Plant Disease • 2017 • 101:1373-1382 • http://dx.doi.org/10.1094/PDIS-03-17-0381-RE

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# *Beet curly top virus* Strains Associated with Sugar Beet in Idaho, Oregon, and a Western U.S. Collection

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## Abstract

Curly top of sugar beet is a serious, yield-limiting disease in semiarid production areas caused by *Beet curly top virus* (BCTV) and transmitted by the beet leafhopper. One of the primary means of control for BCTV in sugar beet is host resistance but effectiveness of resistance can vary among BCTV strains. Strain prevalence among BCTV populations was last investigated in Idaho and Oregon during a 2006-to-2007 collection but changes in disease severity suggested a need for reevaluation. Therefore, 406 leaf samples symptomatic for curly top were collected from sugar beet plants in commercial sugar beet fields in Idaho and Oregon from 2012 to 2015. DNA was isolated and BCTV strain composition was investigated based on polymerase chain reaction assays with strain-specific primers for the Severe (Svr) and California/Logan (CA/Logan) strains and primers that amplified a group of Worland (Wor)-like strains. The BCTV strain distribution averaged 2% Svr, 30% CA/Logan, and 87% Wor-like (16% had mixed infections), which differed from the previously published 2006-to-2007 collection (87% Svr, 7% CA/Logan, and 60% Wor-like; 59% mixed infections) based on a contingency test ($P < 0.0001$). Whole-genome sequencing (GenBank accessions KT276895 to KT276920 and KX867015 to KX867057) with overlapping primers found that the Wor-like strains included Wor, Colorado and a previously undescribed strain designated Kimberly1. Results confirm a shift from Svr being one of the dominant BCTV strains in commercial sugar beet fields in 2006 to 2007 to becoming undetectable at times during recent years.

*Beet curly top virus* (BCTV) in sugar beet (*Beta vulgaris* L.) is an important yield-limiting disease problem in semiarid production areas of the western United States and in Middle Eastern countries (Bennett 1971; Gharouni Kardani et al. 2013; Harveson 2015; Stenger and McMahon 1997; Strausbaugh et al. 2008; Yazdi et al. 2008). BCTV is transmitted in a persistent circulative manner by the beet leafhopper, *Circulifer tenellus* Baker (Hemiptera: Cicadellidae), and can infect over 300 dicotyledonous plant species (Bennett 1971). Yield may be affected in important crops such as common bean, pepper, spinach, sugar beet, and tomato (Blickenstaff and Traveller 1979; Chen and Gilbertson 2009; Creamer et al. 1996; Soto and Gilbertson 2003; Soto et al. 2005). Several distinct, genetically characterized members of the genus *Curtovirus* have been confirmed as causative agents of curly top in sugar beet (Briddon et al. 1998; Gharouni Kardani et al. 2013; Heydarnejad et al. 2007, 2013; Soleimani et al. 2013; Stenger 1998; Strausbaugh et al. 2008; Yazdi et al. 2008). However, reevaluation of the genus *Curtovirus* assigned most of these viruses formerly recognized as distinct *Curtovirus* spp. as strains of BCTV (Varsani et al. 2014a).

*Curtovirus* isolates and strains with greater than 94% sequence identity are now considered variants of the same strain and those with 77% or less sequence identity are considered different species (Varsani et al. 2014a). Therefore, several widely recognized *Curtovirus* spp. affecting sugar beet and vegetable agriculture in the western United States that were previously considered separate species are now recognized as strains of BCTV. These include California/Logan (CA/Logan; also previously referred to as *Beet curly top virus*, California and Logan); Colorado (CO; also previously referred to as *Beet curly top virus* and pCO-95-6-31); Mild (Mld; also previously referred to as *Beet mild curly top virus*, 8-10, SLP1, BMCTV-Mexico, and MX-P24); Pepper curly top (PeCT; also previously known as *Pepper curly top virus*, BV3, and NM); Pepper yellow dwarf (PeYD; also previously known as *Pepper yellow dwarf virus*); Severe (Svr; also previously referred to as *Beet severe curly top virus*, CFH, and BCTV-I); Spinach curly top (SpCT; also previously known as *Spinach curly top virus* and Sp3); and Worland (Wor; also previously referred to as *Beet mild curly top virus* and Worland4) (Baliji et al. 2004; Briddon et al. 1998; Chen et al. 2011; Hernandez and Brown 2010; Lam et al. 2009; Stenger 1994; Varsani et al. 2014a; Velásquez-Valle et al. 2008; Velasquez-Valle et al. 2012). In addition to BCTV, there are two additional *Curtovirus* spp.: *Spinach severe curly top virus* (SpSCTV) and *Horseradish curly top virus* (HCTV) (Hernandez and Brown 2010; Klute et al. 1996; Varsani et al. 2014a). Viruses in the genus *Becurtovirus* also infect sugar beet and cause curly top symptoms. These viruses include both *Beet curly top Iran virus* and *Spinach curly top Arizona virus* (Gharouni Kardani et al. 2013; Hernández-Zepeda et al. 2013; Heydarnejad et al. 2007, 2013; Soleimani et al. 2013, Varsani et al. 2014b, Yazdi et al. 2008).

BCTV almost eliminated sugar beet production in the western United States in the 1920s and early 1930s until resistant cultivars were developed (Bennett 1971; Panella et al. 2014). Resistant cultivars remain an important control measure; however, most commercial cultivars only contain low to moderate levels of resistance, because the resistance is thought to be quantitatively inherited and is difficult to maintain in parental lines used to create commercial hybrids (Gillen et al. 2008; Kaffka et al. 2002; Panella et al. 2014; Strausbaugh et al. 2007). Alternative control measures to supplement host resistance have been investigated and the most effective has been the use of neonicotinoid seed treatments based on the active ingredients clothianidin and thiamethoxam (Strausbaugh et al. 2006, 2010, 2012, 2014). Clothianidin (sold as Poncho and NipsIt) has been shown to increase sugar beet yields by 17% or more in heavily infested commercial fields (Strausbaugh et al. 2006, 2010, 2012, 2014). As a result, some sugar beet production areas now require the use of the neonicotinoid seed treatments (Strausbaugh et al. 2012, 2014). Genetic engineering may offer opportunities for controlling curly top in sugar beet but, to date, disease-resistant transgenic

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*The *e*-Xtra logo stands for "electronic extra" and indicates that two supplementary tables are published online.

Accepted for publication 18 April 2017.

sugar beet cultivars have not been deployed (Ali et al. 2015; Aregger et al. 2012; Golenberg et al. 2009; Hohn and Vazquez 2011; Horn et al. 2011; Ji et al. 2015; Lee et al. 2013; Sahu and Prasad 2015; Sharma et al. 2012; Wang et al. 2012; Zaidi et al. 2016).

Host resistance remains important to sugar beet producers (Strausbaugh et al. 2016). However, some BCTV strains can be more severe than others on sugar beet and resistance can be both general and strain specific, depending on the source of resistance (Montazeri et al. 2016). Therefore, composition and prevalence of strains and variants should be monitored in sugar beet production areas in order to address changes that may influence the performance of commercial cultivars which only contain low to moderate resistance. Several new species or strains associated with curly top have been identified in various agricultural crops. These are likely the result of recombination among strains and variants during mixed infections, and emerge as significant proportions of the virus population due to selection pressures (Bach and Jeske 2014; Briddon et al. 2010; Chen et al. 2010, 2014; Creamer et al. 2005; Hernandez and Brown 2010; Hernández-Zepeda et al. 2013; Lefeuvre and Moriones 2015; Padidam et al. 1999; Razavinejad et al. 2013; Stenger 1998; Stenger and McMahon 1997, Stenger and Ostrow 1996, Stenger et al. 1990, 1994; Strausbaugh et al. 2008). In an effort to increase our knowledge of the species and strains currently associated with curly top in sugar beet production in Oregon and Idaho, commercial fields were sampled from 2012 to 2015. To support the primer-based identification, whole-genome sequencing for the BCTV isolates was used to evaluate and validate performance of strain-specific detection methods, and to determine whether the predominant BCTV variants or strains have changed since the 2006-to-2007 collection (Strausbaugh et al. 2008). Results demonstrated a distinct shift in the presence and prevalence of BCTV strains, including the emergence of new variants that justify increased monitoring.

## Materials and Methods

**Collection.** Curly top symptomatic samples from individual sugar beet plants were randomly collected from commercial fields in southern Idaho and southeastern Oregon from 2012 to 2015, and compared with those collected previously in 2006 and 2007 (Strausbaugh et al. 2008). Samples of new (still expanding) leaves were identified by the presence of typical curly top disease symptoms, including upward and inward rolling of leaves, enations, roughness and thickening of veins, and leaf dwarfing (Wintermantel 2009). In total, 406 plant samples were collected during 2012 (50 plants), 2013 (72 plants), 2014 (131 plants), and 2015 (153 plants) (Supplementary Table S1). These samples were compared with the 266 samples collected in 2006 (119 plants) and 2007 (147 plants). Cultivar names were not tracked during the collections, because the compliment of resistance genes they contain is unknown or not public knowledge and the cultivars available change regularly. Of the 26 cultivars available for the Idaho-Oregon production area in 2012, only 9 were still available to growers in 2015. None of the cultivars in production during the 2006-to-2007 collection were still in production after 2008, because the industry switched to glyphosate-resistant cultivars (Panella et al. 2014).

**Strain distribution.** Leaf tissue was sampled by taking leaf punches with the cap of a sterile 2-ml microcentrifuge tube from each of three leaves per plant (= one sample) and stored at −80°C in the microcentrifuge tube. The frozen leaf samples were lyophilized, then pulverized using a Retch MM301 mixer mill (Retch Inc., Newton, PA) with 5-mm-diameter stainless steel beads. DNA was extracted using the DNeasy Plant Mini Isolation Kit (Qiagen Inc., Valencia, CA), assessed via gel electrophoresis, quantified using a BioPhotometer (Eppendorf AG, Hamburg, Germany), and stored at −20°C. Polymerase chain reaction (PCR) assays for detection of BCTV were performed in 20-μl volumes: 8.8 μl of molecular-grade water (5 Prime Inc., Gaithersburg, MD), 4 μl of 5× Green GoTaq buffer (pH 8.5, with 7.5 mM $MgCl_2$; Promega Corp., Madison, WI), 0.6 μl of 25 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.4 μl of 10 mM dNTP (Promega Corp.), 2 μl of 3 mM each primer (Integrated DNA Technologies, Coralville, IA), 0.2 μl of GoTaq DNA polymerase (Promega Corp.), and 2 μl (approximately 20 ng DNA) of target DNA. The amplification cycle consisted of 3 min at 95°C followed by 35 cycles of 95°C for 30 s, 55 to 57°C (depending on primer pair, as indicated in Supplementary Table S2) for 30 s, and 72°C for 1 min. After the final cycle, the reaction was held at 72°C for 5 min, followed by 6°C. The *Curtovirus* sp.-positive samples were identified using primers BCTV2-F and BCTV2-R designed for amplification of a 496-bp fragment of the coat protein gene, a region genetically conserved among BCTV strains. Strain-specific primer sets for the replication-associated protein (*Rep*) gene were used in the PCR assays: BSCTV-C1 2315F and BSCTV-C1 2740R for the Svr strain (region amplified matches a 426-bp segment from GenBank accession U02311); BMCTV-C1 2213F and BMCTV-C1 2609R for the Wor-like strains (region amplified matches a 397-bp segment from GenBank accession AY134867); and BCTV-C1 2097F and BCTV-C1 2387R for the CA/Logan strain (region amplified matches a 291-bp segment from GenBank accession AF379637). Amplification products were analyzed by agarose gel electrophoresis (1.8% [wt/vol] supplemented with ethidium bromide at 0.01 mg/ml in Tris-borate EDTA [TBE] buffer [89 mM Tris base, 89 mM boric acid, and 2 mM EDTA]). DNA from sugar beet sample CTS07-011 (contains strains CA/Logan, Svr, and Wor) served as a positive control (Strausbaugh et al. 2008). Reactions without template DNA served as negative controls. Comparisons between collections were conducted using a contingency test in SAS (version 9.4; SAS Institute Inc., Cary, NC) via the Proc Freq procedure with the $\chi^2$ statistic.

**Genome sequencing.** Whole-genome sequencing was conducted on 69 sugar beet isolates (Table 1), which were chosen to represent the widest geographic distribution for each strain for the production areas in which they were collected. All the sequenced isolates were from commercial sugar beet fields, except for seven isolates (CTS06-101, CTS06-102, CTS06-103, CTS06-104, CTS07-016, CTS14-1091, and CTS15-1188) collected from sugar beet plants infected with BCTV in the beet leafhopper insectary maintained by the Beet Sugar Development Foundation in Twin Falls, ID. DNA collection, quantification, and storage were as described in the previous section. Amplification of sequencing templates was performed in volumes of 40 μl: 20 μl of molecular-grade water (5 Prime Inc.), 8 μl of 5× Green GoTaq buffer (pH 8.5 with 7.5 mM $MgCl_2$; Promega Corp.), 1 μl of 25 mM $MgCl_2$ (Applied Biosystems), 0.75 μl of 10 mM dNTP (Promega Corp.), 4 μl of 3 μM each primer (Integrated DNA Technologies), 0.25 μl of GoTaq *Taq* DNA polymerase (Promega Corp.), and 2 μl (approximately 10 ng) of target DNA. The amplification consisted of 3 min at 95°C; followed by 35 cycles of 95°C for 30 s, 55 to 62°C (depending on primer pair) for 30 s, and 72°C for 120 s; which was followed by 5 min at 72°C and a holding temperature of 4°C. The primary primer pairs RepQEW-For with CP450-Rev and V2Gen910-For with Rep2GQ-Rev, with overlapping sequences, were used to amplify the entire virus genome (Velasquez-Valle et al. 2012). For the hypervariable C1 region and for plants containing more than one BCTV strain, additional strain-specific primer combinations were utilized. Amplification products were analyzed by agarose gel electrophoresis (1.8% [wt/vol] supplemented with ethidium bromide at 0.01 mg/ml in TBE [89 mM Tris base, 89 mM boric acid, and 2 mM EDTA]). Amplicons were sent to TACGen (Richmond, CA) for PCR cleanup (removes any excess dNTP and unincorporated primers) and were bidirectionally sequenced. Sanger sequencing was repeated to achieve 4× coverage of the entire genome and 8× coverage in the hypervariable C1 region.

Sequences were evaluated using BioEdit, version 7.1.3.0 (Hall 1999) and consensus sequences for each isolate were generated and deposited in GenBank (Table 1). To apply strain demarcation criteria established for BCTV based on sequence identity (Varsani et al. 2014a), sequences were aligned using MUSCLE (Edgar 2004) and compared via SDT v1.2 (Muhire et al. 2014). To graphically illustrate inferred evolutionary history, isolate sequences were compared with BCTV accessions from GenBank. DNA sequences were aligned using ClustalX Ver. 2.0 (Larkin et al. 2007). Using MEGA 7.0.14

(Kumar et al. 2016), the TN93+G+I model (Tamura and Nei 1993) was determined to be the substitution model that best fit the data according to the Bayesian Information Criterion. Using this model, an evolutionary analysis was conducted by the maximum-likelihood method with MEGA 7.0.14. An initial search (two replicates) was used to estimate the model parameters. The parameters were then fixed for a bootstrap analysis of 1,000 replicates. The maximum-parsimony analysis was performed using PAUP 4.0b10 with the heuristic search, simple taxon addition sequences, tree bisection-reconnection branch swapping, and MaxTrees = 100. Statistical support for the analyses was determined using bootstrap values for 1,000 replicates. The Bayesian phylogenetic analyses were conducted with MrBayes 3.2.5 (Ronquist and Huelsenbeck 2003), with searches run until the standard deviation of split frequencies declined <0.01. The analyses were conducted using the default priors (Fraser et al. 2010). The majority-rule consensus was then calculated after removing the first 25% of generations as burn-in. The trees were visualized using FigTree (ver. 1.4.2; Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK). To graphically illustrate differences among strains, a dataset containing 31 isolates (including historical isolates and isolates representing the extremes in the phylogram clades) also was analyzed using SplitsTree 4.14.2 (Huson and Bryant 2006). Because there was a novel strain present in these analyses, this novel strain was compared with five isolates (AF379637, CA/Logan; JN817383, CO; KT583738, Leafhopper71 (LH71); U02311, Svr; and U56975, Wor; these represent the strains determined to be present on sugar beet plus strain LH71) to establish whether recombination occurred. The recombination analyses were conducted using default parameters for the following methods in RDP4 4.80 (Martin et al. 2015): RDP, GENECOV (Padidam et al. 1999), Bootscan (Martin et al. 2005), Maxchi (Smith 1992), Chimera (Posada and Crandall 2001), Siscan (Gibbs et al. 2000), and 3Seq (Boni et al. 2007). Potential recombination events detected with at least three of the seven methods ($P$ values < 0.05) and also supported by phylogenetic analyses were considered credible.

## Results

**Strain distribution.** All samples included in collections to determine BCTV strain variation that produced BCTV-specific amplification products also produced amplification products with the BCTV coat protein primers (BCTV-F and -R). Among the 2006-to-2007 BCTV-positive samples, incidence of the Svr strain was 71 to 92% (78% average for all areas; 87% average for Idaho and Oregon; Table 2). In contrast, the Svr strain was found in only 0 to 8% (2% average) of BCTV-positive samples in the 2012-to-2015 collection. The incidence of the CA/Logan strain in the 2006-to-2007 BCTV-positive samples was 0 to 14% (2% average for all areas; 7% average for Idaho and Oregon; Table 2) whereas, in the 2012-to-2015 BCTV-positive samples, the range varied dramatically from 3 to 76% (30% average), depending on the year. The incidence of the Wor-like strains in the 2006-to-2007 virus-positive samples was 21 to 100% (74% average for all areas; 60% average for Idaho and Oregon; Table 2) whereas, in the 2012-to-2015 virus-positive samples, the range was 78 to 96% (87% average). Based on strain data in Table 2, the contingency test indicated that the 2006-to-2007 and 2012-to-2015 collections differed ($\chi^2 = 179$, $P < 0.0001$). The number of mixed infections during the 2006-to-2007 collection was 59% whereas the number of mixed infections during the 2012-to-2015 collection was only 16%.

**Genome sequencing.** In total, 69 BCTV genomes were sequenced and deposited in GenBank (KT276895 to KT276920 and KX867015 to KX867057; Table 1). When isolates were sequenced from samples positive for CA/Logan and Svr based on the strain-specific primers (BCTV-C1 for CA/Logan and BSCTV-C1 for Svr), all genome sequences had the highest sequence identity with the same strain identified via the primers.

The genome sequences of the 22 isolates classified as Svr strain were all considered variants of the two historical Svr isolates (GenBank accessions U02311 and X97203) based on sequence identity, because they formed a single group with >94% sequence identity among members in the Figure 1 pairwise comparison matrix. Evolutionary relationships among isolates in the maximum-likelihood phylogram in Figure 2 also placed all 22 Svr isolates into a single clade. However, within this clade, the Idaho and Oregon isolates were assigned to two subgroups (Fig. 2; top of the Svr clade), whereas the Colorado, Montana, and Wyoming isolates all fell into a separate subgroup (Fig. 2; bottom of the Svr clade). In the SplitsTree network analysis (Fig. 3), the Svr isolates formed a distinct cluster. Similar clusters were observed for other strains identified in the pairwise and evolutionary relationship analyses. There were also some evolutionary relationships suggested between the Svr isolates and the CA/Logan, PeCT, and SpCT isolates, because these isolates share the same main branch in Figure 2. The SplitsTree network analysis also connected all four of these strains as well.

The 11 CA/Logan sequences were considered variants of the two historical CA/Logan strains (GenBank accessions AF379637 and M24597.2) (Hormuzdi and Bisaro 1993; Stanley et al. 1986) based on sequence identity, because they formed a single group with >94% sequence identity in the Figure 1 pairwise matrix. Evolutionary relationships in the phylogram in Figure 2 placed the CA/Logan isolates into a single clade.

When 36 genomes were sequenced from samples identified by the BMCTV-C1 Wor primers, 56% of the genomes were found to be the CO strain, whereas 39% were Wor and 5% were a novel, previously undescribed strain designated Kimberly1 (Kim1). The designations for these strain identifications were all supported by the pairwise identity matrix (Fig. 1), evolutionary relationships (Fig. 2), and network analysis (Fig. 3). Based on the phylogram, there were some evolutionary relationships between the CO, Mld, Wor, and PeYD strains, because they all share a node. The network analysis also placed these same four strains together at one end of the network. Based on the pairwise identity matrix, there was some overlap in sequence identity between a number of the CO and Wor strains. The 17 CO isolates that shared considerable sequence identity with Wor strains were from Idaho or Oregon, whereas the Idaho CO isolate KT276898 was similar to the California CO isolates. Among the 18 CO isolates that had lower sequence identity with Wor, 15 were California isolates whereas the others were from Colorado or Nebraska. Phylogenetic analyses placed CO and Wor strains on a common node but split the CO and Wor isolates similar to the sequence identity matrix (Fig. 2). However, the two Idaho and Oregon CO isolates from 2007 (KT276898) and 2008 (KU892790) split off with the California CO isolates, whereas all of the more recently (2013 to 2015) collected CO isolates fell into a separate clade.

The two Kim1 isolates (from Idaho and Colorado) were most closely related to the LH71 strain (Chen and Gilbertson 2011, 2016) based on the pairwise identity matrix, phylogram, and network analysis. The distance results in Figure 4 from the RDP4 software also indicate that LH71 was closely related to Kim1. When comparing the Kim1 isolates versus the five reference isolates with the RDP4 software, the Kim1 isolate CTS06-056 (KT276896) was determined to be a potential recombinant with support from all seven methods. The results indicated evidence for the same recombination event to have occurred in Kim1 isolate CTS06-076 (KT276897) as well. The major parent was Wor (U56975), with 97.5% similarity and breakpoints at 1 to 1,633 and 3073 to 3092. The minor parent was Svr (U02311), with 95.4% similarity and breakpoints at 1,634 to 3072 (Fig. 4).

## Discussion

Data from the survey comparisons were the first evidence of a strain shift in sugar beet because 78% of the virus-positive sugar beet samples from the 2006-to-2007 collection were infected with the Svr strain (87% for Idaho and Oregon samples alone) and 59% of the plants had a mixed BCTV infection, whereas only 2% were positive for the Svr strain in the 2012-to-2015 collection and only 16% of the plants had a mixed infection. Sequencing the genomes of 22 samples positive for the Svr strain confirmed that the species-specific primers for Svr were accurate and reliably detected this strain. However, 39 genome sequences from the Wor-like primer-positive samples

identified three strains with the following percentages: 56% CO, 39% Wor, and 5% Kim1, the latter a novel strain identified and genetically characterized in this study. When BCTV strains found in beet leafhoppers were investigated from northeastern Oregon from 2007 to 2008 (Rondon et al. 2016), two of the isolates (accessions KU892789 and KU892791) resembled the Wor strain and one was similar to CO (KU892790) (Fig. 2). This complement of strains was similar to that found in the sugar-beet-growing area of southern Idaho and southeastern Oregon, where CO and Wor have become the dominant BCTV strains.

In California tomato, there was also a transition in the most prevalent BCTV strains. The Svr and Wor strains predominated from the

**Table 1.** Source of the 111 *Beet curly top virus* (BCTV) isolates and sequences utilized in the phylogenetic analyses

| Isolate identification | GenBank accession | Strain[a] | Year | Host | Country | State |
|---|---|---|---|---|---|---|
| This study | | | | | | |
| CTS06-011 | KX867015 | CO | 2006 | *Beta vulgaris* | United States | Nebraska |
| CTS06-012S | KX867016 | Svr | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-012W | KX867017 | Wor | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-013 | KX867018 | CO | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-014 | KT276904 | Svr | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-016 | KT276905 | Svr | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-017 | KT276906 | Svr | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-021 | KX867019 | Svr | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-034 | KT276907 | Svr | 2006 | *B. vulgaris* | United States | Oregon |
| CTS06-053 | KT276895 | CO | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-056 | KT276896 | Kim1 | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-060 | KX867020 | Wor | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-065 | KX867021 | Wor | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-066 | KX867022 | CO | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-068 | KX867023 | Wor | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-070 | KX867024 | Wor | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-073 | KX867025 | Wor | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-076 | KT276897 | Kim1 | 2006 | *B. vulgaris* | United States | Colorado |
| CTS06-078 | KX867026 | Svr | 2006 | *B. vulgaris* | United States | Wyoming |
| CTS06-081 | KX867027 | Svr | 2006 | *B. vulgaris* | United States | Wyoming |
| CTS06-091 | KX867028 | Svr | 2006 | *B. vulgaris* | United States | Montana |
| CTS06-101 | KX867029 | CA/Logan | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-102 | KX867030 | CA/Logan | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-103 | KX867031 | CA/Logan | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-104 | KX867032 | CA/Logan | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-110 | KX867033 | CA/Logan | 2006 | *B. vulgaris* | United States | Idaho |
| CTS06-111 | KX867034 | CA/Logan | 2006 | *B. vulgaris* | United States | Idaho |
| CTS07-016 | KX867035 | CA/Logan | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-019 | KT276898 | CO | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-020 | KT276902 | Wor | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-021 | KT276903 | Wor | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-023 | KT276908 | Svr | 2007 | *B. vulgaris* | United States | Wyoming |
| CTS07-036 | KX867036 | Svr | 2007 | *B. vulgaris* | United States | Wyoming |
| CTS07-043 | KX867037 | Svr | 2007 | *B. vulgaris* | United States | Wyoming |
| CTS07-046 | KT276909 | Svr | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-048 | KT276910 | Svr | 2007 | *B. vulgaris* | United States | Oregon |
| CTS07-053 | KT276911 | Svr | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-056 | KT276912 | Svr | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-057 | KT276913 | Svr | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-059 | KT276914 | Svr | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-088 | KT276915 | Svr | 2007 | *B. vulgaris* | United States | Idaho |
| CTS07-091 | KX867038 | Wor | 2007 | *B. vulgaris* | United States | Colorado |
| CTS07-096 | KT276916 | Svr | 2007 | *B. vulgaris* | United States | Montana |
| CTS07-101 | KT276917 | Svr | 2007 | *B. vulgaris* | United States | Wyoming |
| CTS07-129 | KT276918 | Svr | 2007 | *B. vulgaris* | United States | Montana |
| CTS12-024 | KX867039 | Wor | 2012 | *B. vulgaris* | United States | Idaho |
| CTS13-005 | KX867040 | CA/Logan | 2013 | *B. vulgaris* | United States | Idaho |
| CTS13-028 | KX867041 | CA/Logan | 2013 | *B. vulgaris* | United States | Idaho |
| CTS13-060 | KX867042 | CO | 2013 | *B. vulgaris* | United States | Idaho |
| CTS13-062 | KX867043 | CO | 2013 | *B. vulgaris* | United States | Idaho |
| CTS13-063 | KX867044 | CO | 2013 | *B. vulgaris* | United States | Idaho |
| CTS14-001 | KT276899 | CO | 2014 | *B. vulgaris* | United States | Idaho |
| CTS14-014 | KT276900 | CO | 2014 | *B. vulgaris* | United States | Idaho |
| CTS14-015 | KX867045 | Wor | 2014 | *B. vulgaris* | United States | Idaho |
| CTS14-024 | KT276901 | CO | 2014 | *B. vulgaris* | United States | Idaho |

(*continued on next page*)

[a] BCTV strains California/Logan (CA/Logan), Colorado (CO), Kimberly1 (Kim1), Mild (Mld), Leafhopper 71 (LH71), Pepper curly top (PeCT), Pepper yellow dwarf (PeYD), Severe (Svr; formerly CFH), Severe pepper (SvrPep), Spinach curly top (SpCT), and Worland (Wor).

mid-1990s during the first formal survey of the molecular era (Stenger and Ostrow 1996), through subsequent surveys as late as 2008 (Chen et al. 2010). In contrast, strain evaluations during the 2013 California outbreak, when upward of $100 million was lost (Gordon 2014), suggest the emergence of new variants displacing the older strains. The earliest sequenced CO isolates from Idaho sugar beet in 2007 (KT276898) and a leafhopper in Oregon in 2008 (KU892790) fall in the same clade as the CO isolates associated with the California curly top outbreak in tomato. The CO isolates collected more recently (2013 to 2015) in sugar beet from Idaho and Oregon appear to have diverged and were distributed into a different clade within the phylogram.

**Table 1.** (*continued from preceding page*)

| Isolate identification | GenBank accession | Strain[a] | Year | Host | Country | State |
|---|---|---|---|---|---|---|
| CTS14-054 | KX867046 | CO | 2014 | *B. vulgaris* | United States | Idaho |
| CTS14-067 | KX867047 | CO | 2014 | *B. vulgaris* | United States | Idaho |
| CTS14-070 | KX867048 | CO | 2014 | *B. vulgaris* | United States | Oregon |
| CTS14-071 | KX867049 | CO | 2014 | *B. vulgaris* | United States | Oregon |
| CTS14-124 | KX867050 | CO | 2014 | *B. vulgaris* | United States | Idaho |
| CTS14-1091 | KT276919 | CA/Logan | 2014 | *B. vulgaris* | United States | Idaho |
| CTS15-001 | KX867051 | CO | 2015 | *B. vulgaris* | United States | Idaho |
| CTS15-045 | KX867052 | CO | 2015 | *B. vulgaris* | United States | Idaho |
| CTS15-086 | KX867053 | Wor | 2015 | *B. vulgaris* | United States | Idaho |
| CTS15-091 | KX867054 | Wor | 2015 | *B. vulgaris* | United States | Idaho |
| CTS15-095 | KX867055 | Wor | 2015 | *B. vulgaris* | United States | Idaho |
| CTS15-113 | KX867056 | CO | 2015 | *B. vulgaris* | United States | Idaho |
| CTS15-149 | KX867057 | CO | 2015 | *B. vulgaris* | United States | Idaho |
| CTS15-1188 | KT276920 | CA/Logan | 2015 | *B. vulgaris* | United States | Idaho |
| Other studies | | | | | | |
| BCTV-I | X97203 | Svr | 1986 | *B. vulgaris* | Iran | … |
| BCTV-2007 | KU892789 | Wor | 2007 | *Circulifer tenellus* | United States | Oregon |
| BCTV-2008 | KU892790 | CO | 2008 | *C. tenellus* | United States | Oregon |
| BCTV-2009 | KU892791 | Wor | 2009 | *C. tenellus* | United States | Oregon |
| BMCTV | EU193175 | Mld | 2006 | *Capsicum annuum* | Mexico | … |
| BV3 | JX487184 | PeCT | 2009 | *Solanum lycopersicum* | United States | California |
| B4-2 | KT583729 | CO | 2013 | *B. vulgaris* | United States | California |
| Cal | M24597.2 | CA/Logan | 1985 | *B. vulgaris* | United States | California |
| CFH | U02311 | Svr | 1990 | *B. vulgaris* | United States | … |
| FB1-18 | KT583732 | CO | 2013 | *B. vulgaris* | United States | … |
| FM3-1-10 | KT583733 | CO | 2013 | *Cucumis melo* | United States | California |
| FM4-3-3 | KT583734 | CO | 2013 | *C. melo* | United States | California |
| FP8-9 | KT583735 | LH71 | 2013 | *S. lycopersicum* | United States | California |
| F1-1-2 | KT583728 | CO | 2013 | *S. lycopersicum* | United States | California |
| F1-1-3 | KT583730 | CO | 2013 | *S. lycopersicum* | United States | California |
| F1-2-25 | KT583731 | LH71 | 2013 | *S. lycopersicum* | United States | California |
| KB1-1 | KT583739 | CO | 2013 | *S. lycopersicum* | United States | California |
| K1-2 | KT583736 | CO | 2013 | *S. lycopersicum* | United States | California |
| K2-10 | KT583737 | CO | 2013 | *S. lycopersicum* | United States | California |
| K5 | KT583738 | LH71 | 2013 | *S. lycopersicum* | United States | California |
| LH7-3-5 | KT583740 | LH71 | 2013 | *Circulifer tenellus* | United States | California |
| LH71 | KT583748 | LH71 | 2010 | *C. tenellus* | United States | California |
| Logan | AF379637 | CA/Logan | 1976 | *B. vulgaris* | United States | Utah |
| M1-1-1 | KT583741 | CO | 2013 | *S. lycopersicum* | United States | California |
| M1-3-8 | KT583742 | LH71 | 2013 | *S. lycopersicum* | United States | California |
| M2-4 | KT583743 | CO | 2013 | *S. lycopersicum* | United States | California |
| MXP24-07 | HQ214016 | Mld | 2007 | *Capsicum annuum* | Mexico | … |
| New Mexico | EF501977 | PeCT | 2005 | *C. annuum* | United States | New Mexico |
| pCO-95-6-31 | JN817383 | CO | 1995 | *B. vulgaris* | United States | Colorado |
| PeYD | EU921828 | PeYD | 2007 | *C. annuum* | United States | New Mexico |
| SLP1 | EU586260 | Mld | 2007 | *C. annuum* | Mexico | … |
| SLP2 | EU586261 | Mld | 2007 | *C. annuum* | Mexico | … |
| SJ | KT583749 | SpCT | 2014 | *S. lycopersicum* | United States | California |
| SJ-Y1-37 | KT583746 | Wor | 2013 | *Cucurbita* sp. | United States | California |
| SJ1-2 | KT583744 | CO | 2013 | *S. lycopersicum* | United States | California |
| SJ2-1 | KT583745 | LH71 | 2013 | *S. lycopersicum* | United States | California |
| SK1-2 | KT583747 | CO | 2013 | *S. lycopersicum* | United States | California |
| Sp3 | AY548948 | SpCT | 1996 | *Spinacia oleracea* | United States | … |
| LRME27607 | FJ545686 | SvrPep | 2001 | *C. annuum* | United States | New Mexico |
| Worland | U56975 | Wor | 1996 | *Capsella bursa-pastoris* | United States | Wyoming |
| Worland4 | AY134867 | Wor | 2002 | *B. vulgaris* | United States | … |
| 8-10 | HQ634913 | Mld | 2010 | *Phaseolus vulgaris* | Mexico | … |

Based on the guidelines published by Varsani et al. (2014a), there are two previously unidentified strains among the sequences in GenBank. Genome sequences for the isolates CTS06-056 and CTS06-076 (GenBank accessions KT276896 and KT276897, respectively) submitted as part of this study have less than 94% sequence identity with other strains (Fig. 1) and also fall into a separate group in the phylogenetic (Fig. 2) and network (Fig. 3) analyses. Consequently, these two isolates have been designated Kim1 (for Kimberly, ID, where the original isolate was collected) and are composed of 2,929 to 2,933 nucleotides with seven open reading frames encoding proteins homologous to those of other curtoviruses. Based on these criteria, the beet leafhopper isolates from California (GenBank accessions KT583731, KT583735, KT583738, KT583740, KT583742, KT583745, and KT583748) form another distinct strain, designated LH71 (Chen and Gilbertson 2011, 2016). With the addition of these two strains, this is the first publication to establish 11 recognized strains of BCTV based on current accepted taxonomic criteria (Varsani et al. 2014a).

The sequence analysis of the Idaho and Oregon isolates led to the addition of 69 genome accessions in GenBank, and has identified the predominant strains present in sugar beet during the period during which collections occurred. BCTV, like other viruses, is a collection of dominant and less common strains for which emergence and dominance in a host likely depend on a number of factors. Thus, variants present at very low frequency may not have been identified.

Evaluation of the 111 BCTV genomes available on GenBank assists in clarification of the evolutionary relationships among known BCTV strains. With support from three phylogenetic methods, the maximum-likelihood tree showed the CO, Mld, Wor, and PeYD strains shared a common node, providing evidence for an evolutionary relationship among these strains. The network analysis also demonstrated that these strains are not as divergent from one another as are the other isolates. When evolutionary relationships and recombination events for the CO, Mld, Wor, and PeYD strains were evaluated by Varsani et al. (2014a), similar relationships were established. Similarly, Svr, SpCT, PeCT, and CA/Logan were connected by a common node, whereas Kim1 and LH71 were on other nodes. These nodes were connected at another node, providing evidence for an evolutionary relationship. Similarities in the network analysis among these strains also supported these relationships. Recombination analysis suggests that Kim1 is a recombinant, with the major parent being Wor and the minor parent being Svr. When evolutionary relationships and recombination events for the Svr, SpCT, PeCT, and CA/Logan strains were evaluated by Varsani et al. (2014a), similar relationships were evident.

The comparisons between the 2006-to-2007 and 2012-to-2015 collections provide evidence of a reduction in prevalence of the Svr strain and the number of mixed infections (positive for more than one primer set). The reduction in mixed infections appears to be a result of the reduction in the incidence of the Svr strain. However, these reductions between collections do not necessarily reflect changes in symptom severity on sugar beet. Most of the sugar beet plants sampled between 2012 and 2015 exhibited relatively mild symptoms (slight upward leaf rolling, vein thickening, and stunting). The mild symptoms could have resulted from infection with a mildly virulent strain of the virus, infection after plants achieve significant size (Wintermantel and Kaffka 2006), or both. Historically the Svr and CA/Logan strains have been some of the most virulent on sugar beet and that status may not have changed. However, a significant change in crop management also occurred when neonicotinoid seed treatments became fully labeled for use on sugar beet in 2008. Sugar beet growers planting in the spring in semiarid growing areas rapidly adopted the seed treatments to supplement the low to moderate curly top resistance in commercial cultivars (Panella et al. 2014). Therefore, beet leafhoppers coming off the desert in the spring in Idaho

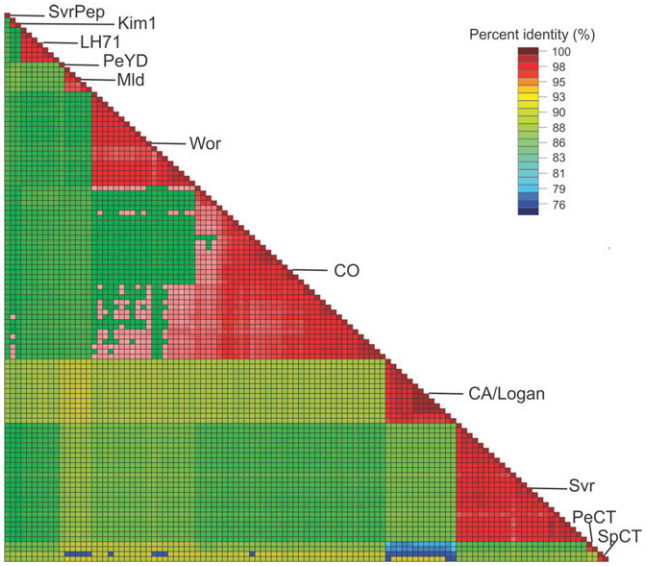


**Fig. 1.** Two-dimensional pairwise identity color matrix with pairwise identities was aligned with MUSCLE and calculated using SDT v1.2 to compare 111 *Beet curly top virus* (BCTV) genomes. BCTV strains are as follows: CA/Logan = California/Logan, CO = Colorado, Kim1 = Kimberly1, LH71 = Leafhopper 71, Mld = Mild, PeCT = Pepper curly top, PeYD = Pepper yellow dwarf, Svr = Severe (formerly CFH), SvrPep = Severe pepper, SpCT = Spinach curly top, and Wor = Worland.

**Table 2.** *Beet curly top virus* (BCTV) strains associated with 406 sugar beet samples collected from 2012 to 2015 in Oregon and Idaho and compared with 246 samples from a 2006-to-2007 collection

| | | | BCTV strain (%)[a] | | |
|---|---|---|---|---|---|
| Year[b] | Sample number | Coat protein (%)[c] | Svr | CA/Logan | Wor-like |
| 2006 (all areas) | 101 | 98 | 71 | 5 | 95 |
| 2006 (ID, OR) | 35 | 93 | 82 | 14 | 100 |
| 2007 (all areas) | 145 | 89 | 84 | 0 | 53 |
| 2007 (ID, OR) | 33 | 73 | 92 | 0 | 21 |
| 2012 | 50 | 56 | 0 | 29 | 79 |
| 2013 | 72 | 71 | 8 | 76 | 78 |
| 2014 | 131 | 97 | 2 | 13 | 95 |
| 2015 | 153 | 67 | 0 | 3 | 96 |

[a] Sugar beet leaf samples positive for coat protein (primers BCTV2-F and -R) were evaluated further with BCTV strain-specific primers to determine the percentage of samples positive for the Severe (Svr; formerly CFH; primers BSCTV-C1 2315F and 2740F) and California/Logan (CA/Logan; primers BCTV-C1 2097F and 2387R) strains. Worland (Wor)-like primers (BMCTV-C1 2213F and 2609R) detected the Colorado, Kimberly1, and Wor strains.

[b] Sugar beet leaf samples symptomatic for BCTV were collected from the Idaho and Oregon production area from 2012 to 2015. These samples were compared with those collected previously from throughout the western United States sugar beet production areas in 2006 and 2007 (Strausbaugh et al. 2008), designated as "all areas." The subset of data from 2006 and 2007 that represented the Idaho and Oregon production area is designated "ID, OR". The 2006 and 2007 data were published previously (Strausbaugh et al. 2008) in a different format and summarized here to facilitate easy comparison with current data sets.

[c] Percentage of samples positive with the primers specific for the BCTV coat protein.

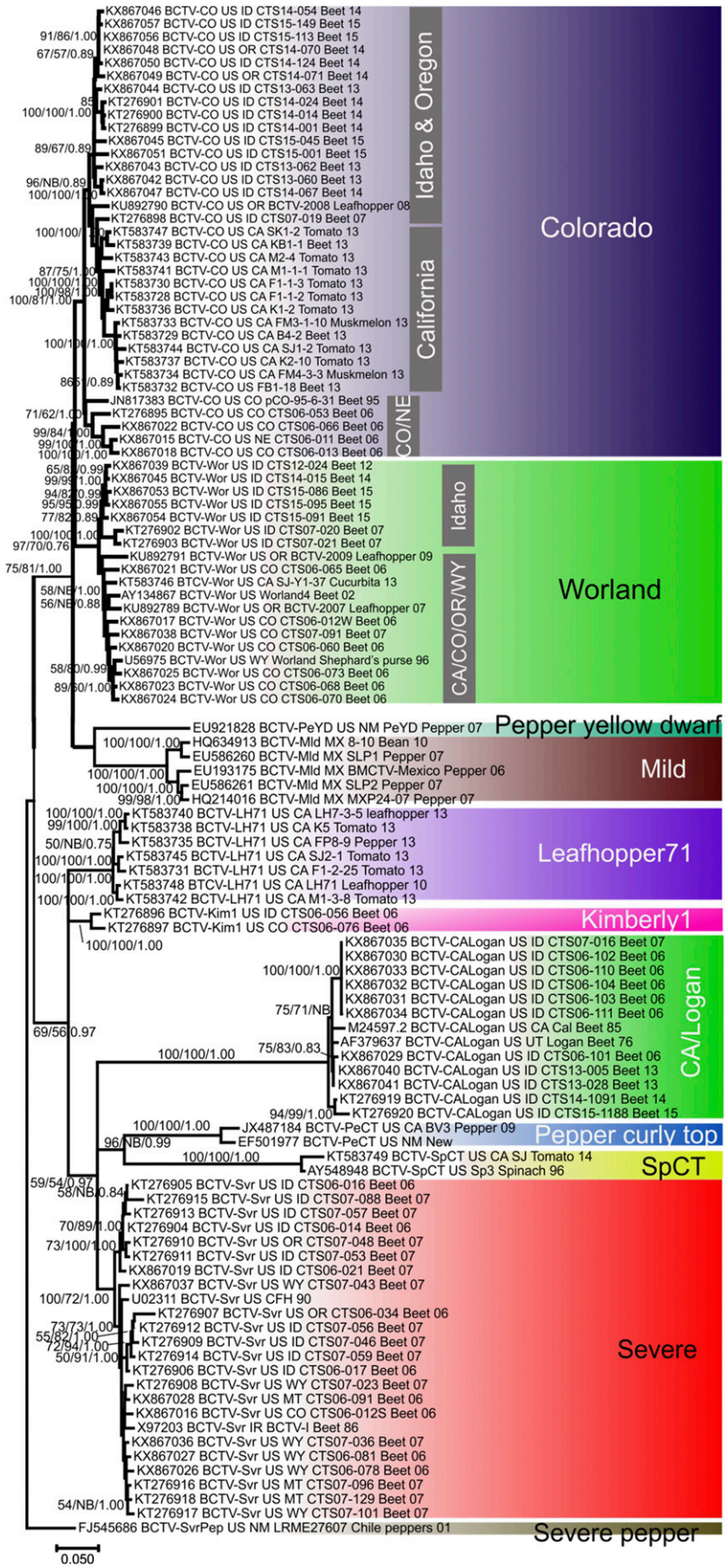


**Fig. 2.** Phylogenetic relationships among 111 *Beet curly top virus* genomes were compared among isolates and strains collected from Idaho, Oregon, a 2006-to-2007 collection, and sequences deposited in GenBank. Numbers on the nodes of the maximum-likelihood (ML) tree represent the statistical support for ML (1,000 replicates, left number), maximum parsimony (MP; 1,000 bootstrap replicates, middle number), and Bayesian method (posterior probabilities, right number). NB = no branch. The tree is drawn to scale, with the branch lengths measured in the number of substitutions per site. Isolates are identified by GenBank accession number followed by strain identification (strain designation, country and state of collection, isolate identification, host, and year of collection).

were likely limited in their ability to feed and transmit BCTV in the sugar beet crop for at least the first two and a half months of the growing season as a result of the neonicotinoid seed treatment. The treatment has been shown to last at least 77 days (Strausbaugh et al. 2016). Therefore, beet leafhoppers surviving in the initial migration from the desert would have to survive on alternate host plants such as common bean or weeds. Preliminary data suggest that mild strains are more effective at establishing in bean and some weed hosts than the Svr strain (Chen and Gilbertson 2009; Wintermantel 2011). These alternate hosts may have favored the CO and Wor strains over the Svr strain, because beet leafhoppers moving to sugar beet might not have been successful at transmitting BCTV until the second generation of beet leafhoppers developed and migrated to sugar beet plants. BCTV transmission is known to be affected by the acquisition host and the ability of the host to maintain high virus titer because BCTV does not replicate in the vector (Chen and Gilbertson 2009; Soto and Gilbertson 2003). Another factor that could have driven a BCTV strain change would be the addition of strain-specific resistance in commercial sugar beet cultivars. Recently, evidence for strain-specific differences in response to sugar beet resistance sources was provided (Montazeri et al. 2016); however, the presence of specific resistance genes or combinations of genes in commercial sugar beet cultivars is not publicly known. Therefore it remains difficult to determine which genes are most effective against specific BCTV strains. Changes in virulence of isolates classified genetically within specific strains on specific host plants, including sugar beet, may have occurred as well but insufficient information is available at this time.

As mentioned above, a large portion of the sugar beet plants sampled between 2012 and 2015 exhibited relatively mild symptoms. However, these mild symptoms were difficult to distinguish from sugar beet plants that were poorly irrigated (wind likely shifted the irrigation sprinkler pattern), because plants that had water deficiencies during the growing season were also stunted and had curled leaves. Thus, the lower percentage of plants positive for the coat protein primers likely reflects the sampling of poorly irrigated plants rather than plants containing a virus variant with a different coat protein.

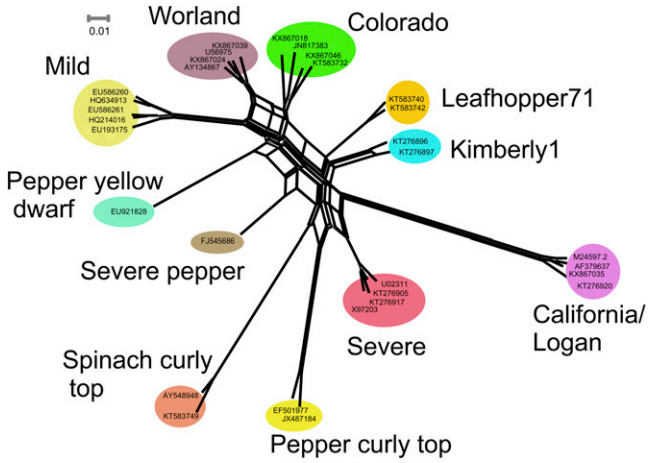


**Fig. 3.** Median-joining network created using SplitsTree4 ver. 4.13.1 was utilized to identify differences among 31 *Beet curly top virus* (BCTV) isolate genomes (designated by their GenBank accession number) representing the 11 BCTV strains. The figure graphically shows the extent of differences among isolates and strains rather than phylogenetic relationships, and takes into account not only nucleotide changes but also gaps in sequence alignment. Isolates selected for this analysis were chosen because they represent historically significant isolates or isolates that were the most divergent in the clades associated with each strain in the phylogenetic analyses.

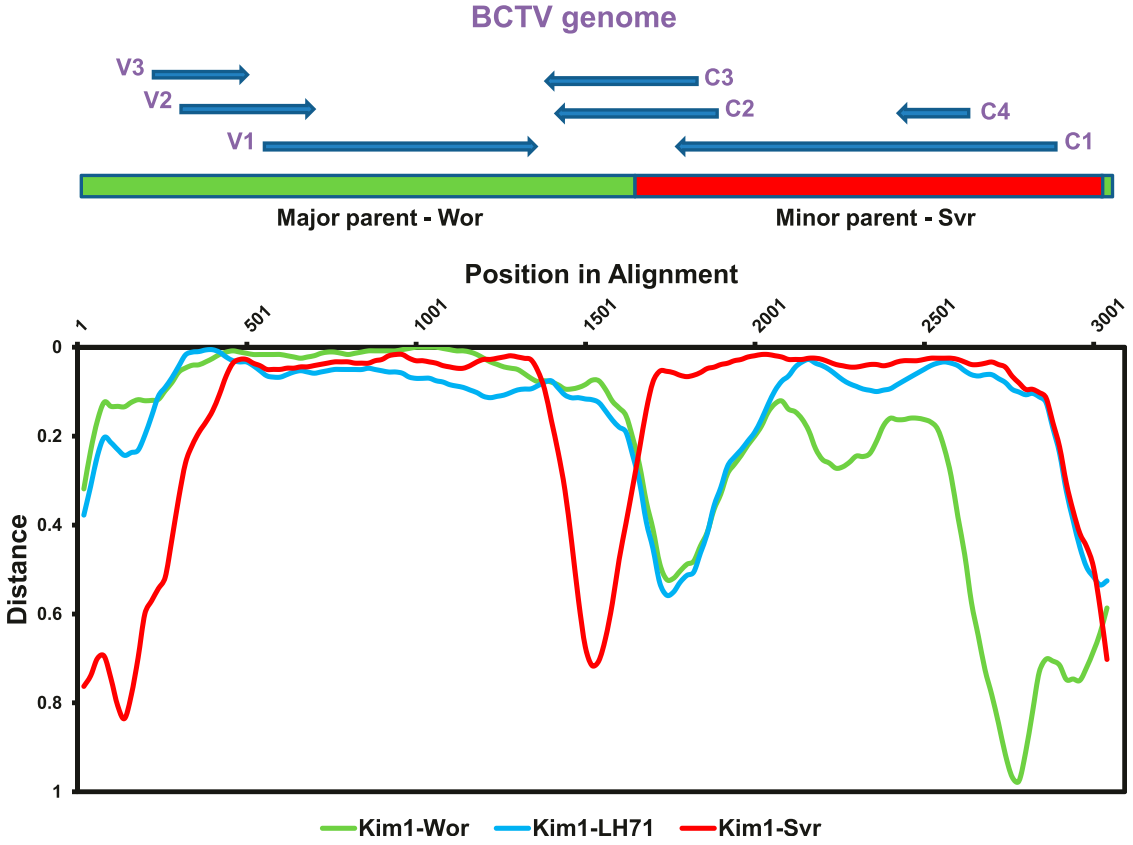


**Fig. 4.** Distance plot based on the aligned *Beet curly top virus* (BCTV) genomes of isolates CTS06-056, for the potential recombinant strain Kimberly1 (Kim1; GenBank accession KT276896); Worland, for major parent strain Worland (Wor; U56975); CFH, for minor parent strain Severe (Svr; U02311); and K5, a strain closely related to Leafhopper 71 (LH71; KT583738). The x-axis represents the nucleotide position in the alignment and the y-axis represents the relative distance from the reference sequence Kim1 calculated using the Kimura model (Kimura 1980). Above the distance plot is a schematic diagram showing the BCTV genome with the gene positions coded in the virion-sense (V) and complementary (C) directions, along with a bar for the Kim1 strain indicating the homologous sequences present in the two parent strains Wor and Svr.

The coat protein nucleotide sequence has historically been conserved among all the BCTV strains.

Prior to the 2006-to-2007 collection, the CA/Logan strain was widely perceived to have become very rare and possibly extinct in nature, because no isolates had been identified in many years (Chen et al. 2010; Creamer et al. 2005). The isolate was believed to exist primarily in laboratory collections. The 2006-to-2007 collection (Strausbaugh et al. 2008) demonstrated an average of 7% incidence of the CA/Logan strain in sugar beet plants evaluated from the Idaho production area, whereas the current survey averaged 30% incidence of CA/Logan among plants evaluated from the same region. If any variants in the sequencing were under represented they may be of the CA/Logan strain because, of all strains, it was the most difficult to obtain the complete genome sequences of this strain using the overlapping primer approach. Furthermore, the 2006-to-2007 collection demonstrated that the few isolates of CA/Logan identified at that time were divergent from one another, supporting the longstanding belief that this strain had been present in the United States for many years and was likely influenced by geographic separation of isolates and locally influenced natural selection (Strausbaugh et al. 2008).

Although insecticide-based control using neonicotinoid seed treatments is quite effective in reducing BCTV incidence and severity, host resistance remains a primary control measure utilized by sugar beet producers for control of curly top (Strausbaugh et al. 2016). However, with new evidence suggesting that some sources of curly top resistance in sugar beet may be more effective against specific BCTV strains (Montazeri et al. 2016), monitoring and evaluating resistance sources against all strains is becoming increasingly important. New strains of BCTV can be expected to continually evolve in response to natural and manmade selection pressure. Screening on a regional basis may also be necessary due to geographic separation of different leafhopper populations and the BCTV isolates each transmits, as well as locally influenced host and environmental conditions.

## Acknowledgments

These data support the objectives of the United States Department of Agriculture–Agricultural Research Service CRIS projects 5368-21220-003-00D and 2038-22000-013-00D. We thank J. Reed for research contributions; the Amalgamated Sugar Co., LLC, Amalgamated Research, LLC, Beet Sugar Development Foundation, and Snake River sugar beet growers for their support; and D. C. Stenger and R. L. Gilbertson for their critical review of the manuscript and helpful suggestions.

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