Short Communication

MULTILOCUS SEQUENCE TYPING OF '*CANDIDATUS* LIBERIBACTER SOLANACEARUM' ISOLATES FROM NORTH AMERICA AND NEW ZEALAND

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SUMMARY

'*Candidatus* Liberibacter solanacearum' is associated with Zebra Chip (ZC) disease of potato. ZC disease has seriously affected potato cultivation in North America and New Zealand. Genotyping '*Ca.* L. solanacearum' isolates in different geographical regions will reveal genetic diversity and the epidemiological relationships of diseases associated with this bacterium. In this study, a panel of 10 multilocus sequence typing (MLST) markers for '*Ca.* L. solanacearum' was developed and used to characterize genetic relationships among '*Ca*. L. solanacearum' isolates in North America (USA and Mexico) and New Zealand. MLST analysis detected two sequence types (ST-1 and ST-2) for each of the 10 loci. Both types are present among US isolates while only one type was detected in Mexico (ST-1) and New Zealand (ST-2) isolates, respectively. This marker system provides a useful tool for genotyping and assessing genetic diversity of '*Ca*. L. solanacearum' populations.

Key words: Zebra chip, '*Candidatus* Liberibacter solanacearum', MLST, genetic structure.

'*Candidatus* Liberibacter solanacearum' is a phloem-limited alpha-proteobacterium and putative causal agent of Zebra Chip (ZC) disease of potato (*Solanum tuberosum*) (Liefting *et al*., 2009; Lin *et al*., 2009; Hansen *et al*., 2008). Potato plants affected by ZC are characterized by multiple symptoms in both the shoot and the tuber. Foliar symptoms include stunting, chlorosis, swollen nodes causing a "zig-zag" appearance of the upper growth, proliferated axillary buds, aerial tubers, browning of the vascular system in below-ground portions of stems, and leaf scorching. Below-ground plant symptoms include enlarged lenticels of the underground stem, collapsed stolons, brown discoloration of the vascular ring, necrotic flecking of internal tuber tissues and occasionally streaking of the medullary ray tissues (Gudmestad and Secor, 2007).

This disease has significantly affected potato production in the USA and Mexico within the past decade, and continues to be a threat to the livelihood of the potato industry. ZC disease was first reported in Mexico in 1994 (Munyaneza *et al*., 2009), and was confirmed to be present in the USA in 2004 (Gudmestad and Secor, 2007; Munyaneza *et al*., 2009). It was also discovered in New Zealand in 2008 (Liefting *et al*., 2008). Apart from its association with ZC disease, '*Ca*. L. solanacearum' has been connected to diseases of other Solanaceae, such as pepper (*Capsicum sp.*) (Wen *et al*., 2009), tamarillo (*S. betaceum*), tomato (*S. lycopersicum*) (Liefting *et al*., 2009; Wen *et al*., 2009; Ling *et al*., 2011), and in one Apiaceae, carrot (*Daucus carota*) (Munyaneza *et al*., 2010). Interestingly, '*Ca*. L. solanacearum' infection was shown to occur latently (asymptomatic) in a number of solanaceous weed species such as wolfberry (*Lycium barbarum*), black nightshade *(Solanum ptychanthum*), and silverleaf nightshade (*Solanum elaeagnifolium*) (Wen *et al*., 2009).

Taxonomically, '*Ca*. L. solanacearum' is closely related to the citrus huanglongbing (HLB)-associated bacteria, '*Ca.* L. asiaticus', '*Ca.* L. africanus' and '*Ca.* L. americanus' (Bové, 2006). While '*Ca.* L. solanacearum' has been determined to be the putative causal agent of potato ZC disease, information regarding the genetic diversity of isolates from different regions and plant hosts is limited. Genotyping '*Ca.* L. solanacearum' isolates will aid understanding the epidemiological relationships of diseases associated with this bacterium. Analysis of informative genes throughout the genome may reveal adaptive features of '*Ca.* L. solanacearum'; these features may include genes that contribute to agricultural success in a given ecosystem.

Multilocus sequence typing (MLST) is a genotyping method that is based on sequence information from a series of housekeeping loci located throughout the genome of the organism of interest (Ibarz Pavon and Maiden, 2009). Housekeeping genes are generally used for MLST since they undergo relatively little change in sequence over time. Moreover, sequence variations that occur within these loci may reflect pathogen adaptation and are

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likely to be informative for identifying discrete groups within a given population. Several studies of human diseases have employed MLST to characterize the population structure of the pathogens and gain insight into their dissemination and control (Deurenberg *et al*., 2007; Maiden *et al*., 1998; Walk *et al*., 2009). More recently, MLST analyses have also been used to understand the genetic structure, variability and evolutionarily relationship of bacteria associated with plant diseases (Almeida *et al.*, 2008; Yuan *et al*., 2010; Feng *et al*., 2009).

Here, we introduce a panel of ten MLST markers from '*Ca*. L. solanacearum' (Table 1). Using these MLST markers, we analyzed the genetic relationships between '*Ca*. L. solanacearum' isolates in North America (USA and Mexico) and New Zealand in order to gain insights into the origin of this pathogen associated with diseases in potato and other crops.

For sample collection, aboveground potato plant parts and tubers with symptoms of ZC were collected from several potato production states in the United States and from several commercial potato fields in Mexico. Plant tissues from each sample were ground under liquid nitrogen. DNA was extracted from 20 mg of freeze-dried tissues using DNeasy Plant Mini Kit (Qiagen, USA). In New Zealand, symptomatic plant material was collected from tomato, tamarillo and potato plants at different locations (Table 2). DNA samples were provided by Dr. Liefting. All samples were confirmed positive for '*Ca.* L. solanacearum' by qPCR prior to performing MLST according to established methods (Lin *et al*., 2011; Wen *et al*., 2009).

To generate a panel of markers suitable for MLST analyses, we examined the '*Ca*. L. solanacearum' genome (GenBank accession No. CP002371) (Lin *et al*., 2011) for orthologs of the 22 housekeeping genes previously used for *Escherichia coli* MLST analysis (Walk *et al*., 2009). From this starting set of 22 sequences, we identified 10 single-copy housekeeping loci that appeared to encode full-length proteins and possessed at least two single nucleotide polymorphisms (SNPs) within the amplified region: ADK (CKC_05260), DnaG (CKC_05195), FumC (CKC_05075), GrpE (CKC_00585), IcdA (CKC_04365), KdsA (CKC_02350), MetG (CKC_02965), MutS (CKC_00815), PurA (CKC_00315), and RecA (CKC_05085). To design primers for MLST, we generated multiple sequence alignments of the coding sequences for each locus using sequences for '*Ca*. L. solanacearum' and orthologous nucleotide sequences for '*Ca* L. asiaticus' psy62 (NC_012985), *Agrobacterium tumefaciens* C58 (NC_003062), *Sinorhizobium meliloti* 1021 (NC_003047), and *Rhizobium etli* CFN42 (NC_007761). We then examined the alignments for regions of sequence variability located between two conserved regions approximately 300-500 base pairs apart and selected primers that would anneal to the conserved segments. Primer sequences, annealing temperatures, amplicon sizes, and SNPs for each '*Ca*. L. solanacearum' MLST locus are shown in Table 1. For each MLST locus (Table 1), we provide the location and identity of each SNP based on its position within the MLST amplicon. The base position shown is relative to the first sequenced position in the sense strand for each MLST locus. The physical location of each of the 10 MLST loci is indicated in Fig. 1 and is based on the '*Ca*. L. solanacearum' genome sequence (Lin *et al*., 2011).

PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, USA) with the following cycle conditions: 94°C denaturation for 4 min, followed by 40-45 amplification cycles (94°C for 45 sec; annealing temperature for 45 sec; and 72°C for 45 sec), and concluding with a final extension step at 72°C for 7 min. Products were separated in 1.5-2.0% TBE-agarose gels and extracted using the Qiaquick Gel Extraction Kit (Qiagen, USA). The concentration of purified PCR products was determined by spectrophotometry and 1 to 20 ng of purified product were used as template for sequencing reactions per the manufacturer's protocol (Applied Biosystems, USA). Sequencing reactions were cleaned up by Big Dye Xterminator Purification kits (Appled Biosystems, USA) prior to sequencing and run on a 3130*xl* Genetic Analyzer (Applied Biosystems, USA). Forward and reverse PCR primers were run for each locus from each isolate (Table 1) to generate unidirectional sequences that were aligned to obtain a full-length sequence for each MLST locus. Quality-trimmed sequences were inspected, analyzed, further trimmed if necessary, and assembled using BioEdit (Isis Pharmaceuticals, USA) or Sequencher software (Genecodes, USA).

Fifty nine DNA samples of '*Ca.* L. solanacearum' that yielded informative data from all 10 MLST loci were selected for analyses. MEGA4 (Tamura *et al*., 2007) using

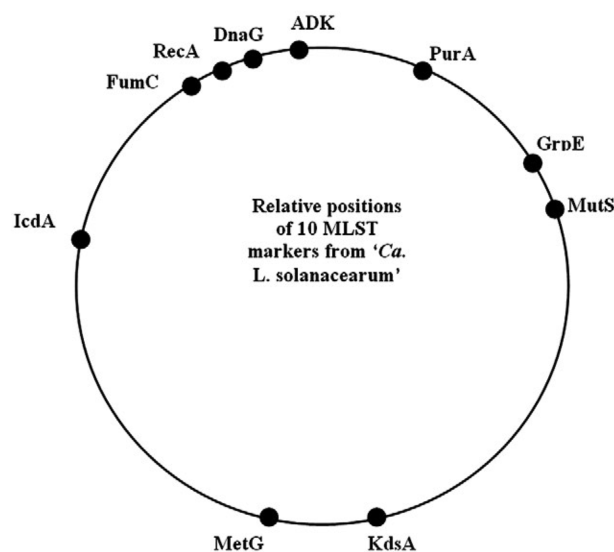

Fig. 1. Relative locations of the 10 MLST loci within the circular chromosome of '*Candidatus* Liberibacter solanacearum'.

Table 1. Characteristics of ten multilocus sequence typing (MLST) markers developed from '*Candidatus* Liberibacter solanacearum'.

Marker	PCR primer sequences	Annealing temperature (°C)	MLST size /Amplicon size (bp) [a]	SNPs	SNP sites [b]
ADK CKC_0526 0	5'-ATGAGAATTATATTTCTAGGCCCTCC -3' 5'-ATCATATTTATCATCTGATCGCACAG -3'	61	383 / 435	10	A4G, **A39G**, A43G, A142G, **T197C**, T211C, T220C, C307T, **A324G**, **C327T**
DnaG CKC_05195	5'-TTGCTATTGACTTTGATTAATCATCC -3' 5'-CAAAGCCTTCTATTATGGCTTCTTG -3'	60	415 / 466	6	T88C, C157A, G199A, **G224A**, **C242A**, **A315G**
FumC CKC_050 75	5'-TTCCTTTAGTCGTCTGGCAAACAGG -3' 5'-ACTTGTGCAGCGTATCCTGAAAATTC -3'	62	307 / 358	3	C121T, C169T, C220T
GrpE CKC_00585	5'-CAGATATGGAAAATATACGCCGTCG -3' 5'-TAGAAATACCAACTAAAGCGGGGCG -3'	61	346 / 396	2	C67T, G190A
IcdA CKC_043 65	5'-GGAAATCCCCTAACGGAACCATTCG -3' 5'-CTACCATATCATCAATGAGACGATGT -3'	62	435 / 486	3	A28G, **G278T**, **A332G**
KdsA CKC_0235 0	5'-GAAAGCCGCGAACATGCTTTTATGAT -3' 5'-GCCATGATAGGAAAAGAACGCATATC -3'	63	453 / 505	4	G111A, T138C, C228A, T441C
MetG CKC_02965	5'-TCGTACGATGATTTTATTCGCACAACGG -3' 5'-GGATCGTTAGGAATTTTTATTCCCCAATC -3'	65	347 / 404	6	**A3C**, G71A, **C150T**. C206T, **G216T**, T239G
MutS CKC_008 15	5'-CCAACAGATTCTAATTATCTCATGG -3' 5'-TCTAAATTGGAACGAGCGGCGGA -3'	58	416 / 464	6	**G242T**, T260C, **C274T**, **G300T**, **T354G**, G407A
PurA CKC_003 15	5'-TGTAGTTGTGGTCGGCTTACAATGG -3' 5'-TATCTTCATAAGCTGGGCCAATACC -3'	61	369 / 419	3	C84T, T201G, **C350A**
RecA CKC_050 85	5'-TTGGAAATAACAGATATGCTGGTGCG -3' 5'-AACCACGCTCCTGATTTATCAACGAT -3'	63	445 / 497	5	A103G, A241G, **A260G**, C400A, C419T

[a] Nucleotide sequence residing between the PCR primers was used for MLST analysis (primer sequences were removed prior to analysis); therefore, the length of the sequence tag for each MLST locus is equal to the size of the amplicon less the length of the two primers used for amplification.

[b] SNP sites are indicated relative to the first nucleotide of the sense strand from the sequenced portion of each amplicon. The first nucleotide indicated is present in Type 1 alleles and matches the '*Ca*. L. solanacearum' genome sequence, while the second nucleotide indicated is present in the Type 2 allele. The complete sequences for each allele are listed in FASTA format in a Supplemental File1. Missense substitutions are indicated in bold. Allele frequencies within the samples are as follows: *ADK* (Type-1 = 0.53; Type-2 = 0.47); *DnaG* (Type-1 = 0.51; Type-2 = 0.49); *FumC* (Type-1 = 0.51; Type-2 = 0.49); *GrpE* (Type-1 = 0.49; Type-2 = 0.51); *IcdA* (Type-1 = 0.53; Type-2 = 0.47); *KdsA* (Type-1 = 0.53; Type-2 = 0.47); *MetG* (Type-1 = 0.53; Type-2 = 0.47); *MutS* (Type-1 = 0.54; Type-2 = 0.46); *PurA* (Type-1 = 0.51; Type-2 = 0.49); and *RecA* (Type-1 = 0.49; Type-2 = 0.51).

ClustalW was used to generate an alignment for the sequences generated from MLST loci. Based on the allelic data obtained from all 10 MLST loci, the sequence type (ST) was identified for each of the isolates. The genetic relationships among the isolates were evaluated by a Neighbor-Joining tree, which was constructed from the concatenated sequences of all of the MLST loci that cover a total of 3,916 base pairs for all 59 isolates. MEGA4 (Tamura *et al*., 2007) software was used for constructing the tree. The percentage of replicate trees in which the isolates clustered together in the bootstrap test (1000 replicates) is shown next to the branches. To provide insight into the origins of '*Ca*. L. solanacearum' and the relationships among isolates, we also used eBURST v3 (http://eburst.mlst.net/) to identify putative founder types and clonal complexes (CCs) from the sequence types identified.

The segments of 10 housekeeping genes with two alleles per locus were present in 59 '*Ca*. L. solanacearum' isolates in this study (Table 2). The sequences of both allelic types of each MLST locus are provided as a supplemental data file in FASTA format. Ten unique STs were detected, with two of the STs occurring in more than 80% of the samples (Table 2).

The neighbor-joining tree (Fig. 2) indicates that there are two major groups (Group I and Group II) among the '*Ca*. L. solanacearum' isolates studied. Isolates obtained from different potato production areas in the USA resided in both groups. Here, no clear genetic association was observed among the isolates based on the

Table 2. *S*equence typing (ST) profile and sample information of '*Candidatus* Liberibacter solanacearum' isolates from North America (USA and Mexico) and New Zealand.

Sequence typing (ST) [a] profile	Number of isolate belongs to each ST (Profile frequency)	Sample location	Host cultivar (Variety [b])
1111111111 (1)	27	**Texas:** Dalhart (4 isolates); Olton (3 isolates); Springlake (1 isolate); Lubbock (1 isolate); Pearsall (2 isolates)	FL 1867 (5 isolates); RN (2 isolates); Unknwon (4 isolates)
		Nebraska: Cody (2 isolates); O'Neill (1 isolate); Southwestern (3 isolates)	FL 1867 (1 isolate); FL 2048 (1 isolate); RN (4 isolates)
		Colorado: Wray (1 isolate)	RN
		Kansas: Garden City (2 isolates)	Atlantic (1 isolate); RN (1 isolate)
		Mexico: 7 isolates	Unknown
2222222222 (2)	23	**Texas:** Dalhart (6 isolates); Olton (1 isolate); Pearsall (1 isolate)	FL 1867 (5 isolates); FL 1291(1 isolate); FL 1922 (1 isolate); Unknown (1 isolate)
		Nebraska: Cody (3 isolates); O'Neill (1 isolate); Columbus (1 isolate); Minden (2 isolates)	FL 1867 (2 isolates); FL 1833 (1 isolate); RN (1 isolate); FL 2053 (2 isolates); Unknown (1 isolate)
		Kansas: Garden City (1 isolate)	FL 1867 (1 isolate)
		California: Bakersfield (1 isolate)	Unknown
		New Zealand: Auckland (2 isolates); Northland (1 isolate); Tom-Waikato (1 isolate); Rillo-South Auckland (1 isolate); Capis-West Auckland (1 isolate)	Infected tomato (1 isolate); Infected tamarillo (1 isolate); Infected capiscum (1 isolate); Infected potato (3 isolates)
2222212222 (3)	1	**Texas:** Olton	RN
1222121122 (4)	1	**Texas :** Olton	Unknown
2222222122 (5)	2	**Nebraska:** Cody **California:** Bakersfield	Burbank Unknown
1212111112 (6)	1	**Nebraska:** Cody	Blazer
1111111211 (7)	1	**Texas:** Olton	FL 1867
1121111111 (8)	1	**Texas:** Dalhart	FL 1867
2212222222 (9)	1	**Nebraska:** Cody	FL 1867
2122222222(10)	1	**Nebraska:** Cody	FL 2084

[a] Sequence typing profiles are listed in the order of gene locus: (1)ADK, (2) DnaG, (3) FumC, (4) GrpE, (5) IcdA, (6) KdsA, (7) MetG, (8) MutS, (9) PurA, and (10) RecA.
[b] RN = Russet-Norkotah.

geographical location or host (Fig. 2). All of the isolates from Mexico clustered in Group I. On the other hand, isolates obtained from all different hosts from New Zealand clustered only in Group II (Fig. 2). Consistent with Neighbor-joining tree, the eBURST program also identified two main groups (clonal complexes, CCs) (Fig. 3). Based on their frequency-of-occurrence, the founders of each group are predicted to be those possessing either all- Type-1 alleles (ST-1) or all-Type-2 alleles (ST-2). The less frequent STs in each group might have been introduced or evolved from recombination amongst the proposed founder types.

The appearance of both of these two lineages in different potato production areas in the USA agree with a recent study of '*Ca.* L. solanacearum' isolates (Nelson *et al.*, 2011) where two haplotypes were reported in the USA from the analyses of 16S rRNA, 16S/23S ISR and 50S rplJ and rplL ribosomal protein genes (Nelson *et al.*, 2011). In our study, only one sequence type was identified in Mexico (ST-1) and in New Zealand (ST-2). Nelson *et al.* (2011) reported two haplotypes in Mexico and one haplotype in New Zealand. The presence of one ST in Mexico (our study) is likely due to the limited number of isolates analyzed. Broader population analyses will be needed for further resolution about the origin and dissemination of this pathogen in different geographical locations.

eBURST analysis was consistent with two primary founder groups of '*Ca*. L. solanacearum'(ST-1 and ST-2). While most of the '*Ca*. L. solanacearum' isolates in MLST analyses appeared to possess one of two genetic makeups (ST-1 or ST-2), the results of this study indicate that a few recombination events have occurred in some isolates from the two major lineages predicted based on this sampling (Table 2). However, the predominant mode of genetic recombination amongst '*Ca*. L. solanacearum' in agricultural settings is unknown.

The polymorphic MLST markers we detected in this

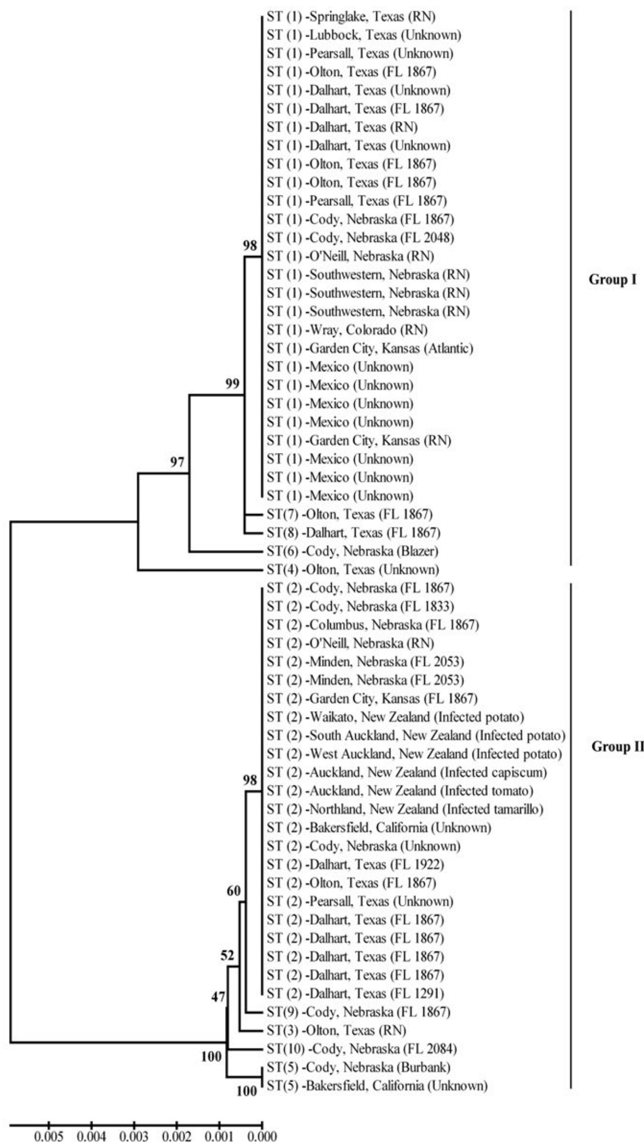

Fig. 2. Neighbor joining tree constructed from the concatenated sequences generated from ten MLST loci among '*Candidatus* Liberibacter solanacearum' isolates in North America (USA and Mexico), and New Zealand. Two groups (group I and group II) are identified from the samples analyzed in this study. Sample label contains geographic location and host cultivar.

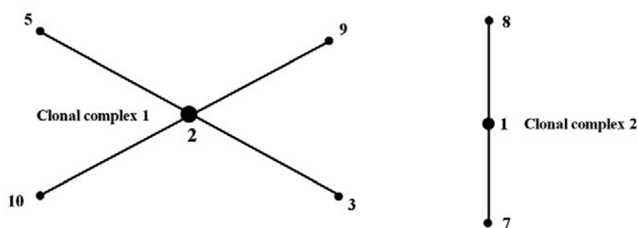

Fig. 3. Clonal complexes and relationship between '*Candidatus* Liberibacter solanacearum' sequence types (STs) based on eBURST grouping. 1(ST1) and 2(ST2) are predicted as founders based on their frequency of occurrence in this study.

study are useful for characterizing '*Ca*. L. solanacearum' and broader epidemiological studies of diseases associated with this bacterium. Further, since '*Ca*. L. solanacearum' has yet to be obtained in axenic culture and is currently intractable to classical genetic approaches and pathogenicity/virulence experiments, this panel of molecular markers may help link segments of the '*Ca*. L. solanacearum' chromosome with varying levels of virulence in the field. For example, if one marker is closely tied to a particularly virulent '*Ca*. L. solanacearum' isolate, the segment of the '*Ca*. L. solanacearum' genome containing that marker may also harbor a gene or gene cluster involved in pathogenesis.

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