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Development of a PCR Assay for the Rapid Detection and Differentiation of '*Candidatus* Liberibacter solanacearum' Haplotypes and Their Spatiotemporal Distribution in the United States

Aimin Wen · Chris Johnson · Neil C. Gudmestad

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Abstract Zebra chip, or zebra complex (ZC) has become an important invasive disease of potato in the United States and New Zealand and is caused by a phloem-limited bacterium, '*Candidatus* Liberibacter solanacearum' (Lso). A PCR assay using a single pair of simple sequence repeat (SSR) primers was developed for simultaneous detection and genotype differentiation of Lso haplotypes associated with zebra chip disease of potato. The sensitivity of the SSR PCR was similar to a 16S PCR assay, with detection limit of 100 copies of the Lso genome in haplotype A infected potato and psyllid samples and 10 copies of Lso genome in haplotype B potato and psyllid samples. The Lso detection frequency of the SSR PCR assay was 79.1 % in potato and 26.4 % in psyllid samples, respectively; whereas the detection frequency of the 16S PCR assay 59.0 % in potato and 25.9 % in psyllid samples, respectively. Samples of Lso positive potato plants and psyllids from multiple states in the US were demonstrated to have either haplotype A or haplotype B Lso and occasionally both haplotypes were found in individual samples. This is the first report that co-infection of the two haplotypes of Lso exists in potato and potato-psyllid samples. Only haplotype A Lso was detected in North Dakota psyllid samples collected in 2010, in Idaho and Washington ZC potato samples sampled from storage in 2011, and in Idaho ZC potato samples in 2012. Haplotype A Lso was also detected in New Zealand ZC affected potato samples and psyllid samples collected in 2010 and 2011. The PCR assay developed is as sensitive as previously developed assays and has the advantage of simultaneously detecting and differentiating Lso haplotypes of the ZC bacterium, thus making it extremely useful for epidemiological studies.

Resumen Zebra chip o el complejo zebra (ZC) se ha convertido en una enfermedad invasiva importante en los Estados Unidos y en Nueva Zelanda, causada por una bacteria limitada al floema, "*Candidatus Liberibacter solanacearum*" (Lso). Se desarrolló un ensayo de PCR utilizando iniciadores de un solo par de secuencia de repetición de genotipo para detección simultánea y diferenciación de genotipo de los haplotipos de '*Candidatus Liberibacter solanacearum*' (Lso) asociados con la enfermedad de zebra chip (ZC) de la papa. La sensibilidad del SSR PCR fue similar al ensayo 16S PCR, con un límite de detección de 100 copias del genomio del Lso en papa infectada del tipo 1 y 10 copias del genomio Lso tipo 2 de muestras de papa y del psílido. La frecuencia de detección del Lso del ensayo SSR PCR fue de 79.1 % en papa y de 26.4 % en muestras del psílido, respectivamente; mientras que la frecuencia de detección del ensayo de 16S de PCR fue de 59 % en papa y de 25.9 en las muestras del psílido, respectivamente. A muestras de Lso de plantas de papa positivas y de psílidos de múltiples estados en los EUA se les demostró tener ya fuera el tipo 1 o el tipo 2 de Lso y ocasionalmente se encontraron ambos haplotipos de biotipos en muestras individuales. Este es el primer reporte de la existencia de co-infección de los dos tipos de Lso en muestras de papa y del psílido de la papa. Solamente se detectó el tipo 1 de Lso en muestras del psílido de Dakota del Norte colectadas en el 2010, en Idaho y Washington en muestras de papa con ZC tomadas del almacén en 2011, y en Idaho ZC en muestras de papa en 2012. También se detectó el tipo 1 de Lso en Nueva Zelanda en muestras de papa afectadas con ZC y en muestras del psílido colectadas en 2010 y 2011. El ensayo de PCR desarrollado

A. Wen · C. Johnson · N. C. Gudmestad (✉)
Department of Plant Pathology, North Dakota State University, Fargo, ND 58108, USA
e-mail: neil.gudmestad@ndsu.edu

es tan sensible como ensayos desarrollados previamente y tiene la ventaja de detectar simultáneamente y de diferenciar haplotipos de Lso de la bacteria ZC, haciéndolo así extremadamente útil para estudios epidemiológicos.

Keywords "*Candidatus* Liberibacter solanacearum" · Zebra chip disease · Simple sequence repeat · Genotyping

Introduction

Zebra chip or zebra complex (ZC) disease is an emerging disease of potato that causes significant losses to potato production. ZC has been reported in the USA, Mexico, Central America, and New Zealand. The putative causal agent has been identified as a phloem-limited bacterium, '*Candidatus* Liberibacter solanacearum' (Lso) and is vectored by the tomato/potato psyllid (*Bactericera cockerelli* Sulc) (Liefting et al. 2009; Lin et al. 2009; Secor et al. 2009; Wen et al. 2009). ZC was first observed in potato fields around Saltillo, Mexico in 1994 (Secor and Rivera 2004), and six years later, it was found in commercial potato fields in the Pearsall area in Texas (Gudmestad and Secor 2007; Munyaneza et al. 2011; Secor et al. 2009). '*Candidatus* Liberibacter psyllaurous' (Lps), suggested as the causal agent of psyllid-yellows (PY) disease of potato and tomato, also vectored by the same tomato/potato psyllid (Hansen et al. 2008), has been shown to be phylogenetically closely related to Lso (Secor et al. 2009; Wen et al. 2009). Moreover, Lso can be detected from both ZC and PY symptomatic potato samples (Wen et al. 2009). Since its first detection, ZC has spread to additional states including New Mexico, California, Nebraska, Kansas, Colorado, and Wyoming (Gudmestad and Secor 2007; Secor et al. 2009), and in 2011 ZC was found in Oregon, Washington and Idaho (Crosslin et al. 2012a, b). Recently, Lso has been reported to be associated with psyllid-affected carrots in Finland, Norway, Spain, and Sweden (Alfaro-Fernández et al. 2012; Munyaneza et al. 2010, 2012a, b) and is vectored by the carrot-psyllid species, *Trioza apicalis* in Finland, Sweden and Norway and by the carrot-psyllid species, *Bactericera trigonica* in Spain.

Several reports have revealed genetic variations among Lso haplotypes based on sequence analysis of 16S-ISR-23S rRNA and 50S ribosomal protein genes (Nelson et al. 2011;

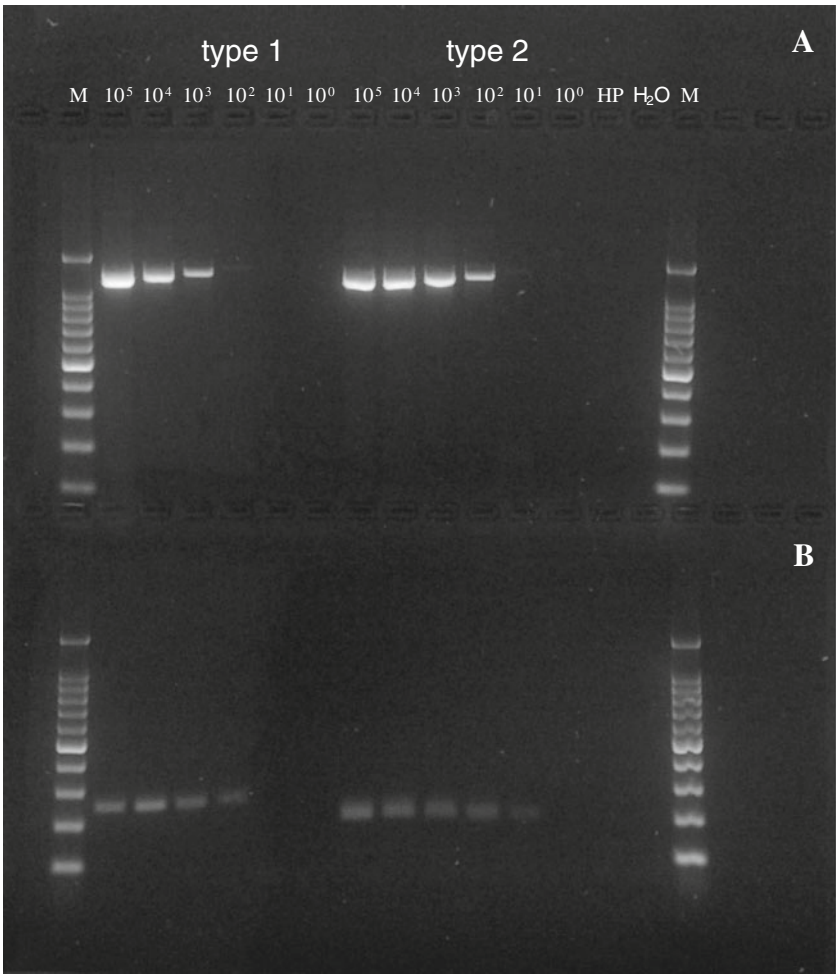

Fig. 1 Sensitivity of SSR and 16S PCR assays in potato samples infected with haplotype A Lso or haplotype B Lso. **a** 16S PCR products, and **b** SSR PCR products on the same set of DNA sample dilutions. Lane1–6: 10-fold dilutions of total DNA extracted from potato sample harboring haplotype A Lso with starting 10^5 copies of Lso genome; lane 7–12: 10-fold dilutions of total DNA extracted from potato sample harboring haplotype B Lso with starting 10^5 copies of Lso genome; lane 13: potato DNA sample free of Lso; lane 14: water, negative control. M: 100 bp plus DNA ladder (Qiagen)

Secor et al. 2009; Wen et al. 2009), and using simple sequence repeat (SSR) and multi-locus sequence typing (MLST) markers (Glynn et al. 2012; Lin et al. 2012). Genotype designations derived from these studies include haplotype A, B and C; Clade 1 and 2; Lineage 1 (type 1) and 2 (type 2); and ST1 and 2. The correlation among these designations is that haplotype A corresponds to Clade 1, Lineage 1 (type 1) and ST-1; whereas haplotype B corresponds to Clade 2, Lineage 2 (type 2) and ST-2. Both haplotypes A and B have been reported in the USA and Central America, which are associated with ZC disease of potato, whereas haplotype C has only been reported in Europe, and is associated with carrot disease. Although there are currently no definitive phenotypical differences assigned to Lso haplotypes, field observations suggest haplotype B (type 2) Lso produces a more severe and destructive disease symptom than haplotype A (type 1) (Gudmestad, personal observation).

Currently, there is no assay to simultaneously detect and genotype Lso. Available Lso detection assays are PCR-based using primers of the 16S rRNA, ISR-23S rRNA or outer member protein gene (Crosslin et al. 2011; Hansen et al. 2008; Li et al. 2009; Liefting et al. 2009; Ravindran et al. 2011; Secor et al. 2009; Wen et al. 2009). Lso haplotype differentiation is either conducted by sequencing the PCR products (Glynn et al. 2012; Nelson et al. 2011; Secor et al. 2009; Wen et al. 2009) or by genotyping PCR products amplified using fluorescent labeled primers, which requires special equipment, and is expensive and time-consuming. Biotyping primers should be designed based on single copy genes. Currently, the 50S rp sequence is the only qualified available sequence, however, the SNPs among the 50S rp gene are so scarce that it is not able to differentiate the two types of Lso using PCR assay. Fortunately, one set of SSR primers (Lin et al. 2012) is a single locus and produces amplicons that can be differentiated on an agarose gel. The purpose of this study was to develop a PCR-based assay that would rapidly and effectively detect and differentiate Lso into haplotypes and to use this assay to evaluate the spatial and temporal distribution of Lso haplotypes in the United States.

Materials and Methods

Plant and Psyllid Samples and DNA Extraction Archived DNA samples obtained from ZC and psyllid yellows (PY) affected potato tissues and collected between 2006 and 2009 were used in this study and were obtained as described previously (Wen et al. 2009). Potato samples displaying

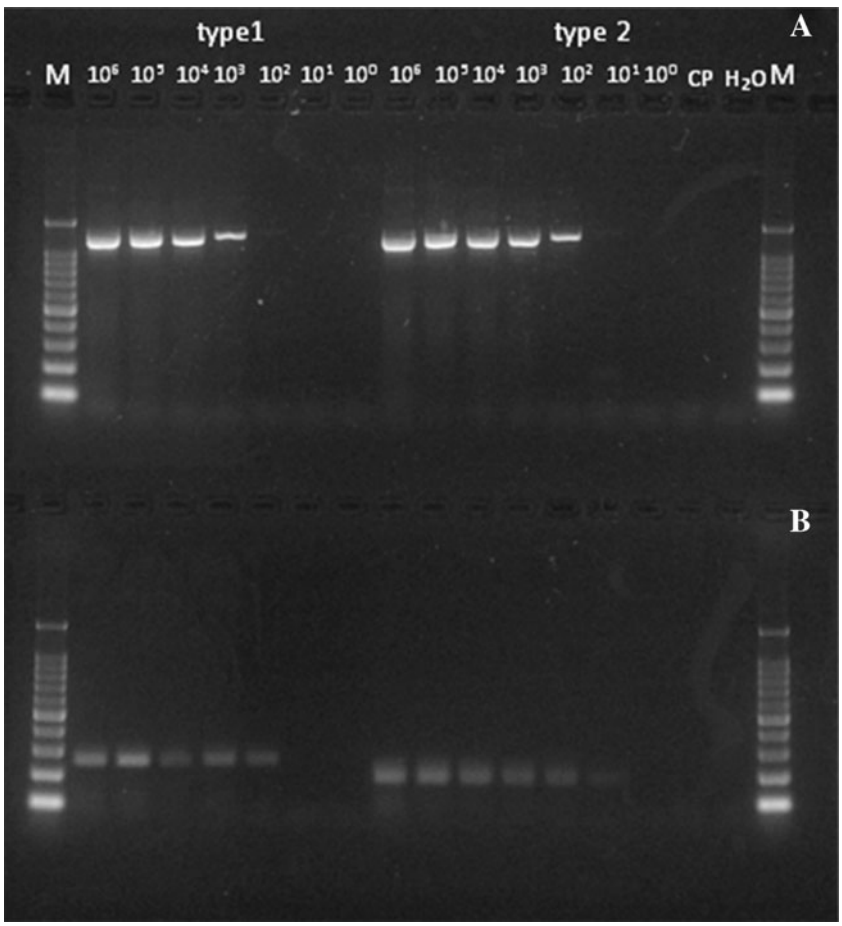

Fig. 2 Sensitivity of SSR and 16S PCR assays in psyllid samples infected with haplotype A or haplotype B Lso. **a** 16S PCR products, and **b** SSR PCR products on the same set of sample dilutions. Lane 1–6: 10-fold dilutions of total DNA extracted from psyllid harboring haplotype A Lso with starting 10^6 copies of Lso genome; lane 7–12: 10-fold dilutions of total DNA extracted from psyllid harboring haplotype B Lso with starting 10^6 copies of Lso genome; lane 13: psyllid DNA sample free of Lso; lane 14: water, negative control. M: 100 bp plus DNA ladder (Qiagen)

ZC and psyllid-yellows (PY) symptoms were collected from commercial potato production regions in Texas from 2010 to 2012, and in Colorado in 2012. Foliar symptoms of ZC include swollen nodes, axillary bud development, reddening of the foliage, leaf scorching, and eventual plant death; whereas PY symptoms include pronounced yellowing of the foliage and shortening of the internodes, resulting in a dwarf rosette appearance. ZC-affected tubers exhibit necrotic flecking and medullary ray discoloration, whereas PY-affected tubers occasionally demonstrate a slight vascular discoloration. Potato tubers displaying ZC symptoms were obtained from storage facilities from Idaho and Washington in 2011, which were kindly provided by Joe Rehder (ConAgra Foods–Lamb Weston, Inc., Pasco, Washington) and Dr. Nora Olsen (University of Idaho, Twin Falls Research and Extension Center, Twin Falls, ID). DNA extraction from petioles, stolons, and tubers, if available, was performed as described previously (Wen et al. 2009). Psyllid (egg, nymph and adult) samples were collected from potato production areas in California, Nebraska, North Dakota, and Texas in 2010. DNA extraction from psyllid was conducted also as previously described (Li et al. 2009). DNA samples derived from Texas psyllid collected in 2011 and 2012, and DNA samples of Mexico psyllid raised in greenhouse in Washington, were kindly provided by Dr. Jim Crosslin (USDA-ARS, Prosser, WA). New Zealand potato and psyllid DNA samples collected in 2010 and 2011 were kindly provided by Drs. Andrew Pitman and Sam Beard (New Zealand Institute for Plant & Food Research, Canterbury Agriculture and Science Centre, Lincoln, New Zealand). Total DNA concentration was quantified using Nanodrop (ThermoScientific).

SSR PCR Assay The PCR assay developed was based on SSR markers developed by Lin et al. (2012). One of the SSR primer pairs, Lso-SSR-1 F and Lso-SSR-1R, produces amplicons of 180 bp and 240 bp, which can be differentiated on an agarose gel. The PCR amplicon of 240 bp was designated as type 1 Lso, correlating to haplotype A and the PCR amplicon of 180 bp was designated as type 2 Lso, correlating to haplotype B. The SSR PCR thermal cycles are 1 cycle at 94 °C for 5 m, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and extension with 1 cycle of 72 °C for 10 m. PCR reaction consists of 1 × of PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM Lso-SSR-F1 and Lso-SSR-R1, and 1 U Platinum DNA Taq polymerase in a 20 μL volume reaction. PCR products were analyzed with electrophoresis on 1.5 % agarose gel stained with GelStar Nucleic Acid Gel Stain (Lonza, Rockland, ME) in 1 × TAE buffer.

Sensitivity of SSR PCR Assay The sensitivity of SSR PCR assay was compared with that of 16S PCR assay with primer set ZCf/OI2c (Wen et al. 2009). Sensitivity tests were performed using 10-fold dilutions of total DNA field potato and psyllid samples. The initial Lso genome copy number in DNA derived from potato and psyllid tissue samples was 10^5 and 10^6, respectively.

Efficiency of SSR-PCR Assay in Lso Detection in Plant and Psyllid Samples SSR PCR was validated by testing Lso from plant and psyllid DNA samples. Detection efficiency of SSR PCR assay was compared to that of 16S PCR assay described above.

Spatial and Temporal Distribution of 'Candidatus Liberibacter solanacearum' haplotypes Lso haplotypes were typed using SSR PCR assay in archived DNA from potato

Table 1 Frequency of '*Candidatus* Liberibacter solanacearum' haplotypes detected in archived DNA from potato samples obtained from seven states of the USA, New Zealand and Mexico in 2006–2012

Year of collection	Origin[a] and symptom[b] (number of samples)	% Lso genotype		
		Type 1	Type 2	Type 1 and type 2
2006	CO, ZC (1)	0	100	0
	NE, ZC (2)	0	100	0
	TX, ZC (73)	52	48	0
2007	TX, ZC (34)	62	38	0
2008	CO, ZC (6)	33	67	0
	KS, PY (7)	29	71	0
	KS, ZC (17)	29	71	0
	NE, PY (4)	100	0	0
	NE, ZC (25)	47	53	0
	TX, PY (6)	50	50	0
	TX, ZC (72)	31	69	0
	MX, ZC (16)	100	0	0
2009	CA, ZC (13)	92	8	0
	NE, ZC (62)	92	8	0
2010	TX, PY (16)	93	7	0
	TX, ZC (110)	63	6	31
2011	ID, ZC (77)	100	0	0
	TX, PY (79)	100	0	0
	TX, ZC (250)	30	23	47
	WA, ZC (77)	100	0	0
	NZ, ZC (11)	100	0	0
2012	CO, ZC (10)	60	40	0
	ID, ZC (23)	100	0	0
	NE, PY (4)	100	0	0
	NE, ZC (11)	100	0	0
	TX, PY (15)	100	0	0
	TX, ZC (30)	88	12	0

[a] NZ stands for New Zealand, and MX stands for Mexico

[b] ZC stands for zebra chip and PY for psyllid-yellows

samples obtained from seven US states, New Zealand and Mexico through 2006 to 2012, and in archived DNA from psyllid samples obtained from five US states, New Zealand, and Mexico through 2010 to 2012.

Results

Detection Sensitivity of SSR PCR Assay The detection limit of the SSR PCR assay was identical to that of the 16S PCR assay. Both assays detected 100 copies of type 1 Lso genome, and 10 copies of type 2 Lso genome in both potato and psyllid samples (Figs. 1 and 2).

Detection Efficiency of SSR PCR in Potato Plant and Psyllid Samples The SSR PCR assay was validated by testing field and storage potato samples obtained between 2008 and 2012. Lso detection frequency of the SSR PCR assay was 70 % in 650 archived potato samples and 88 % in 593 fresh potato samples, respectively; whereas Lso detection frequency of the 16S PCR assay was 58 % in archived potato samples and 63 % in fresh potato samples, respectively. When using psyllid samples obtained in 2010 and 2012 Lso detection frequency of the SSR PCR and 16S PCR assays was 50 % and 45 %, respectively. Thus, the overall Lso detection efficiency of the SSR PCR was demonstrated to be higher than that of the 16S PCR assay.

Spatial and Temporal Distribution of 'Candidatus Liberibacter solanacearum' Haplotypes Haplotype A and haplotype B Lso were detected in archived and fresh DNA from potato samples collected between 2006 and 2012 from Colorado, Kansas, Nebraska, and Texas (Table 1). Interestingly, only haplotype A Lso was detected in ZC-symptomatic potato samples from Idaho, Washington, Mexico and New Zealand; and in potato samples displaying PY symptoms collected in

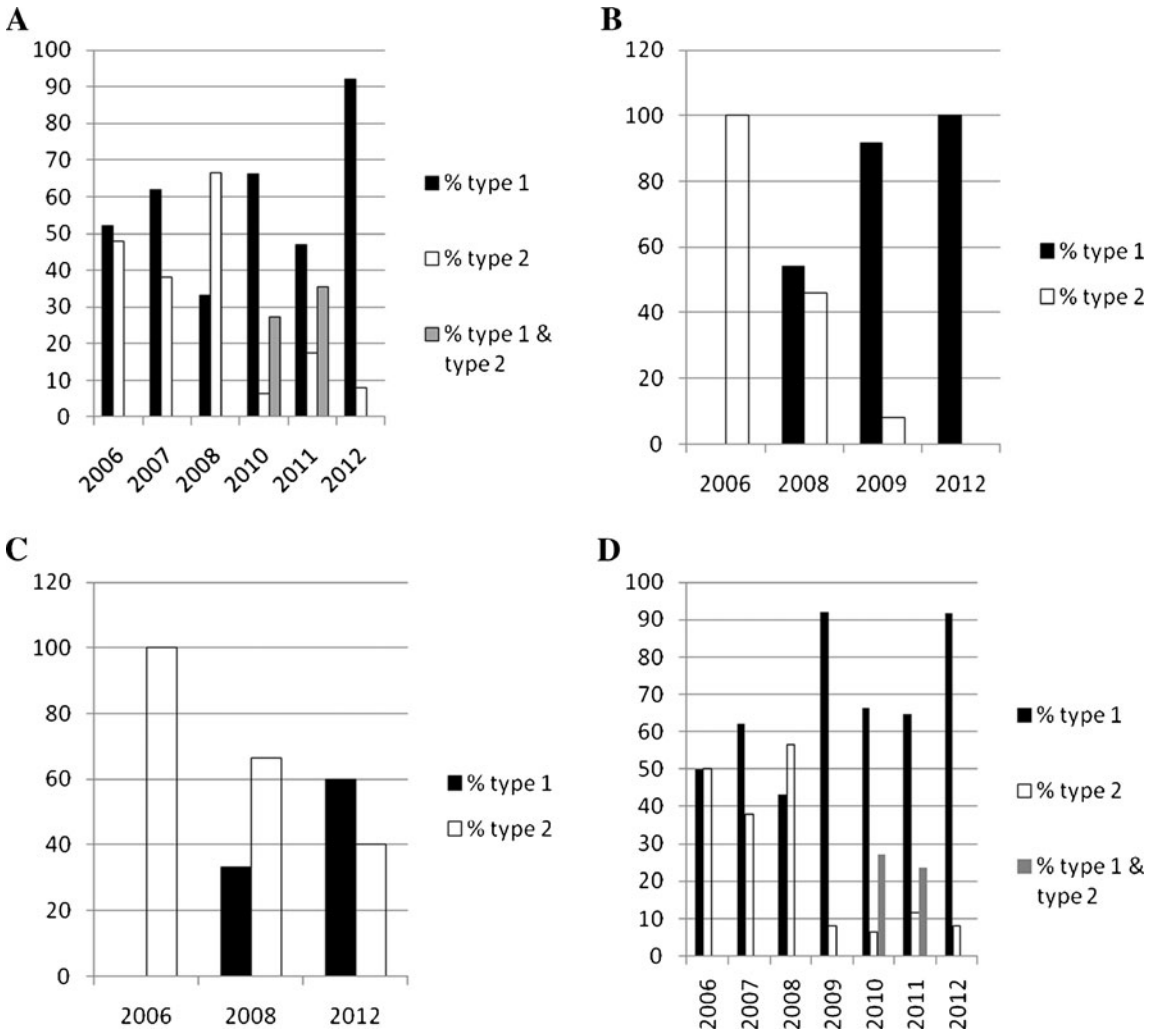

Fig. 3 '*Candidatus* Liberibacter solanacearum' haplotype detection frequency in potato samples collected in California, Colorado, Nebraska, Texas, Mexico and New Zealand from 2006 to 2012. **a** Texas; **b** Nebraska; **c** Colorado; and **d** All samples tested including those from California, USA and from Mexico and New Zealand

2008 and 2012 from Nebraska, and collected from Texas in 2011 and 2012, respectively (Table 1). Both haplotype A and haplotype B Lso were detected simultaneously in individual potato samples displaying ZC symptoms collected in 2010 and 2011 from Texas. Shifts in Lso haplotype detection frequency were noted in samples collected in Texas, Nebraska and Colorado from 2006 to 2012 with the detection of haplotype A Lso increasing in all three states (Fig. 3). In Texas samples from 2006 to 2008, the detection frequency of haplotype B Lso peaked at 67 % in 2008 vs. 48 % and 38 % in 2006 and 2007, respectively (Fig. 3a). However, detection frequency of haplotype B Lso decreased to 6 % in 2010, and remained relatively low in 2011 and 2012 with detection frequencies of 18 % and 8 %, respectively (Fig. 3a). In Nebraska samples, haplotype B Lso detection frequency decreased from 100 % in 2006 to 8 % in 2009, and this haplotype was not detected in 2012 (Fig. 3b). In Colorado samples, detection of haplotype B Lso decreased from 100 % in 2006, to 67 % in 2008 and 40 % in 2012 (Fig. 3c). Overall ZC affected potato tissue collected from the US, Mexico and New Zealand, there has been a marked decrease in the detection frequency of type 2 Lso (Fig. 3d).

In archived DNA from potato psyllids from North Dakota and New Zealand only haplotype A Lso has been detected, while psyllids collected from California were only infected with haplotype B Lso (Table 2). Both haplotype A and haplotype B Lso were detected in potato psyllids from Nebraska, Texas, and Mexico (Table 2). Interestingly, 13 % of the psyllids from Washington in 2010 and 41 % and 25 % of the psyllids from Texas in 2011 and 2012, respectively were found to be simultaneously infected with both haplotype A and haplotype B Lso (Table 2). All of the psyllids from Mexico in 2011 were found also to be infected with both haplotypes of Lso.

Table 2 Frequency of '*Candidatus* Liberibacter solanacearum' haplotypes detected in archived DNA from psyllid samples obtained from five states in the USA, New Zealand and Mexico in 2010–2012

Year of collection	Origin (number of Lso positive samples)	% Lso genotype		
		Type 1	Type 2	Type 1 and type 2
2010	CA (4)	0	100	0
	ND (37)	100	0	0
	NE (54)	98	2	0
	TX (5)	0	100	0
	WA (8)	50	37	13
2011	TX (12)	33	25	42
	NZ[a] (8)	100	0	0
	MX[b] (4)	0	0	100
2012	TX (122)	39	36	25

[a] NZ stands for New Zealand

[b] Mexico psyllids were raised in growth room in Moxie, WA

Discussion

Two bacterial populations of "*Candidatus* Liberibacter solanacearum" have been reported to be associated with zebra chip disease of potato (Glynn et al. 2012; Lin et al. 2012; Nelson et al. 2011; Wen et al. 2009). Nelson et al. (2011) recommended that haplotype should be noted for Lso reports to better understand the biological implications and we agree with that recommendation. However, determination of the haplotypes detected can be very tedious and expensive since it involves sequencing the 16S rRNA gene or other target genes prior to the studies reported here. The application of the SSR primers developed in present study make it possible to simultaneously detect the ZC pathogen and to determine Lso haplotype using a single PCR assay which should facilitate the reporting of Lso haplotypes in future publications.

This study revealed the temporal and spatial distributions of the two haplotypes of Lso in potato displaying either ZC or PY symptoms and potato-psyllid samples collected from 2006 to 2012 in seven US states, Mexico and New Zealand. Haplotype A Lso was found in potato samples from Washington, Idaho, and Wyoming, and in psyllid samples from North Dakota for the first time, whereas haplotype B Lso was found in California for the first time. Haplotype A and haplotype B Lso were detected in potato samples displaying PY, and ZC symptoms in Nebraska, Colorado, Kansas and Texas, whereas only haplotype A was detected in potato and psyllid samples from New Zealand, which is in agreement with previous reports (Nelson et al. 2011; Lin et al. 2012; Glynn et al. 2012; Wen et al. 2009). Temporal shifts in Lso haplotypes were noted during the course of these studies. For example, haplotype B Lso dominated in Texas in 2008 through 2010; however haplotype A Lso has become more prevalent since 2011. It is interesting to note that concomitant with this shift in prevalence of Lso haplotype, ZC disease has become less severe and less prevalent in that state (Gudmestad, personal observation). Another important finding of this study is that two haplotypes of Lso were detected simultaneously in individual potato plants from Texas and Nebraska and potato-psyllid samples from Texas, Washington and Mexico (greenhouse raised psyllids only), which indicates that co-infection of the two Lso haplotypes exist in agricultural ecosystems. This is the first report of co-infection of the two types of Lso in potato and the potato psyllid and it raises interesting questions regarding the efficiency of transmission of each haplotype by a dual infected bacteriliferous vector, *B. cockerelli*. Furthermore, since there are three haplotypes of this insect vector (Swisher et al. 2012), studies that investigate potential Lso haplotype-*B. cockerelli* haplotype interactions are warranted. Research on Lso has been focused on ZC-symptomatic potato plants because of the economic loss ZC caused. Our group had previously detected Lso in PY displaying potato plants (Wen et al. 2009), also we successfully graft-transmitted PY and

detected Lso in scions showing PY symptoms (Wen et al. unpublished). This study further demonstrated that Lso detected in PY-symptomatic potato plants also had two haplotypes. Indeed, the foliar symptoms of ZC and PY are hard to differentiate, however, ZC and PY have distinctive features to potato pathologists. Foliar symptoms of ZC include swollen nodes, axillary bud development, reddening of the foliage, leaf scorching, and eventual plant death; whereas PY foliage symptoms include pronounced yellowing of the foliage and shortening of the internodes, resulting in a dwarf rosette appearance. Moreover, ZC-affected tubers exhibit necrotic flecking and medullary ray discoloration, whereas PY-affected tubers occasionally demonstrate a slight vascular discoloration.

At the beginning of these studies we hypothesized that the two haplotypes might be different in virulence based on observations in the field in Texas over the past two years. Based on, preliminary observations in greenhouse trials (Johnson and Gudmestad, unpublished) we still believe this to be the case, however, it will be important to empirically demonstrate differences in aggressiveness between Lso haplotypes under controlled conditions and these studies are in progress. Nonetheless, the source of the two haplotypes of Lso is still in question and should also be the focus of future research. We believe the focus of that research should be on the source of Lso in overwintering psyllid populations and whether or not the overwintering plant hosts harbor different genetic populations of Lso. Furthermore, it has been established that Lso negatively affects the fecundity of the potato psyllid (Nachappa et al. 2012), however, the haplotype of Lso present in the psyllid population used in that study were not determined and our attempts to secure DNA to determine the Lso haplotype in that colony have failed. Given the decrease in frequency of haplotype B Lso in potato plants and in the potato psyllid in the US over the past four years, this suggests that haplotype B Lso has a more deleterious effect on fitness than haplotype A Lso. But again, studies are warranted to determine if there is a differential effect of Lso haplotype on psyllid fecundity or survival.

The SSR PCR assay developed in this study has its limitations due to the nature of SSR sequences. The difference on detection limit between the two types of Lso (100 copies of haplotype A Lso genome vs. 10 copies of haplotype B genome) might cause errors in detection frequency of the two haplotypes of Lso. With the availability of a haplotype A Lso genome sequence in the near future, more specific primers could be developed which may be more accurate in determining Lso population distribution. Nonetheless, this study provides a tool to detect and type Lso from potato and psyllid samples in a simple PCR assay and should prove useful in future epidemiological studies. This SSR PCR assay is more rapid and less costly compared to conventional genotyping methods since no special equipment is needed to differentiate SSR PCR products. It should be noted, however, that Lso associated with carrot-disease (haplotype C Lso) in Finland cannot be differentiated from haplotype A using this SSR PCR assay because the SSR PCR products have the same length, although the sequences of the SSR products are different (Wen, et al. unpublished). Since haplotype C Lso is associated only with a carrot disease in Europe, the SSR PCR assay can be used for detecting and differentiating Lso haplotypes associated with ZC disease of potato without confusion.

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