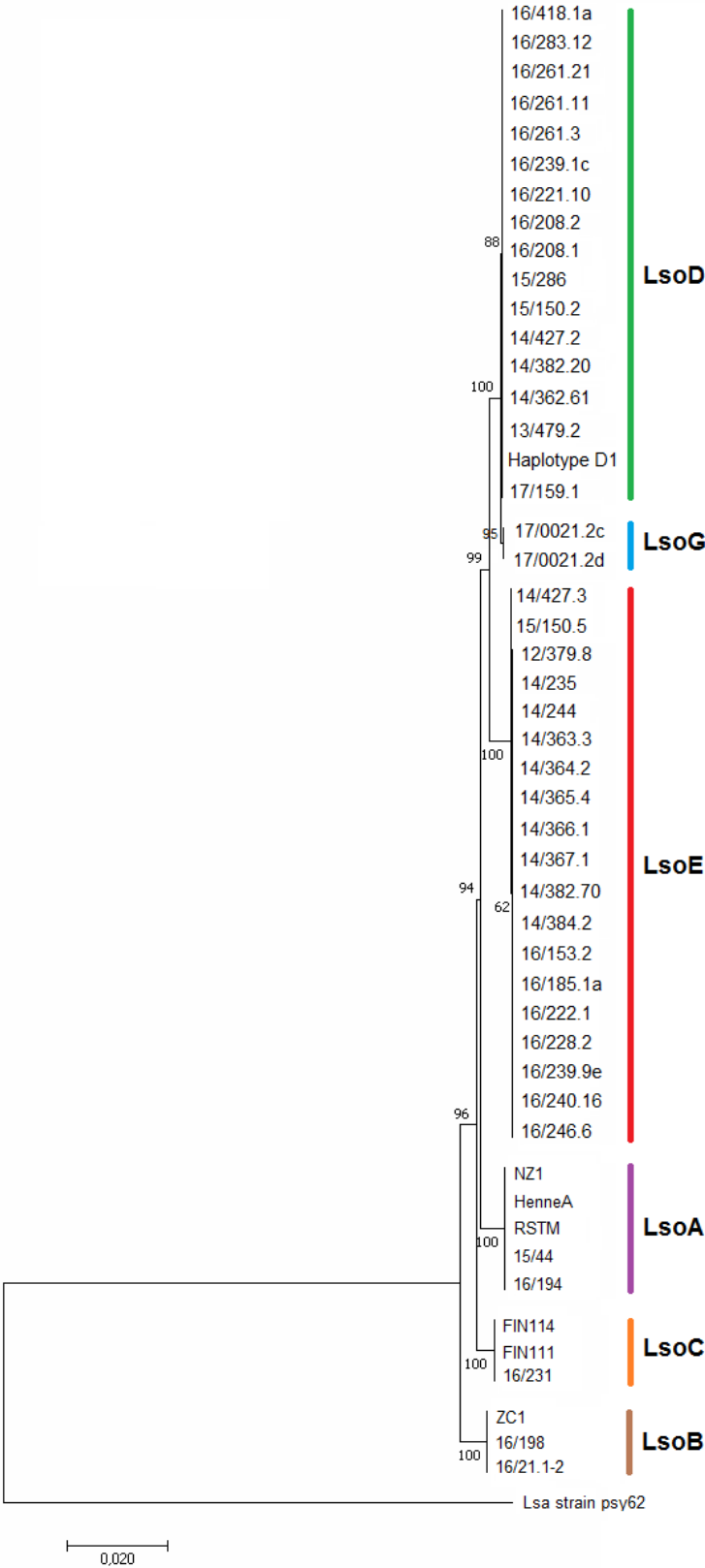
Figure 2. Phylogenetic tree of the MLSA based on concatenated genes (*acnA*, *atpD*, *ftsZ*, *glnA*, *glyA*, *gnd* and *groEL*) among the 49 Lso strains. The tree is based on 5415 bp of common sequence. The tree was constructed using the maximum likelihood method. The confidence of the nodes was tested with 1,000 bootstrap replicates. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Supplementary material

Figure S1. Phylogenetic tree based on the *acnA* gene among the 49 Lso strains. The tree was constructed using the maximum likelihood method. Bootstrap values over 50 (1,000 replicates) were shown at each node. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Figure S2. Phylogenetic tree based on the *atpD* gene among the 49 Lso strains. The tree was constructed using the maximum likelihood method. Bootstrap values over 50 (1,000 replicates) were shown at each node. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Figure S3. Phylogenetic tree based on the *ftsZ* gene among the 49 Lso strains. The tree was constructed using the maximum likelihood method. Bootstrap values over 50 (1,000 replicates) were shown at each node. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Figure S4. Phylogenetic tree based on the *glnA* gene among the 49 Lso strains. The tree was constructed using the maximum likelihood method. Bootstrap values over 50 (1,000 replicates) were shown at each node. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Figure S5. Phylogenetic tree based on the *glyA* gene among the 49 Lso strains. The tree was constructed using the maximum likelihood method. Bootstrap values over 50 (1,000 replicates) were shown at each node. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Figure S6. Phylogenetic tree based on the *gnd* gene among the 49 Lso strains. The tree was constructed using the maximum likelihood method. Bootstrap values over 50 (1,000 replicates) were shown at each node. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Figure S7. Phylogenetic tree based on the *groEL* gene among the 49 Lso strains. The tree was constructed using the maximum likelihood method. Bootstrap values over 50 (1,000 replicates) were shown at each node. The scale bar indicates the number of nucleotide substitutions per site. The tree is rooted with the '*Ca.* L. asiaticus' strain psy62.

Tables

Table 1. List of Lso strains used in this study

Sample ID	Source of isolation	Geographic origin	Year of isolation	Lso haplotype[1]
NZ1	*Bactericera cockerelli*	New Zealand	2010	
RSTM	*Bactericera cockerelli*	United States	2015	
16/19.4	*Bactericera cockerelli*	United States	NA	A
HenneA	*Bactericera cockerelli*	USA	2012	
15/44	*Solanum lycopersicum*	New Zealand	2010	
16/19.8	*Bactericera cockerelli*	United States	NA	
ZC1	*Bactericera cockerelli*	United States	NA	B
16/21.1-2	*Solanum tuberosum*	United States	NA	
FIN111	*Trioza apicalis*	Finland	2012	
FIN114	*Trioza apicalis*	Finland	2012	C
16/231	*Trioza apicalis*	Finland	NA	
16/239-1c	*Bactericera trigonica*	Eure-et-Loir, France	2016	
Haplotype D1	*Bactericera trigonica*	Israel	2017	
15/150.2	*Daucus carota*	Canary Islands, Spain	NA	
13/479.2	*Daucus carota*	Dordogne, France	2013	
16/418.1a	*Daucus carota*	Dordogne, France	2016	
16/221-10	*Daucus carota*	Eure-et-Loir, France	2016	
14/427.2	*Daucus carota*	Gard, France	2014	
16/208-2	*Daucus carota*	Gers, France	2016	
16/261-21	*Daucus carota*	Haute-garonne, France	2016	D
16/283-12	*Daucus carota*	Loiret, France	2016	
16/208-1	*Daucus carota*	Lot-et-Garonne, France	2016	
14/362.61	*Daucus carota*	Maine et Loire, France	2014	
14/382.20	*Daucus carota*	Maine et Loire, France	2014	
17/159.1	*Daucus carota*	Morocco	2013	
16/261-3	*Daucus carota*	Tarn, France	2016	
16/261-11	*Daucus carota*	Tarn, France	2016	
15/286	*Petroselinum crispum*	Aude, France	2015	
14/367.1	*Anthriscus cerefolium*	Maine et Loire, France	2014	
14/363.3	*Apium graveolens*	Maine et Loire, France	2014	
16/239-9e	*Bactericera trigonica*	Loiret, France	2016	
16/153.2	*Bactericera trigonica*	Segovia, Spain	2014	
15/150.5	*Daucus carota*	Canary Islands, Spain	NA	
16/240-16	*Daucus carota*	Cher, France	2016	
14/244	*Daucus carota*	Eure-et-Loir, France	2014	
14/427.3	*Daucus carota*	Gard, France	2014	
14/235	*Daucus carota*	Loiret, France	2014	
12/379.8	*Daucus carota*	Loir-et-Cher, France	2012	E
16/185-1a	*Daucus carota*	Loir-et-Cher, France	2016	
14/382.70	*Daucus carota*	Maine et Loire, France	2014	
16/246-6	*Daucus carota*	Maine et Loire, France	2016	
16/228-2	*Daucus carota*	Vienne, France	2016	
14/366.1	*Foeniculum vulgare*	Maine et Loire, France	2014	
14/365.4	*Pastinaca sativa*	Maine et Loire, France	2014	
16/222-1	*Petroselinum crispum*	Eure-et-Loir, France	2016	
14/364.2	*Petroselinum crispum*	Maine et Loire, France	2014	
14/384.2	*Petroselinum crispum*	Maine et Loire, France	2014	
17/0021-2c	*Daucus carota*	Marne, France	2017	G
17/0021-2d	*Daucus carota*	Marne, France	2017	

[1]: Lso haplotype was determined during previous study (Hajri et al., 2017) using Nelson et al. (2011) or during this study by building the phylogenetic trees including strains LsoA (strains NZ1, HenneA and RSTM), LsoB (strain ZC1), LsoC (strains FIN111 and FIN114) and LsoD (strain haplotype D1); NA, not available

773   **Table 2.** Primers of housekeeping genes used for amplification and sequencing

774

Locus	Primer name	Sequence (5'-3')	Size of amplicon (bp)
acnA	acnA-F	AATCTTGTTGGTTTTGGATGTACAACGTG	949
	acnA-R	CTATCAAATTGGAGCGATGTATACGCT	
atpD	atpD-F	GCGGGAGTTGGGAAAACAGTATTAATCAT	811
	atpD-R	ATTCAGCAACATGAAACGGCTGTGACAT	
glnA	glnA-F	ATGGTTGATGACGCTACCTCTATCATC	905
	glnA-R	AGGGCTTTCGCATGTTTGATAATACCTC	
glyA	glyA-F	CGTTCAACCTCATTCTGGATCTCAGATGA	843
	glyA-R	AATCCTCTCGTTGTACCAGATGGCGTA	
gnd	gnd-F	CTAATGATGATAACAGACGGGAATCC	889
	gnd-R	GTATTATACATCCTGCACGCCAGA	
groEL	groEL-F	CAATCTCGCTGTTCAAGAAGTTGTAGA	927
	groEL-R	TTAACAGATAATGCTTGAGCAGCTCG	
ftsZ	ftsZ-F	ATGGTGGAAAAACACTCTAATGTGGATAT	915
	ftsZ-R	AGCCTCATCAAATGTAGCACCAAGAAT	

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777   **Table 3.** Summary statistics for the seven housekeeping genes and concatenated sequences used
778   in this study
779

Locus	sites[a]	GC%	S[b]	$\theta\pi$[c]	θw[d]	Tajima's D[e]	dN/dS[f]
acnA	816	37.2	25	0.00640	0.00687	-0.22568	0.128
atpD	759	40.7	20	0.00578	0.00591	-0.06851	0.030
ftsZ	774	43.2	12	0.00390	0.00348	0.35732	0.021
glnA	783	35.5	27	0.01123	0.00773	1.49289	0.311
glyA	747	41.8	19	0.00648	0.00570	0.43441	0.199
gnd	744	33.9	22	0.00642	0.00693	-0.24289	0.760
groEL	792	39.9	18	0.00521	0.00510	0.06685	0.000
Concat[g]	5415	38.9	143	0.00649	0.00597	0.31854	0.161

780   [a] number of analyzed sites
781   [b] number of polymorphic sites
782   [c] nucleotide diversity (Nei, 1987)
783   [d] nucleotide diversity with Watterson's estimator (Watterson, 1975)
784   [e] Neutrality test of Tajima (1989); all values are not significant ($P > 0.10$)
785   [f] ratio of non-synonymous to synonymous substitutions
786   [g] data set of concatenated sequences of the seven loci

787

788 **Table 4.** New SNP features of the LsoG haplotype

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Gene region	Haplotypes					
	A	B	C	D	E	G
glnA (778)	G	G	G	G	G	A
glyA (393)	C	C	C	C	C	G
groEL (726)	G	G	G	G	G	T
ftsZ (708)	C	C	C	T	C	C
ftsZ (717)	G	G	G	A	G	G

790

791 Nucleotide numbers are indicated relative to the first nucleotide of the aligned sequence of each
792 gene

793
794