Coevolution of a Persistent Plant Virus and Its Pepper Hosts

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Accepted 13 February 2018.

There are many nonpathogenic viruses that are maintained in a persistent lifestyle in plants. Plant persistent viruses are widespread, replicating in their hosts for many generations. So far, *Endornaviridae* is the only family of plant persistent viruses with a single-stranded RNA genome, containing one large open reading frame. *Bell pepper endornavirus* (BPEV), *Hot pepper endornavirus*, *Capsicum frutescens endornavirus 1* (CFEV 1) have been identified from peppers. Peppers are native to Central and South America and, as domesticated plants, human selection accelerated their evolution. We investigated the evolution of these endornaviruses in different peppers including *Capsicum annuum*, *C. chacoense*, *C. chinense*, *C. frutescens*, *C. baccutum*, and *C. pubescens* using two fragments from the viral helicase (Hel) and RNA dependent RNA polymerase (RdRp) domains. In addition, using single nucleotide polymorphisms, we analyzed the pepper host populations and phylogenies. The endornaviruses phylogeny was correlated with its *Capsicum* species host. In this study, BPEV was limited to *C. annuum* species, and the RdRp and Hel phylogenies identified two clades that correlated with the host pungency. No *C. annuum* infected with CFEV 1 was found in this study, but the CFEV 1 RdRp fragment was recovered from *C. chinense*, *C. frutescens*, *C. baccutum*, and *C. pubescens*. Hence, during pepper speciation, the ancestor of CFEV 1 may have evolved as a new endornavirus, BPEV, in *C. annuum* peppers.

There are many cytoplasmic RNA viruses reported, from plants, that persist for many generations through nearly 100% vertical transmission. These viruses are very poorly studied, even though they are found in many crops and are the most common viruses found in wild plants (Roossinck 2012, 2015). Generally, persistent plant viruses are reported with no effect on their host, although finding an isogenic virus-free plant to use for comparison is not always possible. However, in clover and in a model legume, *Lotus japonicus*, the coat protein gene of the partitivirus *White clover cryptic virus 2* affects nodulation regulation (Nakatsukasa-Akune et al. 2005).

The viruses in the family *Endornaviridae* are found in plants, fungi, oomycetes, and protists. The endornaviruses have

a single-stranded RNA genome of 9,800 to 17,600 nts that contains a single open reading frame (ORF) encoding a large polyprotein (Fukuhara and Moriyama 2008). The virus is not encapsidated and, for this reason, the replicative intermediate double-stranded (ds)RNA is most often found in hosts, but the RNA dependent RNA polymerase (RdRp) indicates that the genome is single-stranded (Roossinck et al. 2011). Additional identified domains in the large polyprotein are methyltransferase (MTR), helicase (Hel), capsular polysaccharide synthase protein, and glycosyltransferase (GT) domains. All members of this family have a highly conserved RdRp at the C terminal end of the ORF, which is most similar to the RdRp of closteroviruses. The other domains are not conserved among all endornaviruses (Roossinck et al. 2011). A Hel domain is reported from most of the endornaviruses, but the origins of the Hel domains are clearly different in different viruses (Roossinck et al. 2011). Endornaviruses are found in many plants, including rice, wild rice, kidney bean, broad bean, barley, cucurbits, yerba mate, Malabar spinach, grapevine, avocado, and peppers (Coutts 2005; Debat et al. 2014; Espach et al. 2012; Moriyama et al. 1995; Okada et al. 2014; Pfeiffer 1998; Valverde and Gutierrez 2008; Villanueva et al. 2012; Wakarchuk and Hamilton 1990; Zabalgoceazcoa and Gildow 1992). All the plants with endornaviruses are reported as healthy and normal, although in *Vicia faba* male sterility is associated with the presence of *Vicia faba endornavirus* (Pfeiffer 1998).

Oryza sativa endornavirus is found in all *japonica* cultivars but not in *indica* cultivars of rice (Horiuchi et al. 2003). *Oryza rufipogon* is the ancestor of domesticated *japonica* rice and has a related persistent virus, *Oryza rufipogon endornavirus*. Around 10,000 years ago, the hosts of these two viruses diverged during the cultivation of rice (Molina et al. 2011). Assuming the virus was already present at the time of domestication, these viruses have diverged by only about 24% over 10,000 years. This is a surprisingly slow evolution rate for RNA viruses with previously reported rates of 10^{-3} to 10^{-4} substitutions per site per year (Roossinck 1997; Roossinck and Ali 2007; Pagán and Holmes 2010).

A large dsRNA was first reported from tissue extracts of healthy bell peppers (*Capsicum annuum*) (Valverde et al. 1990), and in 2007, the partial sequence analysis revealed that the dsRNA is the genome of an endornavirus, now named *Bell pepper endornavirus* (BPEV) (Valverde and Gutierrez 2007). The vertical transmission of BPEV is very high (close to 100%) when both parents are virus-infected (Okada et al. 2011). The single ORF of BPEV starts at nucleotide 225 and ends at nucleotide 14,670, encoding a 4,815-amino acid polyprotein with an estimated molecular mass of 545 kDa (Okada et al. 2011). BPEV contains four identified domains: MTR, Hel, GT, and RdRp (Supplementary Fig. S1) (Roossinck et al. 2011). It is presumed that the large protein is proteolytically cleaved into functional domains, although a protease has not been identified. Analysis of pepper viromes revealed

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Funding: This work was supported by the Pennsylvania State University College of Agricultural Sciences.

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

coinfection with BPEV and several acute viruses in Pusa Jwala and Taiwan 2 pepper cultivars (Jo et al. 2017).

In addition to BPEV, *Hot pepper endornavirus* (HPEV) was reported from a chili pepper (*C. annuum*), with 72% nucleotide identity to the BPEV Yolo Wonder isolate and containing all four identified domains of BEPV (Lim et al. 2015). An additional endornavirus, *Capsicum frutescens* endornavirus 1, was isolated from the tabasco cultivar of *C. frutescens* (R. Valverde personal communication).

Peppers are economically important crops of the *Solanaceae* family. A broad area along the Andes in South America including Peru, Ecuador, and Colombia is thought to be the origin of *Capsicum* spp. (Carrizo García et al. 2016). The genus apparently migrated from South America northward around 6 million years ago, resulting in the dispersal and speciation events. Bolivia and Peru are thought to be the centers of diversification and cultivation of peppers (Carrizo García et al. 2016). The *Capsicum* genus consists of approximately 35 species, of which *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L., and *C. pubescens* (Ruiz & Pavon) are domesticated species that now are grown all over the world (Carrizo García et al. 2013; Qin et al. 2014). Domesticated species of peppers are derived from three distinct genetic lineages. One lineage includes the members of a species complex, *C. frutescens*, *C. annuum*, and *C. chinense*, that were derived independently from wild progenitors, whereas *C. pubescens* and *C. baccatum* are each independent lineages (Eshbaugh 1993; Hill et al. 2013; Pickersgill 1997). The most common cultivated *Capsicum* species is *C. annuum*, which was domesticated in Mexico from chiltepin (*C. annuum* var. *glabriusculum*). Cultivated peppers are divided into two main groups of sweet and pungent peppers. Bell peppers are all sweet *C. annuum* cultivars and are found in a variety of colors with blocky shaped fruits. Pungent peppers, often called chile peppers, refer to a large number of varieties with mild to extreme pungency and belong to *C. annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, and *C. baccatum* (Hulse-Kemp et al. 2016).

Based on the whole-genome sequencing data, domestication has led to an increase in the size of the pepper genome (Qin et al. 2014), but cultivation has resulted in a significant decline in genetic variation. Significant decreases in wild chiltepin populations have occurred over the last few thousand years (González-Jara et al. 2011). Until very recently the origins, relationships, and domestication of peppers were unclear, but markers are now available that allow detailed phylogenetic trees of peppers to be constructed (Hill et al. 2013; Kraft et al. 2014; Qin et al. 2014). For polymorphism detection and expression analysis in *Capsicum* spp., a pepper GeneChipH array was developed, with 40 different *C. annuum* lines and cultivated *C. frutescens*, *C. chinense* and *C. pubescens* tested, resulting in detection of 33,401 single position polymorphism markers. The diversity in nonpungent peppers was reduced three-fold compared with pungent lines (Hill et al. 2013).

Endornaviruses are found in a number of both sweet and pungent pepper cultivars. In this study, we investigated the origin, natural history, and evolution of two persistent viruses, BPEV and *Capsicum frutescens* endornavirus 1 (CFEV 1), in different pepper cultivars. We used a single nucleotide polymorphism (SNP)-based method, Kompetitive Allele Specific PCR (KASP) assay (Ashrafi et al. 2012; He et al. 2014; Hulse-Kemp et al. 2016), to assess the evolutionary history of the hosts of these two endornaviruses.

RESULTS

Screening *Capsicum* spp. for the presence of endornaviruses.

After dsRNA extraction from peppers, the presence of BPEV or CFEV 1 was confirmed by reverse transcription-polymerase

chain reaction (RT-PCR), using specific primers for each virus. The RT-PCR products (BPEV_RdRp, 1,240 bp; BPEV_Hel, 1,162 bp; CFEV 1_RdRp, 1,101 bp; CFEV 1_Hel, 1,016 bp) were analyzed by Sanger sequencing to obtain the consensus sequence for each virus. We detected BPEV in 10 of 33 different *C. annuum* pepper cultivars, including Marengo, Chocolate, Greek Pepperoncini, Hungarian YW, Peter, Joe's Long Cayenne, Padron Hot, Jimmy Nardello's, Neapolitan, and Feher Ozon Paprika (Tables 1 and 2). BPEV has been detected mainly in the samples that were from the United States, except for Chocolate pepper from Guatemala (Fig. 1). Most of the United States virus-positive samples were received from the Hudson Valley and Victory Seeds companies, and we do not have any precise information about their origins and breeding history. Although we considered them as United States samples, the probable origins of some of them are different, specifically, Feher Ozon Paprika and Hungarian YW are probably from Hungary; Greek Pepperoncini is probably from Greece; Padron is likely from Spain; and Neapolitan is likely from Italy.

We did not detect CFEV 1 in any *C. annuum* peppers, but 24 different peppers in diverse *Capsicum* species were infected with this virus; 13 of 16 *C. baccatum*, five of 11 *C. chinense*, five of 18 *C. frutescens*, and one of eight *C. baccatum* were positive for CFEV 1 (Tables 1 and 2). Origins of the seeds for the CFEV1-positive plants are shown in Figure 1.

Using CFEV 1_RdRp primers an 875-bp fragment was amplified from dsRNA extracted from the Ember cultivar (*C. annuum*), which had 94% nucleotide identity to the Cs isolate of HPEV reported from South Korea (Lim et al. 2015). We did not detect BPEV or CFEV 1 in eight accessions of chiltepin (*C. annuum* var. *glabriusculum*) or two accessions of *C. chacoense* in our pepper collection.

Deep sequencing of the Marengo isolate of BPEV.

Using Illumina sequencing, we obtained a contig of 14,290 bp from Marengo peppers, corresponding to nucleotides 223 to 14,512 of BPEV, that differed from the strain amplified using BPEV-specific primers (strain Marengo1). The RdRp and Hel fragments of this isolate (Marengo2) had 90 and 88% nucleotide identity to BPEV Marengo1, respectively. The Marengo2 strain had 99% nucleotide identity to the Maor isolate, while both RdRp and Hel fragments of BPEV Marengo1 showed highest nucleotide identity (99 and 97%, respectively) to the Yolo Wonder isolate.

Virus phylogenies.

In addition to the 10 BPEV isolates detected in this study, the nucleotide sequences of 12 full-length sequences of BPEV available in GenBank (Supplementary Table S3) were included in the phylogenetic analysis. Pairwise identity was 88 to 100% among BPEV_RdRp nucleotide sequences and 87 to 100% among BPEV_Hel sequences. Phylogenetic analysis using the nucleotide sequence of both fragments resulted in two main clades (Fig. 2). In the BPEV_RdRp phylogeny clade 1 (Fig. 2A), viruses isolated from different pepper varieties (Marengo1, Greek Pepperoncini Hungarian YW, Peter, Joe's Long Cayenne, Padron Hot, Jimmy Nardello's, and Neapolitan) are grouped together with Yolo Wonder, Pusa Jwala, and Taiwan 2 isolates (accession numbers are provided in Supplementary Table S1). All isolates in clade 1 are from pungent peppers, except Marengo1 and Yolo Wonder. BPEV isolates from Chocolate, Feher Ozon Paprika, and the Marengo2 strain are grouped in clade 2, which included all sweet pepper isolates (Ij, Phenol, Santa Fe, Kyosuzu, IS, Maor, and Healy) except Chocolate and Santa Fe isolates, which are pungent peppers (Gutiérrez et al. 2017). In the BPEV_Hel phylogeny, the same pattern of two clades was observed; clade 1 contained isolates from pungent peppers and

clade 2 had the sweet pepper isolates (except Chocolate and Santa Fe). However, the BPEV_Hel fragment from Yolo Wonder grouped with the other sweet pepper isolates (Fig. 2B, clade 2), which indicates recombination in this virus, as was previously suggested in another study (Jo et al. 2016).

A Bayesian phylogenetic tree was constructed using nucleotide sequences of pepper endornavirus RdRp regions (BPEV, CFEV 1, and HPEV isolates), with *Phaseolus vulgaris endornavirus 2* as an outgroup (Fig. 3). This tree contains two main clades. Two HPEV isolates (Cs and Ember) were grouped together with BPEV isolates in clade A, all of which are isolated from *C. annuum* species. The second clade contained the CFEV 1 isolates. CFEV 1 from Aribibi Gusano pepper (*C. frutescens*) was distinct from the rest of isolates and, with a posterior probability of 1, may be ancestral to the rest of the CFEV 1 isolates. CFEV 1, isolated from two Habaneros, Royal Gold, and 30040 peppers (all from *C. chinense*), were grouped with three Tabasco peppers (*C. frutescens*) and were placed in clade CF. The BCP clade included isolates mostly from *C. baccatum*, with the exceptions of Ecu (from *C. pubescens*) and Chile Blanco and Lemon Drop (*C. chinense*) (Fig. 3). The CFEV 1 isolates from Aribibi Gusano and Habanero Red showed 83 to 85% nucleotide identity to other CFEV 1 isolates, while there was 94 to 100% nucleotide identity among isolates in the CF clade and 90 to 100% nucleotide identity among isolates in the BCP clade.

Using specific primers, the CFEV 1_Hel fragment was amplified only from Tabasco (Tabasco L-64 and Greenleaf Tabasco, both *C. frutescens*) and 30040 (*C. chinense*) peppers. Also, in the endornaviruses Hel phylogeny all *C. annuum*

isolates (BPEV and HPEV isolates) grouped together and CFEV 1 isolates from *C. frutescens* and *C. chinense* grouped in a second clade (Supplementary Fig. S2).

Pepper phylogenies and population structure.

To examine the population structure in the *Capsicum* spp. using SNP data from the KASP analysis, Bayesian clustering was implemented in STRUCTURE software. The distribution of the 38 *Capsicum* spp. into three distinct clusters is shown in Figure 4A (cluster 1 in red, cluster 2 in green =, and cluster 3 in blue). In cluster 1, Marengo, Jimmy Nardello's, Padron Hot, Ember, Greek Pepperoncini, Hungarian YW, Long Red Cayenne, Feher Ozon Paprika, Joe's Long Cayenne, Chiltepin102, and Diente De Perro are clustered together. Chiltepin54, Serano Hot, Jalapeño, Chiltepin53, Peter, Chocolate, and Jalapeño

Table 2. Number of positive samples in each *Capsicum* species

Species	No. of lines ^a	BPEV ^b	CFEV 1 ^c
<i>C. annuum</i> var. <i>annuum</i>	33	10	0
<i>C. annuum</i> var. <i>glabriusculum</i>	8	0	0
<i>C. baccatum</i>	16	0	13
<i>C. frutescens</i>	18	0	5
<i>C. chinense</i>	11	0	5
<i>C. pubescens</i>	8	0	1
<i>C. chacoense</i>	2	0	0

^a Number of lines tested for *Bell pepper endornavirus* (BPEV) and *Capsicum frutescens* endornavirus 1 (CFEV 1).

^b Number of lines positive for BPEV.

^c Number of lines positive for CFEV 1.

Table 1. Peppers with a detected endornavirus

Names in the tree ^a	Type	Species	PI ^b	Virus
BPEV_Marengo	Sweet	<i>Capsicum annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Jimmy Nardello's	Pungent	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Feher Ozon Paprika	Sweet	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Joe's long cayenne	Pungent	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Greek Pepperoncini	Pungent	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Hungarian YW	Pungent	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Padron Hot	Pungent	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Neapolitan	Pungent	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Peter	Pungent	<i>C. annuum</i> var. <i>annuum</i>	–	BPEV
BPEV_Chocolate	Sweet	<i>C. annuum</i> var. <i>annuum</i>	PI 666471	BPEV
CFEV 1_Habanero Red	Pungent	<i>C. chinense</i>	–	CFEV 1
CFEV 1_Chile Blanco	Pungent	<i>C. chinense</i>	PI 574545	CFEV 1
CFEV 1_30040	Pungent	<i>C. chinense</i>	PI 159236	CFEV 1
CFEV 1_Royal Gold	Pungent	<i>C. chinense</i>	PI 315023	CFEV 1
CFEV 1_Lemon Drop	Pungent	<i>C. chinense</i>	PI 315024	CFEV 1
CFEV 1_Aribibi Gusano	Pungent	<i>C. frutescens</i>	PI 573337	CFEV 1
CFEV 1_Greenleaf Tabasco AI	Pungent	<i>C. frutescens</i>	PI 634826	CFEV 1
CFEV 1_Tabasco L-167	Pungent	<i>C. frutescens</i>	PI 640909	CFEV 1
CFEV 1_Habanero	Pungent	<i>C. chinense</i>	–	CFEV 1
CFEV 1_Ecu	Pungent	<i>C. pubescens</i>	PI 585262	CFEV 1
CFEV 1_Lemon Drop Hot	Pungent	<i>C. baccatum</i>	–	CFEV 1
CFEV 1_WTS-14	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 595905	CFEV 1
CFEV 1_WWMC126	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 632927	CFEV 1
CFEV 1_WW141	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 633756	CFEV 1
CFEV 1_MC145	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 633757	CFEV 1
CFEV 1_MC147	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 633758	CFEV 1
CFEV 1_Malagueta	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 260543	CFEV 1
CFEV 1_Valentine	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 260549	CFEV 1
CFEV 1_Omnicolor	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 260590	CFEV 1
CFEV 1_82	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 337522	CFEV 1
CFEV 1_BGH 4215	Pungent	<i>C. baccatum</i> var. <i>pendulum</i>	PI 441589	CFEV 1
CFEV 1_470	Pungent	<i>C. baccatum</i> var. <i>baccatum</i>	PI 215699	CFEV 1
CFEV 1_No 1553	Pungent	<i>C. baccatum</i> var. <i>baccatum</i>	PI 238061	CFEV 1
HPEV_EMBER	Pungent	<i>C. annuum</i> var. <i>annuum</i>	PI 273426	HPEV

^a Names appear with virus name followed by cultivar name or plant designation. BPEV = *Bell pepper endornavirus*; CFEV 1 = *Capsicum frutescens* endornavirus 1.

^b Plant Introduction number.

Criollo are grouped together in cluster 2. Cluster 3 includes Chiltepin30, Chile Blanco, WWMC 126, Ecu, Lemon Drop Hot, Aribibi Gusano, 30040, Omnicolor, Habanero Red, Valentine, Royal Gold, WW141, Malagueta, No1553, 470, BGH4215, Lemon Drop, Tabasco L167, Greenleaf Tabasco AI, and MC145.

Population structure analysis revealed admixture in Joe's Long Cayenne and Diente De Perro from cluster 1 with peppers in cluster 2. Peter, Chocolate, and Jalapeño Criollo peppers have admixtures with genotypes in cluster 1. Peppers in clusters 1 and 2 belong to *C. annuum*, except Diente De Perro, which

Fig. 1. The distribution map of detected endornaviruses. *Bell pepper endornavirus* (BPEV) and *Capsicum frutescens* endornavirus 1 (CFEV 1). Location indicates the country from which the seeds were obtained, but the probable origins of the seeds are, Marengo, United States; Jimmy Nardello, United States; Feher Ozon Paprika, Hungary; Joe's long cayenne, United States; Greek pepperoncini, Greece; Hungarian YW, Hungary; Padron hot, Spain; Neapolitan, Italy; and Peter, United States.

Fig. 2. Relationships of *Bell pepper endornavirus* (BPEV) isolates. Bayesian analysis of the relationships among aligned nucleotide sequences of BPEV isolated from different peppers. Trees were generated by MrBayes in Geneious 10.0.9, using the best fit model (HKY + I). Posterior probabilities are shown on nodes. *Hot pepper endornavirus* (HPEV) was used as an outgroup. **A**, BPEV_RdRp; **B**, BPEV_Hel.

Ecu pepper (*C. pubescens*) was used as an outgroup for the Bayesian phylogenetic tree for the 38 pepper samples. This tree is in general agreement with the previous population clustering and the endornaviruses phylogenies (Fig. 4B). The clade highlighted in red includes hosts of clade 1 from the BPEV phylogeny, including Feher Ozon Paprika. However, Peter and Chocolate peppers, both positive for BPEV, are in the green-highlighted part of the pepper phylogenies. All pepper lines in the red- and green-highlighted groups belonged to *C. annuum* and are clustered with Chile Blanco (*C. chinense*) and Chiltepin30; Chiltepin102 is the probable ancestor. The final grouping includes the various non-*annuum* species of peppers, in which all of the CFEV 1-positive plants are placed and in which there is less than 2% variation in the SNP data.

In this study, we detected BPEV only in *C. annuum* and in none of the other *Capsicum* species. Previously, BPEV was reported, from several domesticated *Capsicum* species (*C. annuum*,

BPEV has been reported from the United States, Colombia, Canada, China, Japan, Taiwan, India, South Korea, and Israel (Jo et al. 2016; Gutiérrez et al. 2017; Lim et al. 2015; Okada et al. 2011; Valverde and Gutierrez 2007). In this study, all cultivars were from the Americas, and BPEV was found in the United States samples and a Chocolate pepper from Guatemala. Hence, BPEV has been distributed all over the world by pepper dispersal. In structure analysis and pepper phylogenies (Fig. 4), Chocolate pepper was nested within chiltepins, the ancestor of *C. annuum* peppers. While BPEV has not been identified in any chiltepin in this study or previous studies (Okada et al. 2011)), Chocolate pepper may be the origin of introduction of this virus to *C. annuum*.

The admixture observed within *C. annuum* samples (Fig. 4A) revealed the exchange of genetic material between cultivars by breeding and natural cross-pollination. Among four different chiltepins (*C. annuum* var. *glabriusculum*) used in this analysis, Chiltepin102, included in cluster 1, revealed an admixture (approximately 25%) with cluster 3, Chiltepin53 and Chiltepin54 clustered with peppers in cluster 2 and Chiltepin30 in cluster 3

with an admixed genome with cluster 1. This result is consistent with a previous study that used microsatellite markers for pepper (Nicolai et al. 2013).

Phylogenetic analysis of nucleotide sequences of BPEV RdRp and Hel fragments revealed two main clades in both domains, corresponding to the pepper pungency (Fig. 2). Clade 1 included BPEV isolates mainly from pungent pepper and clade 2 from sweet peppers. Two strains of BPEV in Marengo were separated in both BPEV phylogenies. Strain Marengo1 was nested between isolates from pungent peppers, and the Marengo2 strain was placed within sweet pepper isolates. The presence of different strains could result from cross-pollination between peppers, since persistent viruses are transmitted by pollen and ovum (Roossinck 2010). SNP profiles in the transcriptome data from a Maor pepper revealed more than one BPEV variant in this cultivar as well (Jo et al. 2016).

BPEV_Hel from Yolo Wonder showed evidence of recombination in this virus. A schematic diagram of BPEV_Hel

Fig. 4. Population structure of pepper hosts. **A**, Population structure analysis of single nucleotide polymorphism (SNP) data, using STRUCTURE software (2.3.4). The distribution of the 38 *Capsicum* spp. lines into three distinct clusters is shown in red (cluster 1), green (cluster 2), and blue (cluster 3). **B**, Bayesian analysis of the relationships among SNPs in different pepper lines. Ecu (from *C. pubescens*) was used as an outgroup. The tree was generated by MrBayes in Geneious 10.0.9, using GTR + I. Posterior probabilities are shown on nodes.

In Figure 6, the BPEV_RdRp (Fig. 6A) and *C. annuum* peppers (including Chile blanco) (Fig. 6B) phylogenies are shown by connecting viruses to their host; in many samples, congruence is

CFEV 1 was detected in four *Capsicum* species, but in none of our *C. annuum* peppers. Ecu was the only *C. pubescens* positive for this virus; however, it could have been due to our small collection (eight samples) from this species. While our SNP markers did not resolve non-*annuum* phylogenies to verify the species identification for this pepper, the possibility of misclassification of this pepper is low, since morphological traits of *C. pubescens* and *C. baccatum* are quite distinct (Jarret 2008).

Hybrids of *C. pubescens* with the other four *Capsicum* spp. are sterile, so there is no chance for the virus to be transmitted by cross-pollination. Hence, this is evidence for the presence of an endornavirus in the ancestor of all of these *Capsicum* species.

Most of the screened *C. baccatum* peppers were positive for CFEV 1. CFEV 1 isolates from 470 and No 1553 peppers (*C. baccatum* var. *baccatum*), the wild form of this species (Nimmakayala et al. 2016), are grouped together in the BCP clade (Fig. 3), separate from the other isolates. The high incidence of CFEV 1 in *C. baccatum* and its detection in the wild subspecies implies that *C. baccatum* is the original host of CFEV 1. During *Capsicum* speciation, breeding, and dispersal this virus could have been transmitted to *C. frutescens* and *C. chinense*. The interspecific compatibility between these three species also supports this hypothesis (Walsh and Hoot 2001). The locations of *C. baccatum* peppers infected with CFEV 1 were mainly from South America (Peru, Bolivia, Ecuador, Paraguay, Brazil, and Argentina), which is congruent with origin and speciation of *Capsicum* spp. During pepper speciation, the ancestor of CFEV 1 may have evolved as a new endornavirus, BPEV, in *C. annuum* peppers.

Different approaches such as expressed sequence tag-simple sequence repeat (SSR), isozyme, plastid DNA, and SNP analysis confirmed that *C. chinense* and *C. frutescens* were closely related to *C. annuum*, with *C. baccatum* more distant (Jarret 2008; Shirasawa et al. 2013; Walsh and Hoot 2001). It is interesting that we did not detect any BPEV in non-*annuum* capsicum, while CFEV 1 was found in *C. chinense* and *C. frutescens* and *C. baccatum*. It can be concluded that the ancestor of these *Capsicum* species was infected by an endornavirus, and during speciation and domestication of peppers, the virus was transmitted and diverged in different peppers.

MATERIALS AND METHODS

Pepper collection.

We collected 97 different peppers, including *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, *C. pubescens*, and *C. chacoense*, with different improvement levels (cultivars,

landraces, or wild materials). The seed sources and information are found in Supplementary Table S2. Seeds were germinated in wet paper towels and were then transferred to plastic pots containing Sun-Gro Horticulture soil and were grown in an insect-free environmental room at 24°C with fluorescent light (16-h-light and 8-h-dark photoperiod).

Screening *Capsicum* spp. for the presence of BPEV and CFEV 1.

To screen plants for the presence of endornaviruses, dsRNAs were extracted from 5 g of fresh weight leaves, using the protocol described previously (Márquez et al. 2007).

For BPEV, based on the available sequences in GeneBank, primer pairs specific to part of the RdRp (BPEV_RdRp) and Hel (BPEV_Hel) domains were designed to amplify 1,240- and 1,162-bp fragments, respectively. Based on sequence information for CFEV 1 (provided by R. Valverde, Louisiana State University), specific primers for part of the RdRp (CFEV 1_RdRp) and Hel (CFEV 1_Hel) domains were designed to amplify 1,101 and 1,016 bp, respectively. For the RT reaction, we used about 2 µg of dsRNA, mixed with 2 µM reverse primer, 0.5 mM of Tris-EDTA (pH 8.0), and nuclease-free water to a final volume of 12 µl and boiled for 2 min. The mixture was incubated on ice for 2 min and 8 µl of RT mix (200 U of M-MuLV reverse transcription [New England Biolabs], 2 µl of 10× M-MuLV buffer [supplied by the manufacturer] and 10 mM dNTPs) was added and incubation was continued at 42°C for 1 h. Then, cDNA was incubated with 10 µg of boiled RNase A (Sigma) for 15 min at room temperature and was cleaned with E.Z.N.A cycle pure kit (Omega Bio-Tech) according to the manufacture's instruction. About 0.5 µg of cleaned cDNA was used as a template for a 25 µl PCR with *Taq* DNA polymerase (ThermoFisher Scientific), buffer (30 mM MgCl₂, Idaho Technologies), 2 mM dNTPs, and 0.2 µM forward and reverse primers for each fragment. The PCR reactions were completed in capillary tubes with an Idaho Technologies rapid cycler for 40 cycles (94°C denaturation for 0 s, 48°C annealing for 0 s, and 72°C extension for 45 s). The RT-PCR products were separated on a 1.2% agarose gel in 0.5× Tris-borate-EDTA, and

Fig. 6. Congruence between the A, BPEV_RdRp and B, pepper phylogenies. For pepper, *Capsicum annuum* samples and Chile blanco were included and Chiltepin102 was used as an outgroup. The tree was generated by MrBayes in Geneious 10.0.9, using GTR + I. Posterior probabilities are shown on nodes.

appropriate bands were excised and purified using E.Z.N.A. gel extraction kit (Omega Bio-Tech). Sequence analysis of the PCR products was done by the Genomic Core Facility of Pennsylvania State University, University Park, PA, U.S.A. The sequences have been deposited in GenBank under the accession numbers listed in Supplementary Table S4.

Nucleotide sequences were aligned with Clustal W using default settings in the program Geneious 10.0.9 (Kearse et al. 2012). The alignment was visually corrected as necessary. Phylogenetic analysis was performed using MrBayes (Ronquist and Huelsenbeck 2003) implemented as a Geneious plug-in. The nucleotide sequences of 12 full-length sequences of BPEV available in GenBank were included in the phylogenetic analysis. HPEV was used as an outgroup for the phylogenies of BPEV regions. For the phylogenies of all pepper endornaviruses *Phaseolus vulgaris endornavirus 2* was used as an outgroup. For BPEV_RdRp and BPEV_Hel phylogenies, the HKY 98 + I substitution model and, for *Endornaviridae* _RdRp and _Hel phylogenies, GTR + G + I were selected as the best fit models, according to JModelTest (Darriba et al. 2012); burn-in and chain length were 100,000 and 1,100,000, respectively.

Deep sequencing of the Marengo isolate of BPEV.

The dsRNA extracted from Marengo bell pepper was treated with RQ1 DNase (Promega) and, after cDNA preparation as described above using tagged primers for multiplexing, was sequenced as part of a pool of 96 samples at the Oklahoma

Medical Research Foundation, Clinical Genomic Center, using the Illumina HiSeq 3000 system. The raw data were trimmed using trimmomatic and quality was assessed by fastqc. Reads were assembled using the Velvet (1.2.10) program and were mapped against the BPEV Yolo Wonder isolate (JN019858.1) as a reference sequence. The consensus sequence was used for the analysis.

Pepper phylogenies and population structure.

To determine the phylogeny of peppers, DNA of plants listed in Table 3 was extracted, using cetyltrimethylammonium bromide (CTAB) as described previously (Healey et al. 2014), with some modifications. Plant tissue (0.5 g) was flash-frozen in liquid nitrogen, was ground to a fine powder, and was mixed with 5 ml of preheated extraction buffer (100 mM Tris-HCl, pH 7.5, 25 mM EDTA, 1.5 M NaCl, 2% [wt/vol] CTAB, and 0.3% [vol/vol] β -mercaptoethanol). The sample was incubated at 65°C and was mixed by inversion every 10 min for 30 min, followed by centrifugation for 5 min at 5,000 $\times$ g. The supernatant was transferred to a fresh tube and was mixed with an equal volume of phenol/chloroform (1:1, wt/wt). After mixing by inversion for 5 min, the sample was centrifuged for 10 min at 5000 $\times$ g and the upper phase was transferred to a new tube and was treated with 5 μ l of RNase A (10 mg/ml) (Sigma-Aldrich) for 15 min at 37°C, followed by an additional extraction as above with phenol/chloroform. The final aqueous phase was precipitated with 0.3 M sodium acetate and two volumes of

Table 3. Peppers used for Kompetitive Allele-Specific PCR (KASP) analysis

Line name ^a	Species	Location ^b	PI ^c
Marengo	<i>Capsicum annuum</i> var. <i>annuum</i>	United States	–
Jalapeno	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Serrano Hot	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Long Red Cayenne	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Jalapeno Criollo	<i>C. annuum</i> var. <i>annuum</i>	Guatemala	PI 666462
Jimmy Nardello's	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Fehér Ozon Paprika	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Joe's Long Cayenne	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Greek Pepperoncini	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Hungarian YW	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Padron Hot	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Peter	<i>C. annuum</i> var. <i>annuum</i>	United States	–
Chocolate	<i>C. annuum</i> var. <i>annuum</i>	Guatemala	PI 666471
Ember	<i>C. annuum</i> var. <i>annuum</i>	United States	PI 273426
Chiltepin30	<i>C. annuum</i> var. <i>glabriusculum</i>	Guatemala	PI 632932
Chiltepin53	<i>C. annuum</i> var. <i>glabriusculum</i>	Mexico	–
Chiltepin54	<i>C. annuum</i> var. <i>glabriusculum</i>	Mexico	–
Chiltepin102	<i>C. annuum</i> var. <i>glabriusculum</i>	Mexico	–
Habanero Red	<i>C. chinense</i>	United States	–
Chile Blanco	<i>C. chinense</i>	Mexico	PI 574545
30040	<i>C. chinense</i>	United States	PI 159236
Royal Gold	<i>C. chinense</i>	Peru	PI 315023
Lemon Drop	<i>C. chinense</i>	Peru	PI 315024
Aribibi Gusano	<i>C. frutescens</i>	Bolivia	PI 573337
Greenleaf Tabasco AI	<i>C. frutescens</i>	United States	PI 634826
Tabasco L-167	<i>C. frutescens</i>	United States	PI 640909
Ecu	<i>C. pubescens</i>	Ecuador	PI 585262
Lemon Drop Hot	<i>C. baccatum</i>	United States	–
WWMC126	<i>C. baccatum</i> var. <i>pendulum</i>	Paraguay	PI 632927
WW141	<i>C. baccatum</i> var. <i>pendulum</i>	Paraguay	PI 633756
MC145	<i>C. baccatum</i> var. <i>pendulum</i>	Paraguay	PI 633757
Malagueta	<i>C. baccatum</i> var. <i>pendulum</i>	Brazil	PI 260543
Valentine	<i>C. baccatum</i> var. <i>pendulum</i>	Peru	PI 260549
Omnicolor	<i>C. baccatum</i> var. <i>pendulum</i>	Peru	PI 260590
BGH 4215	<i>C. baccatum</i> var. <i>pendulum</i>	Brazil	PI 441589
470	<i>C. baccatum</i> var. <i>baccatum</i>	Peru	PI 215699
No 1553	<i>C. baccatum</i> var. <i>baccatum</i>	Bolivia	PI 238061

^a Cultivar name or other reference name.

^b Location of the seed source.

^c Plant Introduction number.

absolute ethanol. The pellet was washed with 70% ethanol, was dried, and was resuspended in 100 µl of Tris-EDTA buffer. DNA samples were sent to TraitGenetics Company in Germany for a KASP assay with 96 SNPs. The KASP genotyping assay is a fluorescence-based reporting system for detection of the alleles (SNPs) at a specific locus within the genomic DNA (Ashrafi et al. 2012; He et al. 2014). The SNP information is provided in Supplementary Table S5.

SNPs within different pepper lines identified in the KASP analysis were used for a Bayesian cluster estimation of population structure, using the STRUCTURE software (version 2.3.4) (Pritchard et al. 2000). STRUCTURE was run for $K = 1$ to $K = 10$ (K = number of clusters), and for each K value, 10 replicates were performed with a burn-in of 35,000 followed by 30,000 Markov chain Monte Carlo iterations after burn-in. The replicate with highest probability of K was selected. In addition, to reveal the phylogenetic origin of these pepper lines, a phylogenetic tree was generated with the SNP data, using MrBayes in Geneious. JModelTest suggested a Sym substitution model, but since it is not implemented in MrBayes, several models were tested that all resulted in the same tree, and the GTR + I substitution model was selected as the best fit model; burn-in was 100,000 and total chain length was 1,100,000.

ACKNOWLEDGMENTS

We thank R. Valverde for providing the CFEV 1 RdRp and Hel sequences. We appreciate all the people who helped us to collect different peppers, F. García-Arenal (providing chiltepins samples), M. D. Orzolek, R. Bello-Bedoy, A. Stephenson, USDA-GRIN (most of the samples). Special thanks to R. Jarret for his guidance in pepper information, H. Ashrafi and T. Hill for their discussions about pepper markers and analysis.

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