Diversification of an emerging bacterial plant pathogen; insights from the global spread of *Xanthomonas euvesicatoria* pv. *perforans*

Short title: Intercontinental dissemination of *X. euvesicatoria* pv. *perforans*

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Abstract

Emerging and re-emerging plant diseases continue to present multifarious threats to global food security. Considerable recent efforts are therefore being channeled towards understanding the nature of pathogen emergence, their spread and evolution. *Xanthomonas euvesicatoria* pv. *perforans (Xep*)*,* one of the causal agents of bacterial spot of tomato, rapidly emerged and displaced other bacterial spot xanthomonads in tomato production regions around the world. In less than three decades, it has become a dominant xanthomonad pathogen in tomato production systems across the world and presents a model for understanding diversification of recently emerged bacterial plant pathogens. Although *Xep* has been continuously monitored in Florida since its discovery, the global population structure and evolution at the genome-scale is yet to be fully explored. The objectives of this work were to determine genetic diversity globally to ascertain if different tomato production regions contain genetically distinct *Xep* populations, to examine genetic relatedness of strains collected in tomato seed production areas in East Asia and other production regions, and to evaluate variation in type III effectors, which are critical pathogenicity and virulence factors, in relationship to population structure. We used genome data from 270 strains from 13 countries for phylogenetic analysis and characterization of *Xop* effector gene diversity among strains. Our results showed notable genetic diversity in the pathogen. We found genetically similar strains in distant tomato production regions, including seed production regions, and diversification over the past 100 years, which is consistent with intercontinental dissemination of the pathogen in hybrid tomato production chains. Evolution of the *Xep* pangenome, including the acquisition and loss of type III secreted effectors, is apparent within and among phylogenetic lineages. The apparent long-distance movement of the pathogen, together with variants that may not yet be widely distributed, poses risks of emergence of new variants in tomato production.

109 **Introduction/Main**

110 Emerging and re-emerging plant diseases are a constant threat to global food security [1-3]. Bacterial
111 plant pathogens cause some of the most intractable diseases of crops worldwide [4-7]. Novel emergence
112 and re-emergence of bacterial diseases continue to be reported across the globe and is associated with an
113 upsurge in efforts devoted to understanding the nature of pathogen emergence, spread, and evolution [8-
114 17]. A bacterial plant pathogen that emerged in the last few decades and is of global epidemiological
115 consequences is *Xanthomonas euvesicatoria* pv. *perforans,* one of the causal agents of bacterial spot of
116 tomato [18].

117 Bacterial spot disease of tomato affects all aboveground plant parts including leaves, stems, flowers
118 and fruit. Under optimal environmental conditions, fruit lesions and/or extensive defoliation can
119 dramatically limit marketable yields and poses a continuous challenge to tomato production [19-21]. Once
120 epidemics are initiated, growers have limited management tools and have relied heavily on copper-based
121 bactericides. However, reliance on copper compounds has led to widespread copper tolerance [22-30].
122 Alternative bactericides are often costly, provide insufficient control when the weather favors rapid
123 disease development, and rarely improve yields. While historically four taxa have caused this disease,
124 *Xanthomonas euvesicatoria* pv. *perforans* (*Xep*) [31] (syn. *X. perforans* [32, 33]) has emerged rapidly and
125 become a major player on tomato [18, 27, 34-41]. *Xep* was first reported in 1991 in Florida, USA [32] and
126 is now found in all tomato production areas of the world, including regions with no history of the disease
127 [42]. *Xep* has been isolated from tomato seed [32]; therefore, a plausible hypothesis for new outbreaks of
128 *Xep* is pathogen movement with seeds and planting materials [43].

129 Tomato production is characterized by a high seed replacement rate (99.3%), meaning that growers
130 require seeds each season, which in turn requires large-scale seed production [44]. Tomato hybrid seed
131 production is concentrated in geographic areas where environmental conditions minimize seed
132 contamination by pathogens and seed production costs are low. These seed production regions supply
133 hybrid seeds globally for commercial production of tomato fruits for the fresh market or for processing
134 into tomato products (e.g., sauce, paste, and diced tomatoes). The long-distance movement of seeds poses

135 a high risk for dissemination of seed borne pathogens to commercial tomato production areas. Seedlings
136 are typically grown in transplant facilities and then transplanted into fields for the regional and
137 international transplant markets, potentially amplifying and further disseminating seed-borne pathogens
138 [45].

139 The success of *Xep* as a pathogen has been attributed to its production of bacteriocins against
140 competing bacterial spot species, rapid genome evolution via recombination affecting the chromosome,
141 and introduction of genes via horizontal gene transfer that contribute to fitness in tomato fields [46-52].
142 Distinct genetic lineages of *Xep*, each with unique patterns of allelic variation among core genes (genes
143 present in all strains), were identified in fresh market and processing tomato production fields in the
144 United States [47, 50, 51, 53, 54]. Additional lineages of *Xep* were found in Nigeria, Iran, Italy, and the
145 Southwest Indian Ocean islands [42, 48, 55].

146 *Xanthomonas perforans* strains, like other xanthomonads, acquire nutrients through colonization of
147 compatible hosts. The type III secretion system (T3SS) and type III effector (T3E) proteins are critical for
148 suppression of host defenses and virulence by *Xep* [56]. Effector content varies among *Xanthomonas*
149 species and distinct lineages of *Xep* have distinguishable effector content [23, 48, 50, 57, 58]. Strains of
150 *Xep* isolated in the 1990s were limited to tomato [59], but now strains of *Xep* are causing bacterial spot
151 disease of pepper [50, 58, 60]. Host range expansion was attributed, in part, to loss of effectors that act as
152 avirulence factors in pepper and other genomic changes as a result of recombination with other
153 *Xanthomonas* lineages, including pepper pathogenic *X. euvesicatoria* pv. *euvesicatoria*. Effector variation
154 may cause differences in disease epidemiology in addition to host range [49, 57, 61]. For example,
155 wildtype strains with the acquired effector XopJ2 showed three times faster spread in the field than
156 isogenic mutant strains without the effector [51].

157 Emerging pathogens may show limited genetic variation if they experienced a bottleneck during the
158 ecological and evolutionary processes that often precede emergence (e.g., host jump or introduction
159 event) [62]. *Xep* appears genetically diverse but it is not known how this variation is structured across
160 global tomato production regions. The first objective of this work was to determine if different tomato

161 production regions contain genetically distinct *Xep* populations. Second, we asked if there was evidence
162 for long-distance pathogen dissemination, as would be indicated by genotypes shared among distant
163 regions. Specifically, we obtained strains from tomato seed production areas in East Asia and determined
164 if they resembled strains from fruit production fields elsewhere in the world, which would be expected if
165 strains are being disseminated in seeds. Third, we estimated the timing of *Xep* population expansion
166 relative to its first report in 1991. Finally, we evaluated T3SE content and allelic variation in the context
167 of geography and core genome variation as a proxy for genetic variation in virulence. Overall, we found
168 extensive genetic diversity within *Xep;* genetically similar strains in distant geographic regions, inclusive
169 of seed production regions; evidence of diversification prior and subsequent to the first report of
170 emergence; and lineage-specific T3SE repertoires. Together, these results illustrate the capacity for this
171 pathogen to rapidly evolve and strongly support the potential for intra- and intercontinental movement of
172 pathogens in tomato production systems.
173
174 **Results**
175 *X. euvesicatoria* pv. *perforans* strains from seed and commercial fruit production areas
176 A total of 270 *Xep* genomes from 13 different countries – representing seed and fruit production –
177 were used in this study (Table 1). We generated new genome sequence data for 153 strains (S1 Table;
178 NCBI BioProject PRJNA941448). *Xep* strains were differentiated from other tomato-pathogenic
179 xanthomonads using a real-time qPCR assay that specifically amplifies the *hrcN* (*hrpB7*) gene in *Xep* [63]
180 and inoculated on tomato cv. 'Bonny Best' to confirm pathogenicity. Strains from China, Thailand, and
181 Vietnam were collected from seed production areas (n = 31) and all other strains (n = 239) were collected
182 in commercial fruit production areas from Australia, Brazil, Canada, Ethiopia, Iran, Italy, Mexico,
183 Nigeria, South Africa, and the United States. Within the US, strains were collected from seven different
184 states in the Midwest and Southeast, including strains collected since 1991 from Florida.
185

186 **Table 1. *Xanthomonas euvesicatoria* pv. *perforans* strains used in this study.**

Country	Locality	Year	Strain (original name, if applicable)
Australia [57]	Queensland	2015	Aus3, Aus7, Aus14
		2016	Aus5, Aus10, Aus11
		2017	Aus1, Aus15, Aus16
Brazil [39]	São Paulo	2011	Bzl1 (2011-107), Bzl2 (2011-132)
	Goiás	2012	Bzl3 (2012-08)
	Goiás, São Paulo	2013	Bzl5 (2013-16), Bzl6 (2013-42)
	Goiás, Minas Gerais	2014	Bzl7 (2014-10), Bzl8 (2014-17)
	Minas Gerais	2015	Bzl10 (2015-53), Bzl11 (2015-56)
	Goiás	2016	Bzl13 (2016-08)
	Goiás	2017	Bzl14 (2017-21)
Canada	Ontario	2016	4A, 4D, 12A, 14A
China		2016	CHI-3, CHI-5, CHI-6, CHI-7, CHI-8, CHI-10, CHI-12, CHI-15, CHI-18
Ethiopia [36]		2011	ETH5, ETH11, ETH21, ETH25, ETH33
Iran [41]		2013	K41, F210, F215, TOM801, TOM816
Italy [64]		2011	1P6S1, 2P4S1, 2P4S1D, 2P6S1, 1P4S1D
Mexico			Mexico-1, Mexico-3, Mexico-LT1, Mexico-LT3, Mexico-LT5
Nigeria [37, 65]		2014	NI-1, NI-2, NI-4, NI-7, NI-12, NI-13
		2015	KS3, KS5, KS9, KS28
South Africa	Pretoria		X2-B14, X10-B85, X59-BD1351, X47-BD167

Vietnam			SEA-3, SEA-5, SEA-21, SEA-23
Thailand		2016	THA-8, THA-14, THA-40, THA-45, THA-54, THA-72, THA-81A, THA-100, THA-112, THA-116, THA-119, THA-120, THA-126, THA-127, THA-128, THA-132, THA-135, THA-157A
United States	Alabama [66]	1996	Xp1861
	Indiana [25]	2016	16-1165A1, 16-1181-2, 16-1182A, 16-1184A, 16-1187A, 16-1205A, 16-1402A, 16-974C, 16-990A, 16-990C
		2014	14-463-1A
	Florida [32, 33, 43, 47, 58, 67]	1991	XV0938, Xp91-118, Xp894, Xp909, Xp1183
		1992	Xp1118, Xp1144
		1993	Xp1241, Xp1268, Xp1275
		1994	Xp1550, Xp1564
		1995	Xp1797, Xp1805
		1996	Xp1856
		1997	Xp1912
		1998	Scott-1, Xp1920
		2006	Xp1-5, Xp1-6, Xp3-12, Xp3-15, Xp3-16, Xp3-8, Xp4-20, Xp5-14, Xp5-6, Xp5-9, Xp7-12, Xp8-16, Xp9-5, Xp10-13, Xp11-2, Xp15-11, Xp17-12, Xp18-15
		2007	Xp4B
		2010	Xp2010
		2011	GEV485

		2012	GEV839, GEV872, GEV893, GEV904, GEV909, GEV915, GEV917, GEV936, GEV940, GEV968, GEV993, GEV1001, GEV1026, GEV1044, GEV1054, GEV1063
		2013	TB6, TB9, TB15
		2015	GEV2047, GEV2048, GEV2049, GEV2050, GEV2052, GEV2055, GEV2058, GEV2059, GEV2060, GEV2063, GEV1989, GEV1991, GEV1992, GEV1993, GEV2004, GEV2009, GEV2010, GEV2011, GEV2013, GEV2015, GEV1911, GEV1912, GEV1913, GEV1914, GEV1915, GEV1916, GEV1917, GEV1918, GEV1919, GEV1920, GEV1921
		2016	GEV2065, GEV2067, GEV2072, GEV2087, GEV2088, GEV2089, GEV2097, GEV2098, GEV2099, GEV2108, GEV2109, GEV2110, GEV2111, GEV2112, GEV2113, GEV2114, GEV2115, GEV2116, GEV2117, GEV2118, GEV2119, GEV2120, GEV2121, GEV2122, GEV2123, GEV2124, GEV2125, GEV2126, GEV2127, GEV2128, GEV2129, GEV2130, GEV2132, GEV2133, GEV2134, GEV2135
	Louisiana [24]	2013	mli-2
	North Carolina [27]	2015	NC-14, NC-47, NC-67, NC-101, NC-112, NC-204
		2016	NC-242, NC-252, NC-282, NC-289, NC-350, NC-373, MRS-30P-011

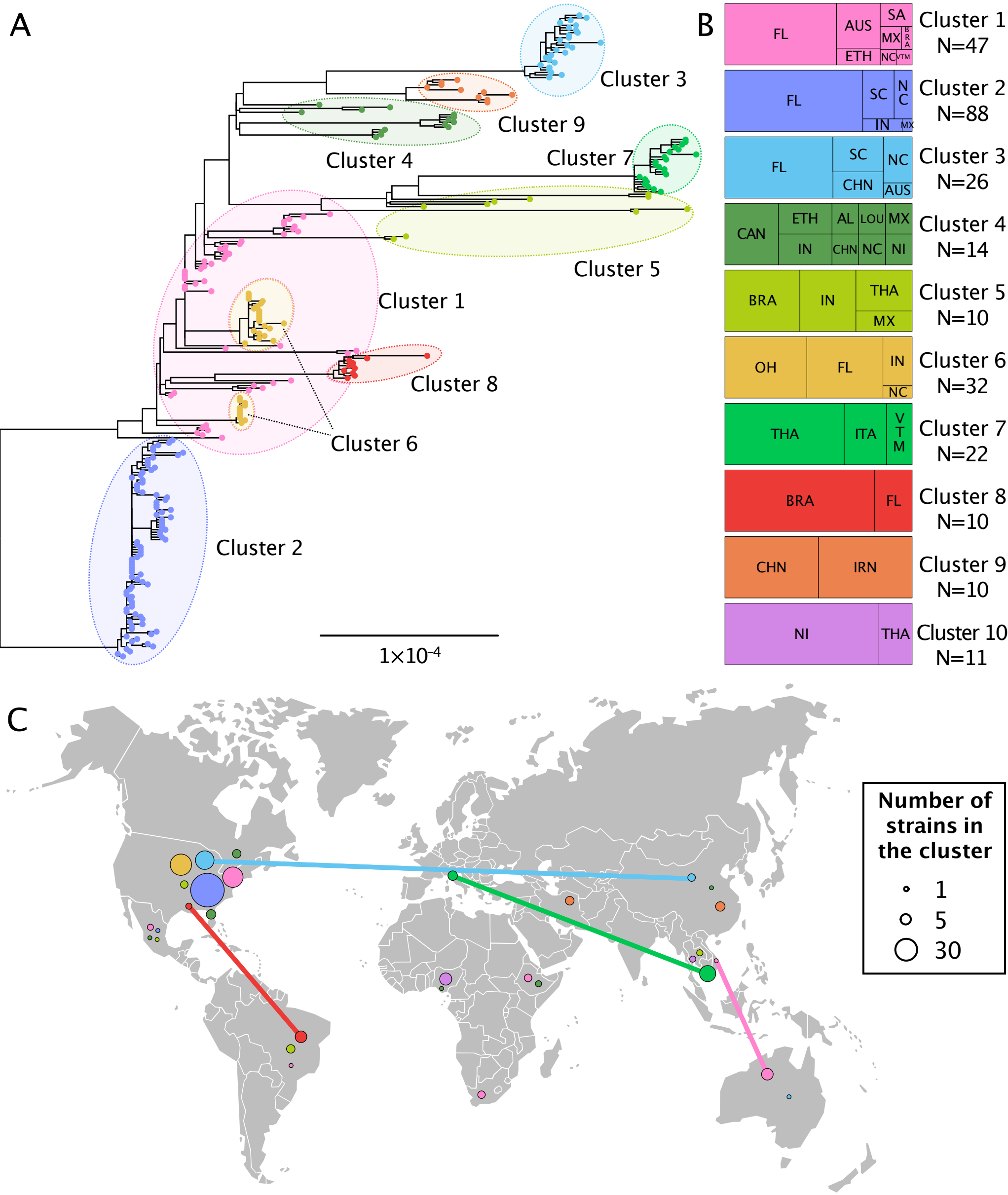
	South Carolina [43]	2016	GEV2407, GEV2408, GEV2384, GEV2388, GEV2389, GEV2390, GEV2391, GEV2392, GEV2393, GEV2396, GEV2397, GEV2399, GEV2400, GEV2403, GEV2410, GEV2420
	Ohio [53]	2017	SM-1806, SM-1807, SM-1808, SM-1809, SM-1810, SM-1811, SM-1812, SM-1813, SM-1814, SM-1815, SM-1828, SM-1829, SM-1830, SM-1831

Genomic diversity in *X. euvesicatoria* pv. *perforans*

To examine genetic diversity in the core genome, we curated a set of 887 genes that were present in all 270 *Xep* genomes based on IMG/JGI gene annotation. The aligned sequence length of concatenated core genes was 617,855 bp, which contained 14,427 polymorphic sites after removing ambiguous nucleotides and any alignment gaps (S1 Data). Maximum likelihood phylogenetic analysis showed a distinct and especially diverged lineage of 11 strains from Nigeria and Thailand (S1 Figure), that included a previously defined atypical strain – NI1 – from Nigeria [48]. Grouping strains by state within the United States and country elsewhere produced an FST [68] of 0.66. The number of core gene SNPs by geographic location represented by more than one strain ranged from 15 to 5929 (S2 Table). Nucleotide diversity (average number of differences among sequences) ranged from 3 to 1287, with both extremes in nucleotide diversity coming from the Midwestern U.S., Ohio and Indiana respectively (S2 Table). Tajima's D [69] by geographic location ranged from –2.0 to 1.7 (S2 Table).

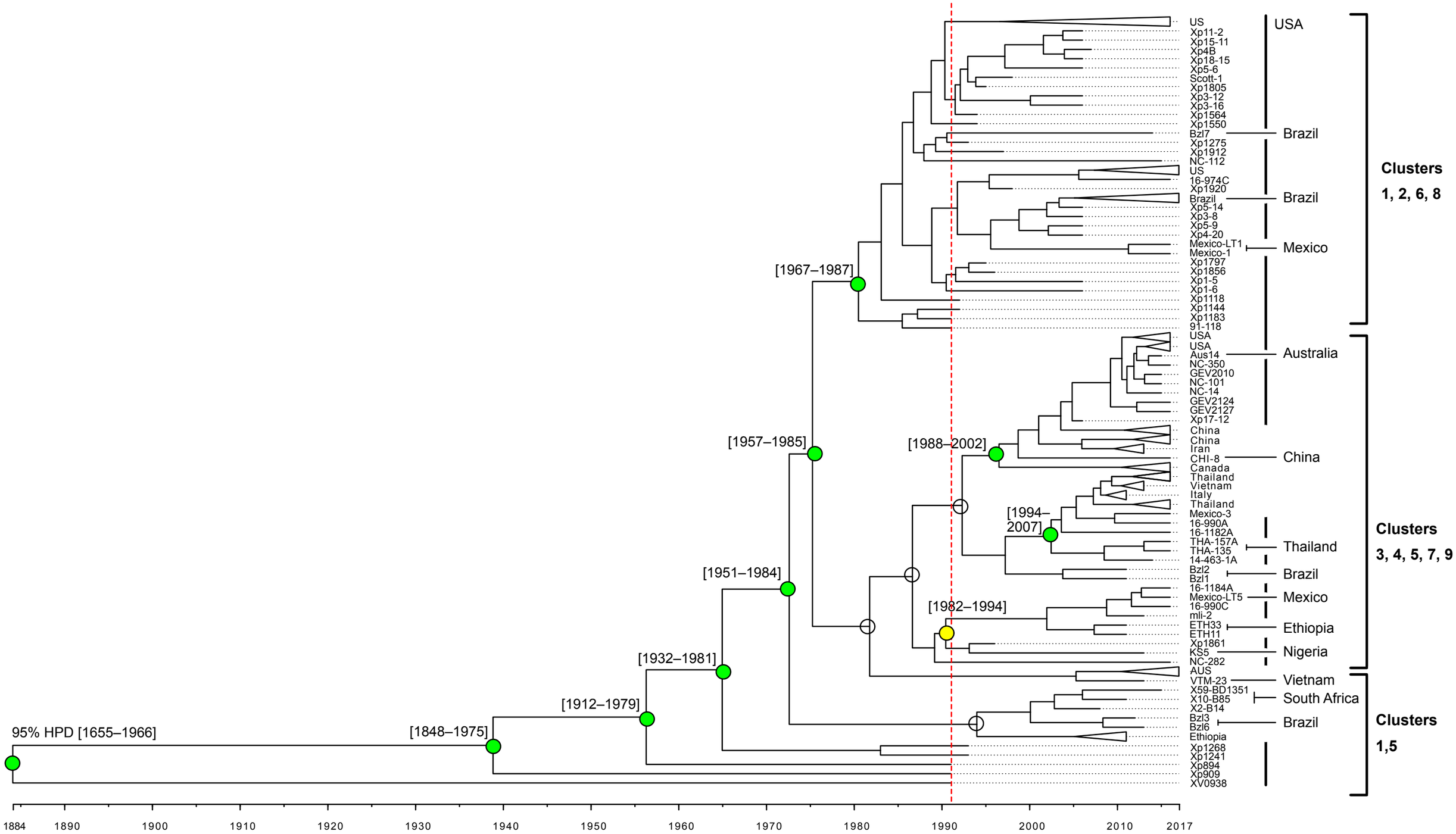
Phylogenetic analysis of core SNPs, followed by correction of branch lengths for recombination, showed diversifying lineages of *Xep* (Figure 1; S1 Figure). After excluding a particularly diverged lineage of 11 strains (S1 Figure), ClonalFrameML [70] estimated an overall ratio of recombination rate to mutation rate (R/theta) of 0.60, with recombination causing approximately seven times more base changes than mutation (delta = 231; nu = 0.05). There were an estimated 221 recombination events that

205 affected more than 96 Kbp in terminal branches and 494 recombination events detected in internal
206 branches encompassing 190 Kbp.
207
208 **Figure 1. Population structure of *Xanthomonas euvesicatoria* pv. *perforans* strains collected from**
209 **tomato production regions.** (A) Maximum likelihood phylogenetic tree of 259 *X. euvesicatoria* pv.
210 *perforans* strains constructed with nucleotide sequences from 887 core genes, corrected for recombination
211 by ClonalFrameML. Tips are colored according to clusters identified by hierBAPS. Cluster 10 strains
212 (n=11) were genetically distant and excluded from the tree (see S1 Figure). Nucleotide alignment is
213 available as S1 Data. (B) Distribution of 270 strains in each hierBAPS cluster by country or state of
214 collection. Abbreviations are as follows: AUS – Australia; BRA – Brazil; CAN – Canada; CHN – China;
215 ETH – Ethiopia; FL – Florida, USA; IN – Indiana, USA; IRN – Iran; ITA – Italy; LOU – Louisiana,
216 USA; MX – Mexico; NC – North Carolina, USA; NI – Nigeria; AL – Alabama; OH – Ohio, USA; SA –
217 South Africa; SC – South Carolina, USA; THA – Thailand; VTM – Vietnam. (C) Map showing
218 distribution of clusters by country of collection. Countries connected by lines show selected instances of
219 genetically similar strains collected in different countries. (D) Pairwise comparison of whole genome
220 average nucleotide identity (ANIb) of selected strains shows high identity between strains isolated from
221 different continents. For each comparison, genome coverage is shown by grayscale in boxes, scale shown
222 to the right. Values for each comparison are for genomes in rows when compared to genomes in columns.
223 See S3 Table for additional ANI output.
224
225 To summarize population structure based on core gene SNPs, we used hierBAPS [71], which
226 assigned individual strains to 9 clusters using allele frequencies (Figure 1; S1 Table). This analysis
227 excluded the 11 highly diverged strains from Nigeria and Thailand, which we designated as cluster 10.
228 F_{ST} among clusters was 0.80. In some cases, clusters corresponded to phylogenetic lineages, including
229 clusters 2, 3, 7, 8, and 9 (Figure 1). The remaining clusters were polyphyletic, encompassing multiple
230 diverged clades or individual strains. Nucleotide diversity within clusters ranged from 13.8 to 675.9 and


A
Cluster 3
Cluster 9
Cluster 4
Cluster 7
Cluster 5
Cluster 1
Cluster 8
Cluster 6
Cluster 2
1×10-4
B
FL
AUS
SA
MX
BRA
ETH
NC
VTM
Cluster 1
N=47
FL
SC
NC
IN
MX
Cluster 2
N=88
FL
SC
NC
CHN
AUS
Cluster 3
N=26
CAN
ETH
AL
LOU
MX
IN
CHN
NC
NI
Cluster 4
N=14
BRA
IN
THA
MX
Cluster 5
N=10
OH
FL
IN
NC
Cluster 6
N=32
THA
ITA
VTM
Cluster 7
N=22
BRA
FL
Cluster 8
N=10
CHN
IRN
Cluster 9
N=10
NI
THA
Cluster 10
N=11
C
Number of strains in the cluster
1
5
30
D
VTM-5 (cluster 7)
THA-5 (cluster 7)
1P4S1D (cluster 7)
VTM-23 (cluster 1)
Aus3 (cluster 1)
CHI-5 (cluster 3)
NC350 (cluster 3)
GEV2099 (cluster 3)
VTM-5 (cluster 7) 100.00 99.94 99.95 99.65 99.66 99.69 99.55 99.54
THA-5 (cluster 7) 99.99 100.00 99.99 99.75 99.75 99.75 99.75 99.75
1P4S1D (cluster 7) 99.99 99.99 100.00 99.73 99.74 99.74 99.75 99.74
VTM-23 (cluster 1) 99.74 99.76 99.76 100.00 99.99 99.80 99.77 99.77
Aus3 (cluster 1) 99.74 99.76 99.76 99.98 100.00 99.81 99.77 99.77
CHI-5 (cluster 3) 99.74 99.75 99.75 99.78 99.79 100.00 99.99 99.98
NC350 (cluster 3) 99.57 99.69 99.68 99.69 99.70 99.92 100.00 99.98
GEV2099 (cluster 3) 99.57 99.71 99.70 99.70 99.70 99.93 99.99 100.00
Vietnam Thailand Italy Vietnam Australia China USA USA
Coverage
100%
98%
96%
94%
92%
90%

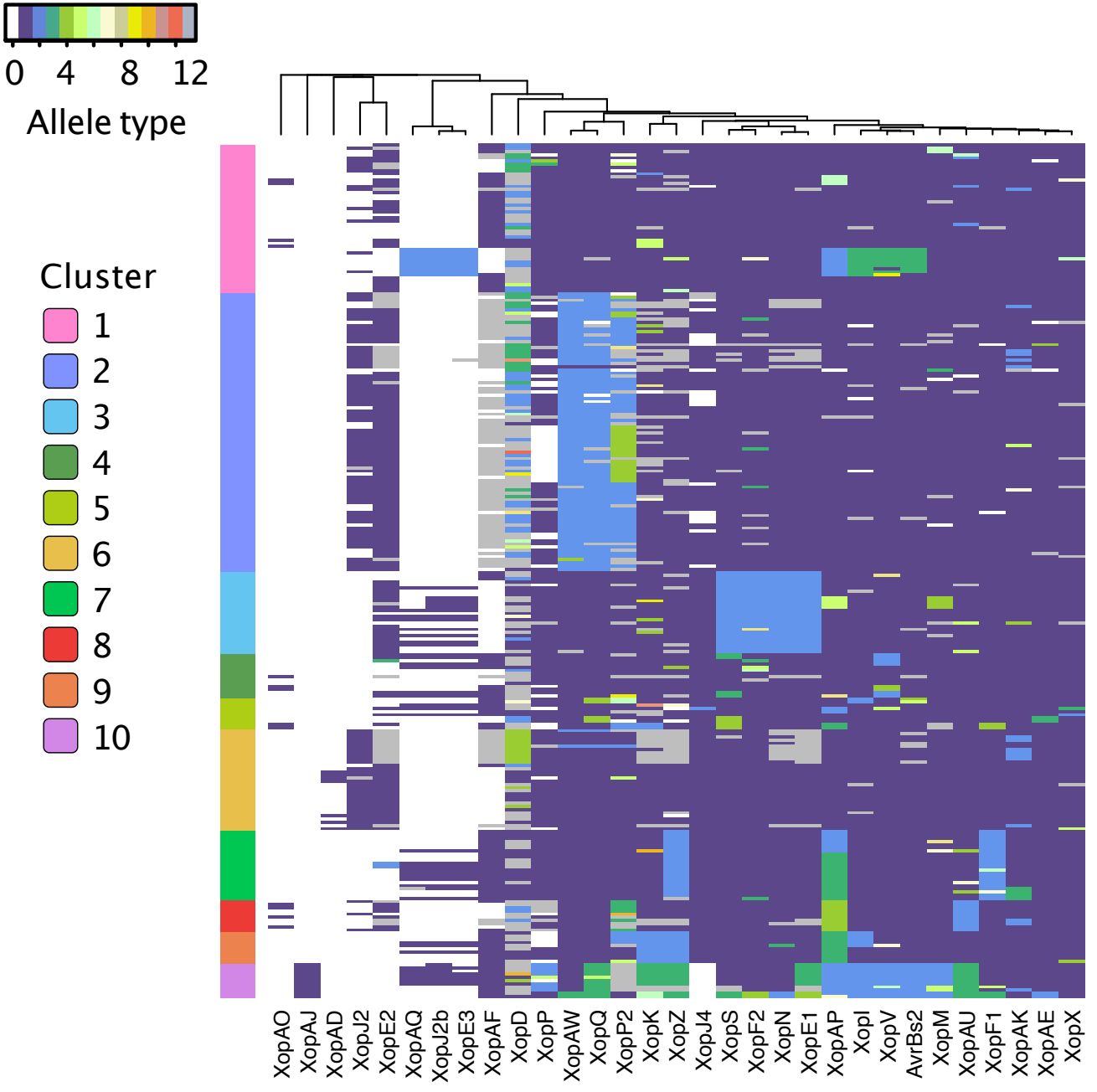
231 Tajima's D from –2.6 to 1.0. Presence-absence gene variation in the pangenome largely paralleled the
232 phylogenetic diversity of core genes in that polyphyletic clusters 1, 4, and 5 also showed the most
233 variation in gene content (S2 Figure).
234
235 Geographic distribution of *X. euvesicatoria* pv. *perforans* core gene clusters
236 Cluster 1 encompasses genetically diverse strains from seven countries, including most of the
237 strains from Australia, all four strains from South Africa, and one strain from Southeast Asia (Figure 1B).
238 All USA strains assigned to cluster 1 were isolated in or before 2006 from Florida except for one strain
239 from North Carolina. Cluster 2 contains 88 strains from the United States and one from Mexico, while
240 Cluster 3 includes strains isolated from Florida, North and South Carolina, China, and Australia. Cluster 4
241 encompasses multiple lineages of strains from the United States, Canada, Ethiopia, China, and Nigeria.
242 Cluster 5 is polyphyletic with diverged strains from three continents. Cluster 6 was isolated only within
243 the United States from Florida, Indiana, North Carolina, and Ohio. Cluster 7 is a monophyletic group of
244 strains from Southeast Asia and Italy. Cluster 8 is another monophyletic group found only in Brazil and
245 Florida. Cluster 9 includes two clades of strains, one from China and the other from Iran and Nigeria.
246 Cluster 10 comprises the atypical strains from Nigeria and similar strains from Thailand. Most countries
247 contained strains from more than one core gene cluster (Figure 1C).
248 Clusters 1, 3, 4, 5, 7, 9, and 10 contain strains isolated from both seed production and commercial
249 fruit production regions, whereas strains in clusters 2, 6, and 8 were only isolated from commercial fruit
250 production regions. Some strains found on different continents were nearly identical in core gene
251 sequences (Figure 1D) with very high average nucleotide identity. Strains in cluster 1 from Australia
252 differed by 6 to 10 SNPs in more than 617 Kbp of core gene sequence from strain VTM-23 from
253 Vietnam. Pairwise average nucleotide identity (ANIb) between VTM-23 to Aus3 was 99.99% with
254 alignment fraction of 0.998 (Figure 1D). Strains from the USA had up to 99.87 ANIb with strains from
255 Australia and Vietnam (S3 Table). A different strain from Vietnam, VTM-5 in cluster 7, had as few as
256 four SNPs in the core genome when compared to strains from Italy and ANIb of 99.95% to Italian strain

257 1P4S1D (Figure 1D). Likewise, strains collected in a seed production region in China had ANIb up to
258 99.99% with strains from Florida and North Carolina. We also found similar strains between Brazil and
259 USA, for example Bzl-10 (Minas Gerais) and Xp3-8 (Florida) had greater than 99.9% ANI (S3 Table).
260 Other strains were similar between countries in core genes only after correction for recombination.
261
262 Timing of *X. euvesicatoria* pv. *perforans* lineage emergence
263 We used the years of strain collection to estimate the timing of diversification of our sample of
264 *Xep*, excluding the cluster 10 strains. We inferred dated phylogenies using whole genome alignments with
265 inferred recombinant sites removed by Gubbins [72]. Due to recombination with other *X. euvesicatoria*
266 lineages, we did not include an outgroup (S1 Figure, part B; [48]). Sampling year was significantly
267 correlated with root-to-tip distance ($R^2 = 0.20$ for the whole genome alignment, $P < 1\times10^{-4}$, S3 Figure).
268 The root inferred by the BactDating R package [73] was placed between strains isolated in Florida in
269 1991. The most recent common ancestor (MRCA) of all strains was dated to 1884 (95% HPD: 1655–
270 1966). Notably, strains that were isolated in the early 1990s, when *Xep* was first detected in U.S. tomato
271 production [32, 37], represented multiple lineages (Figure 2). The MRCA of the clade representing core
272 gene clusters 1, 2, 6, and 8 (including strains from USA, Brazil, and Mexico) was dated to 1980 (95%
273 HPD: 1967–1987). A major clade, encompassing strains in clusters 3, 4, 5, 7, 9, which were collected in
274 Africa, the Americas, Asia, Australia, and Europe, did not have a significant temporal signal across the
275 clade. We repeated the analysis with BEAST, which inferred a different rooting. The tree inferred by
276 BEAST placed the root between two strains isolated in 2011 from Brazil and all other strains (S4 Figure,
277 part B). The MCRA of the BEAST tree was dated to 1868 (95% HPD: 1862–1919), which was similar to
278 the root date estimated using BactDating (S4 Figure).
279
280 **Figure 2. Dated phylogeny of 259 *X. euvesicatoria* pv. *perforans* strains.** BactDating analysis estimated
281 an approximately 130-year history for *Xep* strains in core gene clusters 1 through 9 (Figure 1). Red dotted
282 line indicates the first documented isolations in 1991. Internal nodes were collapsed for clades containing


US
Xp11-2
Xp15-11
Xp4B
Xp18-15
Xp5-6
Scott-1
Xp1805
Xp3-12
Xp3-16
Xp1564
Xp1550
Bzl7
Xp1275
Xp1912
NC-112
US
16-974C
Xp1920
Brazil
Xp5-14
Xp3-8
Xp5-9
Xp4-20
Mexico-LT1
Mexico-1
Xp1797
Xp1856
Xp1-5
Xp1-6
Xp1118
Xp1144
Xp1183
91-118
USA
USA
Aus14
NC-350
GEV2010
NC-101
NC-14
GEV2124
GEV2127
Xp17-12
China
China
Iran
CHI-8
Canada
Thailand
Vietnam
Italy
Thailand
Mexico-3
16-990A
16-1182A
THA-157A
THA-135
14-463-1A
Bzl2
Bzl1
16-1184A
Mexico-LT5
16-990C
mli-2
ETH33
ETH11
Xp1861
KS5
NC-282
AUS
VTM-23
X59-BD1351
X10-B85
X2-B14
Bzl3
Bzl6
Ethiopia
Xp1268
Xp1241
Xp894
Xp909
XV0938
USA
Brazil
Brazil
Mexico
Australia
China
Thailand
Brazil
Mexico
Ethiopia
Nigeria
Vietnam
South Africa
Brazil
Clusters
1, 2, 6, 8
Clusters
3, 4, 5, 7, 9
Clusters
1,5
[1967–1987]
[1957–1985]
[1988–2002]
[1994–
2007]
[1951–1984]
[1982–1994]
[1932–1981]
[1912–1979]
[1848–1975]
95% HPD [1655–1966]
1884
1890
1900
1910
1920
1930
1940
1950
1960
1970
1980
1990
2000
2010
2017

283 strains from a single country with branch tips indicating country or strain (for full tree see S4 Figure).
284 Vertical line to the right of tip labels indicates strains from USA; other countries are labeled. Temporal
285 signal was assessed using Phylostems and results are shown for major nodes (for full results see S3
286 Figure). Empty circles indicate no significant temporal signal. Colored circles indicate nodes with
287 statistically significant temporal signal based on adjusted R^2 values: green – 0.13-0.19; yellow – 0.45. The
288 95% highest posterior density (95% HPD) of date estimates for major nodes with significant temporal
289 signals are shown in brackets.
290
291 Type III effector content
292 We detected 32 predicted type III effectors in our collection of 270 strains (S5 Figure, S4 Table).
293 The diversity in amino acid sequences of predicted effectors ranged widely from a single conserved allele
294 to 8 or more alleles per locus (Figure 3). None of the effectors were present and intact in 100% of our
295 genomes, in part due to our analysis of draft genomes. The following effector genes were present in more
296 than 95% of strains and can be considered "core effectors": *avrBs2*, *xopF1*, *xopF2*, *xopI*, *xopM*, *xopQ*,
297 *xopS*, *xopV*, *xopX*, *xopAE*, *xopAK*, *xopAP*, *xopAU*, and *xopAW*. The genes for *xopD*, *xopE1*, and *xopN*
298 were present in some form in all genomes but more than 5% of strains contained a contig break within the
299 gene. A closer examination of *xopD* by PCR and Sanger sequencing showed this to be an assembly issue
300 due to the repeats within the gene. Effectors at low frequency in our *Xep* strains (<25%) were *xopE3*,
301 *xopAD*, *xopAJ*, *xopAO*, and *xopAQ*. Transcription activator-like (TAL) effectors typically do not assemble
302 in draft genomes due to their characteristic repeat sequences, but there were BLAST hits to previously
303 described TAL effectors in 65 strains. We Sanger sequenced the TAL effector gene in strain 2P6S1
304 collected in Italy (NCBI accession number OQ588696), which confirmed that it had the same repeat
305 variable diresidues as PthXp1 reported in *Xep* strains from Alabama [50]. Previous phenotyping and
306 sequencing indicated that the strain isolated in Louisiana, USA origin has AvrHah1 [16].
307

308 **Figure 3. Variation in type III effectors (Xop proteins) in *Xanthomonas perforans*.** Type III effectors
309 are in columns and 270 *X. euvesicatoria* pv. *perforans* strain in rows. Effector status is shown by allele
310 type: absence is indicated by allele type 0 (white), while the most frequent allele observed when the
311 effector is present is allele type 1 (purple), second most frequent is allele type 2 (blue), and so on. Putative
312 pseudogenized effectors are shown as allele 13 (gray). The order of columns was determined by
313 hierarchical clustering analysis, placing similarly distributed effectors adjacent to each other. Genomes
314 showing BLAST hits to TAL effector(s) are indicated in Supporting Information Table S3 and not shown
315 in heatmap. *X. euvesicatoria* pv. *perforans* strains (rows) are organized by core gene cluster.
316
317 The T3E effector XopAF (AvrXv3), which is targeted by the tomato resistance gene *Xv3* [74],
318 was missing or pseudogenized in 64% of strains. Most strains examined from the United States did not
319 have a complete copy of this gene, whereas it was intact in many strains collected in Asia and Africa. The
320 gene for XopJ4 (AvrXv4), recognized by resistance gene *RXopJ4* from *S. pennellii* [75], was present in
321 88% of strains and absent in all cluster 10 strains and 19 of 88 cluster 2 strains. XopJ2 (AvrBsT), which
322 elicits an HR in pepper but increases virulence in tomato [49], was present in less than half of strains
323 examined (43%) and overwhelmingly in strains from the United States. A homolog of XopJ2, recently
324 designated XopJ2b [76], was present in 50 strains, including two strains from Australia that carried both
325 copies of XopJ2 (S4 Table).
326 We tested for evidence of positive selection in T3E by estimating synonymous and non-
327 synonymous (dN/dS) substitution rates using a Bayesian approach for detecting pervasive selection
328 (FUBAR, [77]) and maximum likelihood approach for detecting episodic selection (MEME, [78]). We
329 found evidence of pervasive positive selection affecting at least one amino acid in AvrBs2, XopD,
330 XopE1, XopF2, XopK, XopM, XopP and its paralog XopP2, XopQ, XopS, and XopAQ (S4 Table). We
331 found evidence of episodic selection affecting at least one amino acid in XopF2, XopK, XopP, XopP2,
332 XopQ, XopV, and XopAP (S4 Table).


0
4
8
12
Allele type
Cluster
1
2
3
4
5
6
7
8
9
10
XopAO
XopAJ
XopAD
XopJ2
XopE2
XopAQ
XopJ2b
XopE3
XopAF
XopD
XopP
XopAW
XopQ
XopP2
XopK
XopZ
XopJ4
XopS
XopF2
XopN
XopE1
XopAP
XopI
XopV
AvrBs2
XopM
XopAU
XopF1
XopAK
XopAE
XopX

333 We defined the effector profile of each strain as the predicted presence or absence of each
334 effector and its allelic state, excluding TAL effector hits. Grouping effector profiles according to core
335 gene cluster revealed that allelic variation of effectors often paralleled core genome variation (Figure 3).
336 For example, specific alleles of effectors XopAW, XopQ, and XopP2 were mostly limited to strains in
337 cluster 2. Cluster 3 strains carried unique alleles for effectors XopF2, XopS, XopN, and XopE1, and
338 strains from cluster 7 shared unique alleles for effectors XopF1 and XopZ. Strains from highly diverged
339 cluster 10 had rare alleles in many effectors, and it was the only cluster in which effector XopAJ was
340 found (Figure 3). To visualize variation among strain effector profiles independent of core gene clusters,
341 we transformed dissimilarities between profiles into distances represented in a two-dimensional plot and
342 defined eight effector profile clusters (S6 Figure, part A). A lack of low frequency effectors characterized
343 effector cluster A, containing 188 strains from 11 of 13 countries (S6 Figure). The remaining effector
344 clusters were defined by the presence of one to three low frequency effectors (S7 Figure). While most
345 effectors were found in multiple countries and continents (S6 Figure), populations in Brazil, Ethiopia,
346 Nigeria, Thailand, South Africa, and the United States contained low frequency effectors that were not
347 widely distributed.
348
349 Copper resistance genes
350 Xanthomonads, including *Xep*, have acquired genes conferring copper tolerance, likely in
351 response to exposure to copper-based bactericides [30, 79-81]. In *Xep*, copper tolerance is conferred by an
352 operon containing the copper resistance genes *copA* and *copB*, and regulator *copL* (*copLAB*) [80]. BLAST
353 analysis showed that these genes were present in 73% of the genomes in our sample (S5 Table). Copper
354 resistance genes are prevalent in the USA; only the genomes from strains isolated from Florida in the
355 early 1990s and a strain from Louisiana lacked *copLAB*. The genes were also missing in the genomes of a
356 few strains from Australia (1), Brazil (2), Ethiopia (2), Mexico (1), and Vietnam (2). In contrast, the
357 genes were absent in all genomes of all strains from Nigeria, China, Iran, Italy, and Thailand.
358

359 **Discussion**

360 Emerging plant pathogens have the potential for global outbreaks, exacerbated by complex trade
361 networks. Hybrid tomato production relies on international breeding and production chains with a global
362 network to deliver seeds to growers. Global trade associated with vegetable seed production provides a
363 pathway for global spread of pathogens, with quantities traded that challenge even strong phytosanitary
364 measures [82, 83]. Over 100 countries import seeds of tomatoes and other vegetables; for example, 11.7
365 million kg of vegetable seed were imported to the USA in 2019, with China being the biggest supplier at
366 2.4 million kg [84]. *Xanthomonas* species can infest pepper and tomato seed [85], and *Xep* has been
367 isolated from tomato seed [32, 86], supporting the hypothesis that seeds can be a source of inoculum for
368 bacterial spot outbreaks [87, 88]. Thirty years after its first report, *Xep* has been identified in tomato
369 production areas around the world [18]. Our results showed extensive genetic diversity in the pathogen,
370 but also genetically similar strains in distant tomato production regions. Furthermore, we found
371 genetically similar strains in seed production and fruit production regions on different continents, as
372 would be expected if the pathogen was being moved in shared production chains. Dated phylogenies
373 indicate multiple waves of diversification of the *Xep* population, before and since its first detection in
374 1991. Variation in gene content confirms that *Xep* acquired and lost type III effectors during its
375 diversification, which will continue to challenge sustainable management of tomato bacterial leaf spot
376 [49, 66].

377 Using our broad strain collection, we found *Xep* variants in seed production regions in Asia that
378 were previously reported in Australia, Italy, Nigeria, and the United States [43, 47, 48, 57]. Strains from
379 Italy were nearly identical in core genes and very similar in accessory genomes to strains collected from
380 Thailand and Vietnam, both major seed production regions. The atypical bacterial spot strains from
381 Nigeria, recently designated as race T5 [21], were genetically similar to strains from Thailand. A recently
382 described variant of *Xep* in Florida [cluster 3; [43, 47]], which was also found in Australia [57], was
383 similar in the core genome to strains found in China; however, these strains showed divergence in the
384 pangenome, consistent with accessory genome evolution in emergent populations. Beyond previously

385 described variants, we found strains in Iran that were closely related to strains from China; multiple
386 instances of genetic similarity between strains from North America and Ethiopia; and highly similar
387 strains shared between USA and Brazil, USA and Australia, and between Australia and Vietnam. Given
388 the variation of *Xep* across our sample, genetic similarity in core genes and gene content across continents
389 is strong evidence of international dissemination. Genetically similar strains of bacterial spot pathogens *X.*
390 *euvesicatoria* pv. *euvesicatoria* and *X. hortorum* pv. *gardneri* collected from different continents similarly
391 suggest intercontinental dissemination in tomato and pepper seed [15, 58, 89]. Whole genome analysis of
392 *X. hortorum* pv. *pelargonii* strains from a 2022 epidemic of bacterial blight of geranium in the USA
393 showed zero to seven chromosomal SNPs among isolates of the emergent strain that was distributed to
394 multiple states in plant cuttings [9, 90].

395 Other *Xep* genotypes indicated a more limited distribution. We did not find core gene cluster 2
396 strains in the seed production regions sampled (China, Thailand, Vietnam), while this lineage was highly
397 represented in our USA sample. To date, strains in this cluster have been found only in the southeastern
398 and midwestern USA [43, 47, 50, 53, 54] and Mexico. Seedling nurseries in the southeast USA produce
399 tomato transplants for growers in multiple states. Interstate movement of strains on seedlings is likely
400 responsible, at least in part, for disseminating genetically similar strains to different states [43, 53]. We
401 previously reported extensive recombination with *X. euvesicatoria* pv. *euvesicatoria* in cluster 2 strains
402 [47] and this cluster had a diverse accessory genome, perhaps suggesting that it has a larger geographic
403 distribution than represented in our sample, which is biased towards the USA.

404 The *Xep* strains we examined from the USA were assigned to core gene clusters 1, 2, 3, 4, 5, 6,
405 and 8, representing several distinct genetic lineages. To better understand the initial emergence of *Xep*, we
406 used calibrated phylogenies to examine the timing of lineage divergence. International trade in F1 hybrid
407 tomato seed surged in the second half of the 20th century, after the first hybrid tomato cultivars were
408 released by 1940 [91, 92]. There was a 300-fold increase in hybrid tomato seeds exported from Asia
409 between 1962 and 1977 [93, 94] and subsequent rapid growth in tomato production. Our analyses
410 estimate the most recent common ancestor of our sample to ~150 years ago, while the major ancestral

411 lineages diverged during or after the early expansion in the hybrid seed trade. We hypothesize that the
412 emergence and geographic distribution of lineages may be associated with the multinational structure of
413 tomato breeding and seed production, in which parental lines and geographic locations of seed production
414 change over time [95].

415 The roles of T3E in pathogenicity and virulence make them important members of both the core
416 and accessory genomes. We found up to 16 putative core effector genes, most of which exhibited allelic
417 variation. The impact of allelic variation in *Xep* effectors on pathogen fitness, if any, is unknown. Low
418 frequency effectors were found across core gene clusters, suggesting acquisition of new effectors and
419 their exchange among *Xep* lineages. For example, some strains in clusters 3, 4, and 5 from the United
420 States, Canada, and Mexico carried the same alleles of low frequency effectors XopAQ and XopE3 as
421 strains from Asia, Nigeria, and Italy. BLAST analysis suggested the geographically widespread presence
422 of transcription activation-like (TAL) effectors in *Xep*. Both TAL effectors described in *Xep*, *avrHah1*
423 and *pthXp1*, are associated with increased disease severity on tomato [16, 50]. Acquisition of T3Es could
424 increase the fitness of *Xep* relative to other bacterial spot pathogens and cause more damaging disease
425 outbreaks [49, 51, 66].

426 The release of new plant varieties that carry disease resistance genes can have dramatic effects on
427 pathogen population structure due to selection to overcome host resistance [96-98], and we have
428 previously reported on the loss of function of effector AvrXv3 (XopAF) across lineages [27, 53, 66].
429 Examination of T3E content at a global scale puts variation previously observed in Florida into a larger
430 context. XopAF was present in strains collected in the 1990s (cluster 1), but absent or non-functional in
431 most strains from Florida, Indiana Ohio, and North Carolina, USA [23, 25, 27, 53, 66]. Here, we found
432 that *xopAF* was intact in many strains from seed production areas, suggesting strong selection for loss of
433 function in commercial fruit production. The effector XopJ4 is a possible resistance target [66], but Klein-
434 Gordon et al. [23] reported that it was missing from 3.2% of Florida strains collected in 2017 and, here,
435 we found that it was absent in one North Carolina and 20 Florida, USA strains. All strains collected
436 outside the USA contained *xopJ4*, except for cluster 10 strains. Another XopJ family member, *xopJ2*, is a

437 virulence factor in tomato [49, 51]. It is common in North America, particularly in cluster 2 and 6 strains,
438 but absent or infrequently detected in *Xep* populations elsewhere. An alternative form of this effector,
439 recently described as XopJ2b [76], is more common in strains from outside North America.

440 Bacterial spot is a destructive disease in areas where tomatoes are grown under humid conditions
441 and growers in the USA have relied heavily on copper bactericides to manage this disease. In response,
442 *Xep* strains have developed copper tolerance [99]. Most strains isolated from Florida in the 1990s lacked
443 the *copLAB* genes, but they are now common in strains collected in the USA. A recent study of Florida
444 strains found that these copper resistance genes are more frequently present on the chromosome than on a
445 plasmid, suggesting selection for vertical inheritance of copper tolerance [30]. In contrast, strains from
446 other countries lacked copper resistance genes, indicating little or no local selection for the acquisition of
447 *cop* genes.

448 In summary, we found strong evidence for intercontinental movement of *Xep*, consistent with the
449 international nature of tomato breeding and hybrid tomato seed production. We also found notable
450 diversity in our global sample of *Xep*, including in seed production regions, and multiple variants of *Xep*
451 that do not appear to be widely distributed. The genomic diversity of *Xep* in seed and fruit production
452 regions creates the opportunity for recombination among strains and subsequent dissemination of high
453 fitness variants of *Xep*.

454

455

456 **Materials and Methods**

457 Bacterial strains, genome sequencing, and assembly

458 *Xep* strains were collected from 13 different countries (Table 1; S1 Table). Strains from the
459 United States were collected from seven states between 1991 to 2016 and comprised 181 strains. The
460 remaining 89 strains were collected from Canada, Mexico, Brazil, Italy, Ethiopia, Nigeria, South Africa,
461 Iran, China, Thailand, Australia, and Vietnam. Strains from China, Thailand, and Vietnam were collected
462 from fields designated for production of tomato seed for the global market. Strains from Brazil were

463 obtained from both staked fresh-market and processing tomato commercial fields. Strains from Italy were
464 isolated from tomato pith in greenhouse tomato showing wilting symptoms [64, 100]. Strains from South
465 Africa were collected from commercial seed lots. Strains from Nigeria were obtained from fields
466 cultivated for both subsistence and commercial purposes. Strains from other countries were collected
467 from fields designated for commercial fruit production.

468 A total of 270 *Xep* genome sequences were used during this study (S1 Table). Draft and whole
469 genomes of 117 strains were generated and published previously [43, 47, 48, 57, 58, 67, 100]. The
470 remaining 153 strains were sequenced for this study using Illumina platforms. Genomic DNA was
471 extracted from single colony cultures grown for 24-hr in nutrient broth using the Wizard Genomic DNA
472 Purification Kit (Promega, Chicago, IL) following manufacturer instructions. Genomic libraries for
473 sequencing were prepared using the Nextera DNA library preparation kit from Illumina (Illumina, San
474 Diego, CA). Sequencing was performed at the Interdisciplinary Center for Biotechnology Research,
475 University of Florida, using an Illumina MiSeq to generate 250 bp paired end reads for each strain.
476 Additional genomic sequence data were generated for five strains for the ANI analysis (S1 Table, part B).
477 Genomic DNA was extracted using the above methods except that extracted genomic DNA was sent to
478 SeqCenter (Pittsburg, PA) for sequencing with Illumina NovaSeq 6000, producing 150 bp paired end
479 reads.

480 Raw reads were trimmed of adapters and paired with Trim Galore
481 (https://github.com/FelixKrueger/TrimGalore)[101], then assembled into contigs with Spades version
482 3.10.1 [102], with k-mers 21, 33, 55, 77, 99, and 127 with read error correction and "--careful" switch.
483 Reads were then aligned to the assembled contigs using Bowtie 2 v. 2.3.3 [103]. Inconsistencies were
484 identified and polished using Pilon [104]. Contigs smaller than 500 bp and with less than 2.0 k-mer
485 coverage were filtered out. Quality of genomes were assessed with CheckM [105]. Assembled genomes
486 were annotated using the IMG/JGI platform [106]. The genome data generated for this study are available
487 in NCBI BioProject PRJNA941448.
488

489 Core gene phylogeny

490 In a previous study, we defined a set of 1,356 'core genes' from 58 genomes of *Xep* strains
491 isolated from Florida [47]. The core genes were determined based on amino acid sequence homology
492 using GET_HOMOLOGUES software package [107]. We used the core genes from a representative *Xep*
493 genome, Xp91-118, as query to search the remaining 269 genomes using local BLAST [108]. BLAST
494 results were filtered using query coverage and pairwise nucleotide sequence alignment thresholds of 70%
495 each and the sequence was checked for the presence of standard start and stop codons at either end of the
496 gene and gene was removed if both were not present. A total of 887 genes were found to be intact in all
497 270 genomes. Genes were individually parsed and aligned using MAFFT [109] and concatenated using
498 sequence matrix [110]. The result was a 617.854 Kbp alignment, hereafter referred to as core genes.

499 The concatenated core gene sequence was used to construct a maximum likelihood (ML)
500 phylogenetic tree using RAxML v.8.2.12 [111]. General time reversible model with gamma distributed
501 rates and invariant sites (GTRGAMMA) was used as the nucleotide substitution model. To account for
502 recombination, the ML tree output from RAxML and concatenated core genome alignment were used as
503 was input for ClonalFrameML v1.12 [70].

504

505 Population structure

506 SNPs were extracted from core genes for hierarchical clustering based on Bayesian analysis of
507 population structure (hierBAPS) algorithm [112], implemented in the 'rhierBAPS' R package v 1.0.1 [71,
508 113]. For visualization, hierBAPS clusters were added to the phylogenetic tree generated from
509 ClonalFrameML using the 'ggtree' package in R [114]. The treemap function in plotly [115] was used to
510 show the relative distribution of clusters across geographic locations. R package 'ggplot2' was used to
511 map hierBAPS clusters to countries [116]. The 'PopGenome' R package [117] was used to calculate FST,
512 nucleotide diversity (pi), and Tajima's D statistic by geographic location and by hierBAPS cluster.

513 Assembled genomes were used for calculating average nucleotide identity and pangenome
514 analysis. Average nucleotide identity (ANIb) between strains was calculated using assembled genomes

515 with Pyani version 0.2.10 [118]. The pangenome was estimated using Roary v3.12.0 [119] after
516 annotation from Prokka v1.12 [120]. The gene presence absence matrix from Roary (S4 Data) was used
517 as input for generation of NMDS plots using the 'dplyr' and 'ggplot2' packages from tidyverse [116] and
518 to generate gene accumulation curves for each cluster using package 'micropan' [121].
519
520 Bayesian analysis of *X. euvesicatoria* pv. *perforans* divergence times
521 A whole genome alignment was generated using split k-mer analysis version 2 (SKA2) [122] for
522 all 270 *Xep* strains plus outgroup *X. euvesicatoria* pv. *euvesicatoria* strain 85-10 (NCBI Accession
523 GCA_000009165.1; S2 Data). The alignment was reduced to variable sites only using Geneious 2023.2.1
524 (BioMatters Ltd.) A phylogenetic network was calculated from the resulting SNPs using the NeighborNet
525 2004 algorithm in SplitsTree5 [123, 124]. Phylogenetic conflict was indicated between the 259 strains,
526 cluster 10 strains, and outgroup (S1 Figure, part B). Removing the cluster 10 strains did not remove the
527 conflict between *Xep* and *Xee* outgroup. As a result, we limited our dating of the phylogeny of *Xep* to the
528 259 strains in BAPS clusters 1 through 9. We used Gubbins v. 2.4.1 [72] to remove putative recombinant
529 sites from whole genome alignments generated using SKA2 [122] and the complete genome of Xp91-118
530 as a reference (GCF_000192045.2). The resulting alignment was used to infer a phylogenetic tree using
531 the GTRGAMMI model in RAxML version 8.2.10 [125]. The temporal analysis was conducted with
532 BactDating v1.1.1 [73]. The inputs to the BactDating analysis were the maximum likelihood tree and
533 dates of isolation assigned as dates of tips. The rooting of the tree was estimated using the initRoot
534 function, which maximizes the correlation between tip date, the year the strain was collected, and root-to-
535 tip branch lengths. Dates of nodes were inferred using the bactdate function on the re-rooted tree using a
536 relaxed molecular clock with Markov chain Monte Carlo (MCMC) chains of 10^6 iterations. Phylostems
537 [126] was used to assess the temporal signals within internal clades for interpretation of node date
538 inferences.
539 We also used BEAST v. 1.10.4 (Suchard et al. 2018) to infer a dated phylogeny. The XML file
540 was manually edited to include the 'ascertained' flag in the alignment block (S3 Data). The HKY

541 nucleotide substitution model with empirical base frequencies and gamma distribution of site-specific rate
542 heterogeneity was used with coalescent Bayesian skyline priors with an uncorrelated relaxed clock for
543 Bayesian phylogenetic inference over MCMC chains of 200 million generations. Adequate mixing was
544 assessed based on a minimum effective sample size of 200 for parameter estimates as calculated by
545 Tracer v. 1.10.4. A maximum clade credibility tree was inferred from the posterior distribution of trees
546 using TreeAnnotator v. 1.10.4, specifying a burn-in of 10% and the 'keep' option for node heights. Trees
547 were visualized in iTOL version 6.9.1 [127].
548
549 Type III Effector Analysis
550 A T3E effector database was curated using amino acid sequences of 66 *Xanthomonas* effectors
551 [128] (S6 Table). When available, functional annotations were retrieved from NCBI and Pfam databases
552 [129]. Orthologous sequences were identified with the software BLASTp [130, 131], by querying the
553 curated effectors database against the amino acid sequences of the annotated genomes of 270 *Xep* strains.
554 Sequences (BLAST hits) were considered effector orthologs when at or above a threshold of 70 percent
555 identity and 50 percent query coverage. When multiple sequences from the same strain had hits above the
556 thresholds to a particular effector, a weighted calculation of the identity and coverage was used to select
557 the best hit. Sequences with homology to multiple effectors and sequences with evidence of contig breaks
558 were manually removed. Assignment of sequences as effector orthologues was confirmed by performing
559 a clustering analysis of all sequences using the software USEARCH v. 11.0.667 and the algorithm HPC-
560 CLUST [132]. For the duplicated effector XopP, we used a phylogenetic analysis of all sequences to
561 distinguish likely orthologous alleles from the more genetically distant paralogous sequences, which were
562 assigned to XopP2.
563 Orthologous sequences from each effector were extracted from the annotated genomes, aligned
564 with MAFFT [109], and allelic variants identified [133] to generate a numeric matrix representing
565 presence and allelic variant or absence. Hierarchical clustering analysis of effectors was performed by
566 calculating a distance matrix with function 'dist' with the method 'manhattan', and the function 'hclust'

567 with the method ‘complete’ from the R package ‘vegan’ [113, 134]. The results were displayed as a
568 heatmap with the package ‘gplots’ and the function heatmap.2 [135].
569 To investigate the presence of positive selection acting on the effector sequences, we used the
570 software HyPhy (Hypothesis Testing using Phylogenies) implementing the methods FUBAR (Fast,
571 Unconstrained Bayesian AprRoximation) and MEME (Mixed Effects Model of Evolution) [77, 78]. The
572 Bayesian method FUBAR evaluates pervasive selection, assuming the same rates of synonymous and
573 nonsynonymous substitution per site on all branches. The method MEME uses a maximum likelihood
574 approach to evaluate episodic selection, i.e., selection only a subset of branches of the phylogeny. For
575 each effector gene, a codon-aware alignment was generated with the software PRANK using the codon
576 flag ‘-c’ as settings [136]. RAxML [111] was used to infer a phylogenetic tree with the GTRGAMMA
577 (gamma time-reversible) model of nucleotide substitution. The codon-aware alignment and phylogenetic
578 tree were used as the input files for FUBAR and MEME.
579 To determine the relationship of the effector profiles with respect to core gene cluster, geographic
580 and temporal distribution, we transformed the dissimilarities in the matrix of effector profiles into
581 distances with non-metric multidimensional scaling (NMDS). We used the Bray-Curtis dissimilarity
582 index, a robust index able to handle missing data that considers the presence and absence of effectors as
583 equally informative, calculated with the package ‘vegan’ and the function ‘metaMDS’ [134]. We used a
584 low number of dimensions (K=2) and set try=30 and trymax=500 for random starts to avoid the NMDS
585 getting trapped in local optima. NMDS plots were created with the packages ‘ggrepel’ and ‘ggplot2’ [137,
586 138]. Based on the NMDS analysis, we assigned strains to effector clusters, which were plotted on a
587 worldwide map with the packages ‘ggplot2’ and ‘scatterpie’ [138, 139]. The map was created in R with
588 the packages ‘cowplot’, ‘ggrepel’, ‘ggspatial’, ‘libwgeom’, ‘sf’, ‘rgeos’, ‘memisc’, ‘oz’, ‘maptools’ and
589 ‘rnaturalearth’ with the function ‘ne_countries’ [137, 140-147]. Geographic coordinates (longitude,
590 latitude) of countries and states (for USA) of collection were obtained with the R package ‘googleway’
591 [148] and the function ‘mutate_geocode’ from Google maps.

592 To sequence the putative TAL effector from 2P6S1, native plasmid DNA was isolated using the
593 alkaline lysis method [149]. *Eco*RI digested DNA of the plasmid prep was ligated into vector pLAFR3
594 [150] restricted with the same enzyme for transformation into *E. coli* DH5α. Clones containing the TAL
595 effector were identified by PCR and analyzed by restriction digest. One clone, designated as p7.1,
596 contained an approx. 5 Kbp *Eco*RI fragment and was selected for Sanger sequencing and phenotype
597 testing. For Sanger sequencing of the TAL repeat region, DNA of p7.1 was restricted with *Nsi*I and the
598 internal fragment was ligated into vector pBluescript restricted with *Pst*I. Additional pBluescript
599 subclones were made using *Bam*HI (~3 Kbp and ~1.1 Kbp) and *Bam*HI/*Eco*RI (~1 Kbp) in order to cover
600 the entire cloned region in p7.1. All clones were transformed into DH5α for sequencing using vector
601 primers T3 and T7.
602 Copper resistance genes in assembled genomes were identified with BLASTn analysis using *copL*
603 (MBZ2440241.1), *copA* (MBZ2440240.1), and *copB* (MBZ2440239.1)
604 from *Xep* strain Xp2010 as reference sequences [30].
605
606 **Acknowledgements**
607 We thank D. Ritchie and C. Mauney for providing reference strains and collecting disease samples,
608 respectively. Tina Simonton and Phyllis May provided assistance with strain collection in Ontario. This
609 research was supported by USDA NIFA awards 2015-51181-24312, 2020-67013-31921, and 2022-
610 51181-38242, Ontario Agri-Food Innovation Alliance, Ontario Tomato Research Institute, and the Fresh
611 Vegetable Growers of Ontario, the North Carolina Tomato Growers Association. Plant diagnostic
612 laboratories receive funding from USDA-NIFA through the National Plant Diagnostic Network. FIB was
613 supported by Conacyt Mexico. MK was supported by Swedish International Development Cooperation
614 Agency.
615
616 **Author Contributions**

617 ST, FIB, MOJ, PDR, GEV, JBJ, and EMG conceived the study. MOJ, GVM, PA, TBA, GC, LBTdlB,
618 EB, TCC, DTKT, TAC, DSE, RFG, DMF, MK, MLI, FJL, LL, ETM, SAM, NTTN, EO, AMQD, RR,
619 FR, GER, VMS, PT, CT, JBJ, and GEV collected bacterial strains. ST, MOJ, GVM, and JKG prepared
620 genomic libraries. ST, FIB, MOJ, JHT, A Sharma, A Subedi, AK, and EMG performed data analysis. ST,
621 FIB, MOJ, A Sharma, A Subedi, GEV, JBJ, and EMG drafted the manuscript. All authors reviewed the
622 manuscript.
623
624 **Data summary**
625 The raw read files and genome assemblies for all strains sequenced in this study are deposited in NCBI
626 under BioProject PRJNA941448. The sources of genome assemblies acquired from public databases are
627 listed in Table 1.
628
629

630 **Supporting Information**

631

632 **S1 Figure. Phylogenetic analysis of 270 *X. euvesicatoria* pv. *perforans* strains.** (A) Maximum likelihood
633 phylogenetic tree of *Xanthomonas euvesicatoria* pv. *perforans* strains based on aligned nucleotide sequences of
634 887 core genes. The tree was inferred using RAxML using a GTRGAMMAI substitution model. The tree was
635 rooted using the 11 genetically diverged strains that make up core gene cluster 10. (B) NeighborNet network
636 inferred using SNPs from aligned whole genome sequences, including *X. euvesicatoria* pv. *euvesicatoria* strain
637 85-10 (bolded) as an outgroup. Core gene cluster 10 strains are highlighted. Reticulations in the network indicate
638 conflicting phylogenetic relationships.

639

640 **S2 Figure. Accessory genome variation in *Xanthomonas euvesicatoria* pv. *perforans*.** (A) Visualization of
641 pangenome variation by non-metric multidimensional scaling of gene presence-absence for all 270 *X. perforans*
642 strains by BAPS cluster. Ellipses assume a multivariate t-distribution. (C) Increase in gene count with increasing
643 number of strains sampled. Clusters 1 and 2 were represented by the most strains, but other clusters showed
644 similar rates of increase in the pangenome of the cluster. Pangenome matrix used for analysis is available as S4
645 Data.

646

647 **S3 Figure. Temporal signal in phylogenetic tree of 259 *X. euvesicatoria* pv. *perforans* strains.** (A)
648 Correlation between sampling year and root-to-tip distance in maximum likelihood phylogenetic tree
649 inferred from alignment of whole genome sequences. Output was generated from BactDating R package.
650 (B) Temporal signal within the phylogenetic determined using Phylostems tool. Nodes with significant
651 temporal signals are indicated with colored circles. Adjusted R-squared values by color are: dark green 0–
652 0.2; light green 0.2–0.4; yellow 0.4–0.6; orange 0.6–0.8; red 0.8–1.

653

654 **S4 Figure. Dated phylogenies of 259 *X. euvesicatoria* pv. *perforans* strains.** (A) Dating using
655 BactDating relaxed clock analysis on RAxML-generated phylogeny. This is the tree shown in Figure 2,

656 shown here without collapsed nodes. (B) Dating of same dataset using BEAST with coalescent Bayesian
657 skyline priors and an uncorrelated relaxed clock.
658
659 **S5 Figure. Frequency of Xop effectors among 270 *Xanthomonas euvesicatoria* pv. *perforans* strains.**
660 The most common allele observed was assigned to allele type 1, second most frequent allele to allele type
661 2, and so on. Note that alleles classified as pseudogenes included contig breaks, which include assembly
662 errors. For example, all strains appear to have *xopD*, but a repeat caused a contig break in the gene in
663 nearly half of the genomes.
664
665 **S6 Figure. Clustering of 270 *Xanthomonas perforans* effector profiles by non-metric**
666 **multidimensional scaling and distribution of resulting clusters among geographic regions.** Analysis
667 did not include TAL effectors. (A) The most frequently observed group of effector profiles form cluster
668 A. This cluster of 188 strains is represented as a star in plots B-C, as it is represented in most BAPS core
669 gene clusters (B), most of the sampled tomato production regions (C), and in collections from 1991 to
670 2017 (D). Clusters were largely defined by low frequency effectors (S7 Figure). (E) Distribution of strains
671 by effector clusters among sampled countries.
672
673 **S7 Figure. Variation in type III effector profiles in 270 *Xanthomonas euvesicatoria* pv. *perforans***
674 **strains ordered according to NMDS of effector profiles.** Analysis did not include TAL effectors. Type
675 III effectors are in columns and *Xep* strains in rows. Effector status is shown by allele type: absence is
676 indicated by allele type 0 (white), while the most frequent allele observed when the effector is present is
677 allele type 1 (purple), second most frequent is allele type 2 (blue), and so on. Putative pseudogenized
678 effectors are shown as allele 13 (gray). The order of columns was determined by hierarchical clustering
679 analysis, placing similarly distributed effectors adjacent to each other. Order of rows is based on NMDS
680 clustering analysis of effector profiles (see S6 Figure).
681

682 **S1 Table. Genome data and metadata for *X. euvesicatoria* pv. *perforans* strains.** (A) BAPS cluster
683 assignment for each strain and NCBI information for each genome. Genome assembly statistics are given
684 for newly sequenced strains. (B) Depth of coverage relevant to ANI comparisons in Fig. 1D.
685
686 **S2 Table. Genetic diversity statistics by geographic region and BAPS group.** (A) Statistics by country
687 and U.S. state. (B) Statistics by BAPS group.
688
689 **S3 Table. Average nucleotide identity (ANIb) comparisons between strains with highly similar core**
690 **gene sequences collected across continents.** (A) Proportion nucleotide identity. (B) Alignment fraction.
691
692 **S4 Table. Putative type III effectors (Xop proteins) found in 270 *X. euvesicatoria* pv. *perforans***
693 **assembled genomes.** (A) Summary for each locus. (B) Results by strain. Each different amino acid
694 sequence per gene was assigned a numerical allele type, such that the most common allele observed was
695 assigned to allele type 1. Potential pseudogenes are indicated with "pseudo" and absence indicated with
696 zero. Locus tags refer to JGI IMG annotations (https://img.jgi.doe.gov). Reference sequences used for
697 BLAST searches are given in S5 Table. The final column shows the result of BLAST searches for TAL
698 effectors.
699
700 **S5 Table. Presence or absence of copper genes (*copLAB*) in 270 *X. euvesicatoria* pv. *perforans***
701 **assembled genomes**. Symbols represent gene presence '+' or absence '-'. Contig break in gene is
702 indicated by (+).
703
704 **S6 Table. Type III effector database used to query assembled genomes for effector genes.**
705
706 **S1 Data. Nucleotide alignment of 887 core genes from 270 *X. euvesicatoria* pv. *perforans* strains.**
707 Alignment is 617,855 bp in FASTA format.

708

709 **S2 Data. Nucleotide alignment of variable sites from whole genome alignment of 270 *X.***

710 ***euvesicatoria* pv. *perforans* strains and *X. euvesicatoria* pv. *euvesicatoria* strains 85-10.**

711

712 **S3 Data. XML file used for BEAST analysis.**

713

714 **S4 Data. Pangenome matrix for 270 *X. euvesicatoria* pv. *perforans* strains**

715

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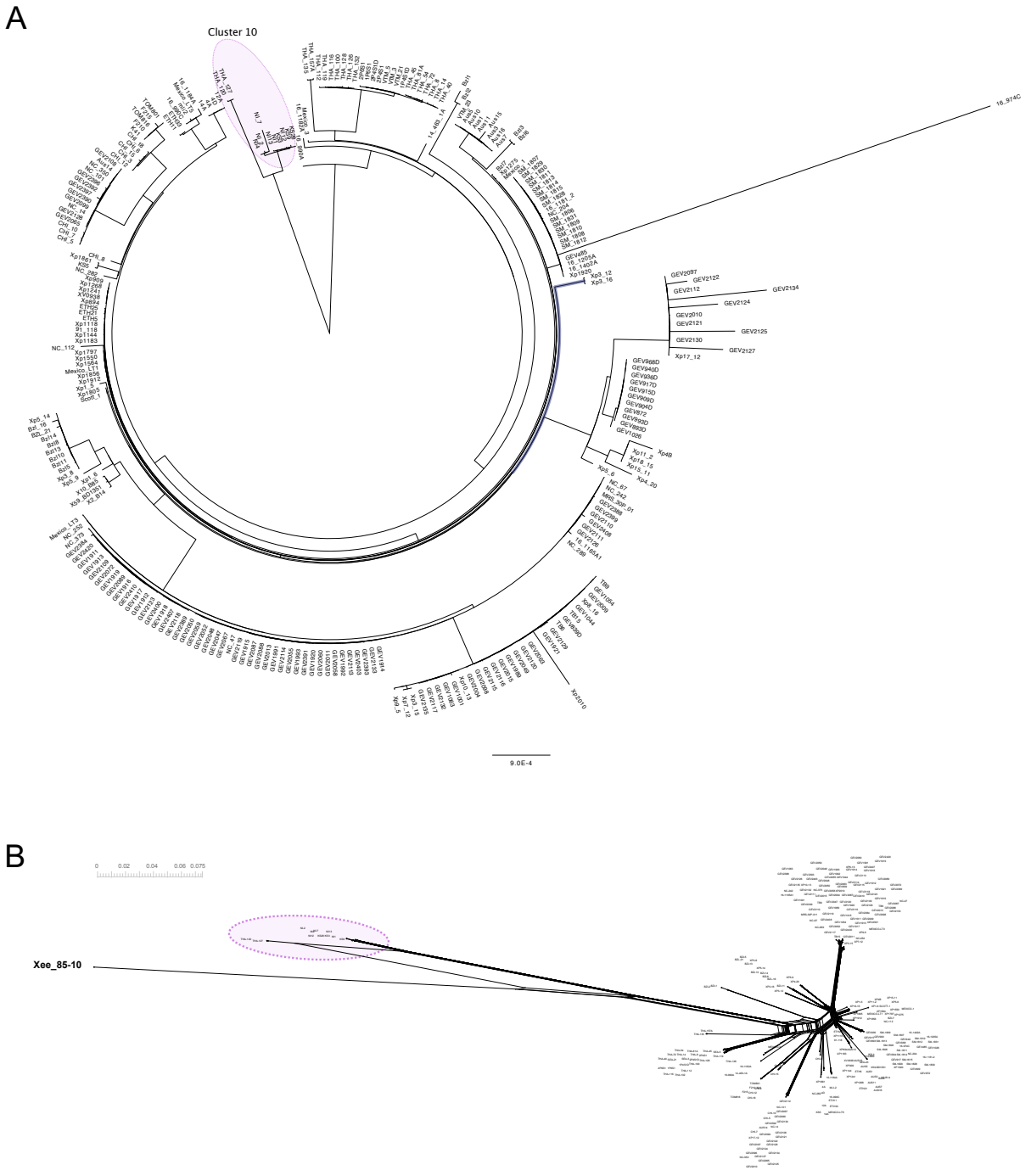
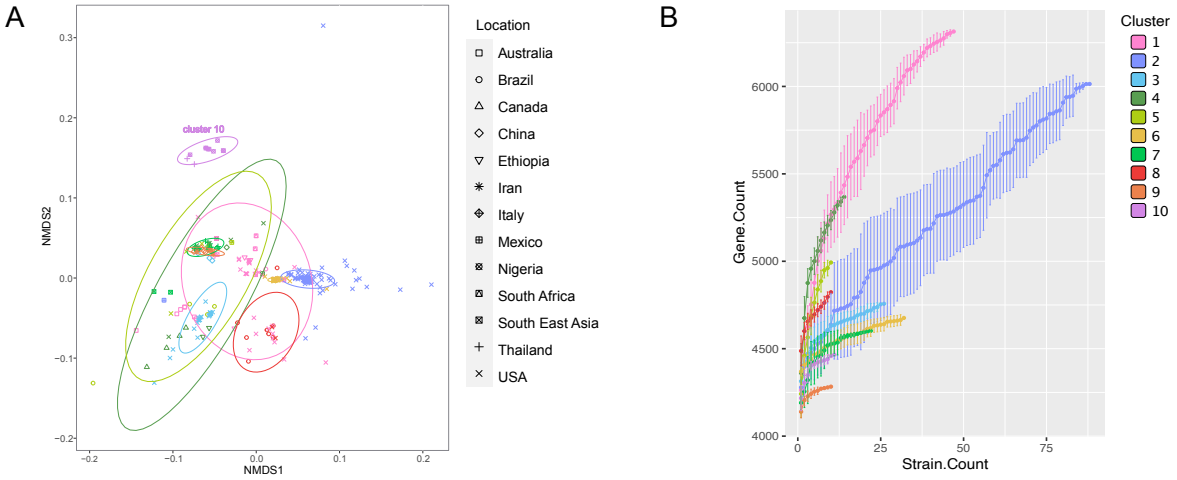
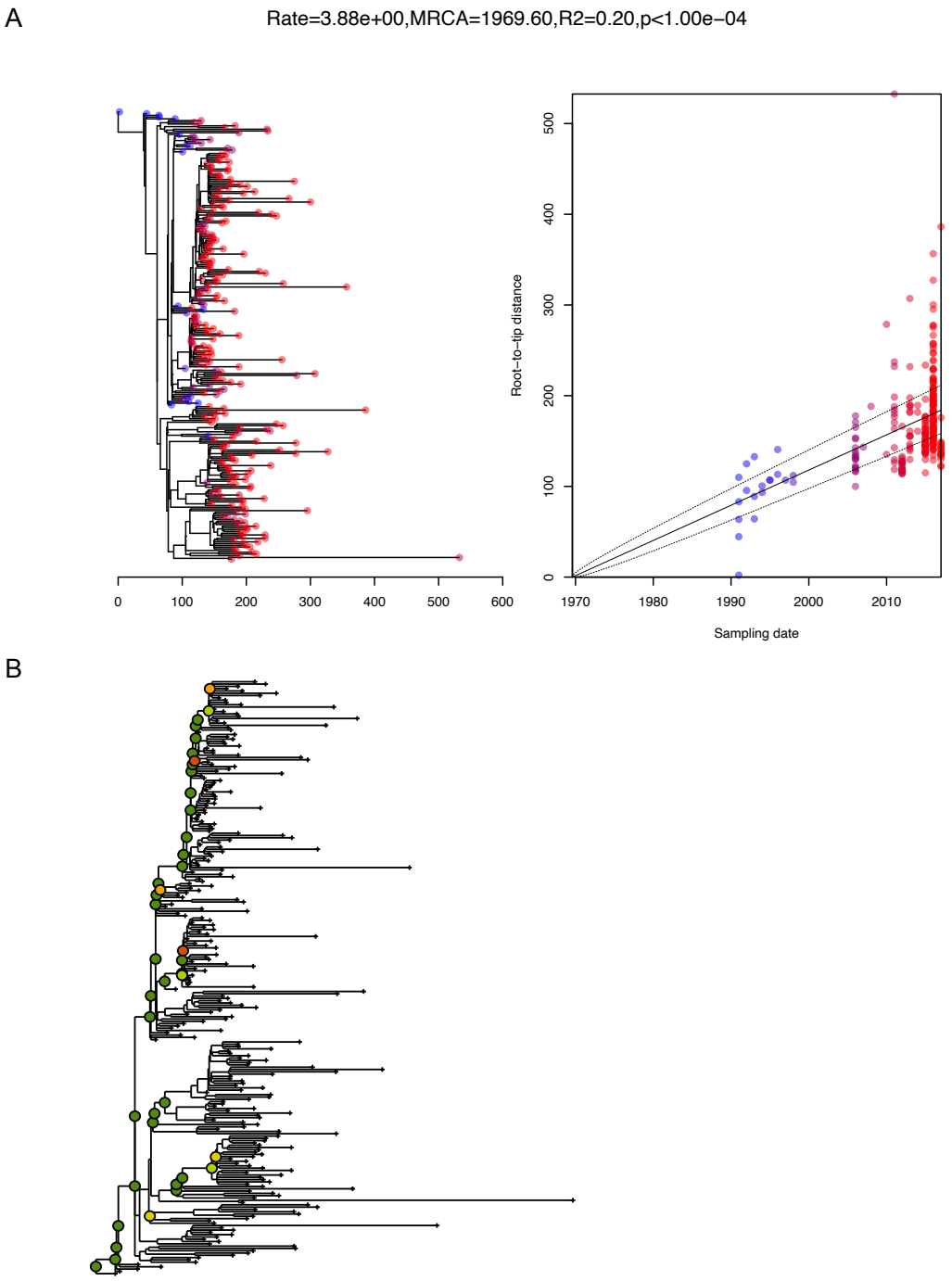


S1 Figure. Phylogenetic analysis of 270 *X. euvesicatoria* pv. *perforans* strains. (A) Maximum likelihood phylogenetic tree of *Xanthomonas euvesicatoria* pv. *perforans* strains based on aligned nucleotide sequences of 887 core genes. The tree was inferred using RAxML using a GTRGAMMAI substitution model. The tree was rooted using the 11 genetically diverged strains that make up core gene cluster 10. (B) Neighbor-net network inferred using SNPs from aligned whole genome sequences, including *X. euvesicatoria* pv. *euvesicatoria* strain 85-10 (bolded) as an outgroup. Core gene cluster 10 strains are highlighted. Reticulations in the network indicate conflicting phylogenetic relationships.



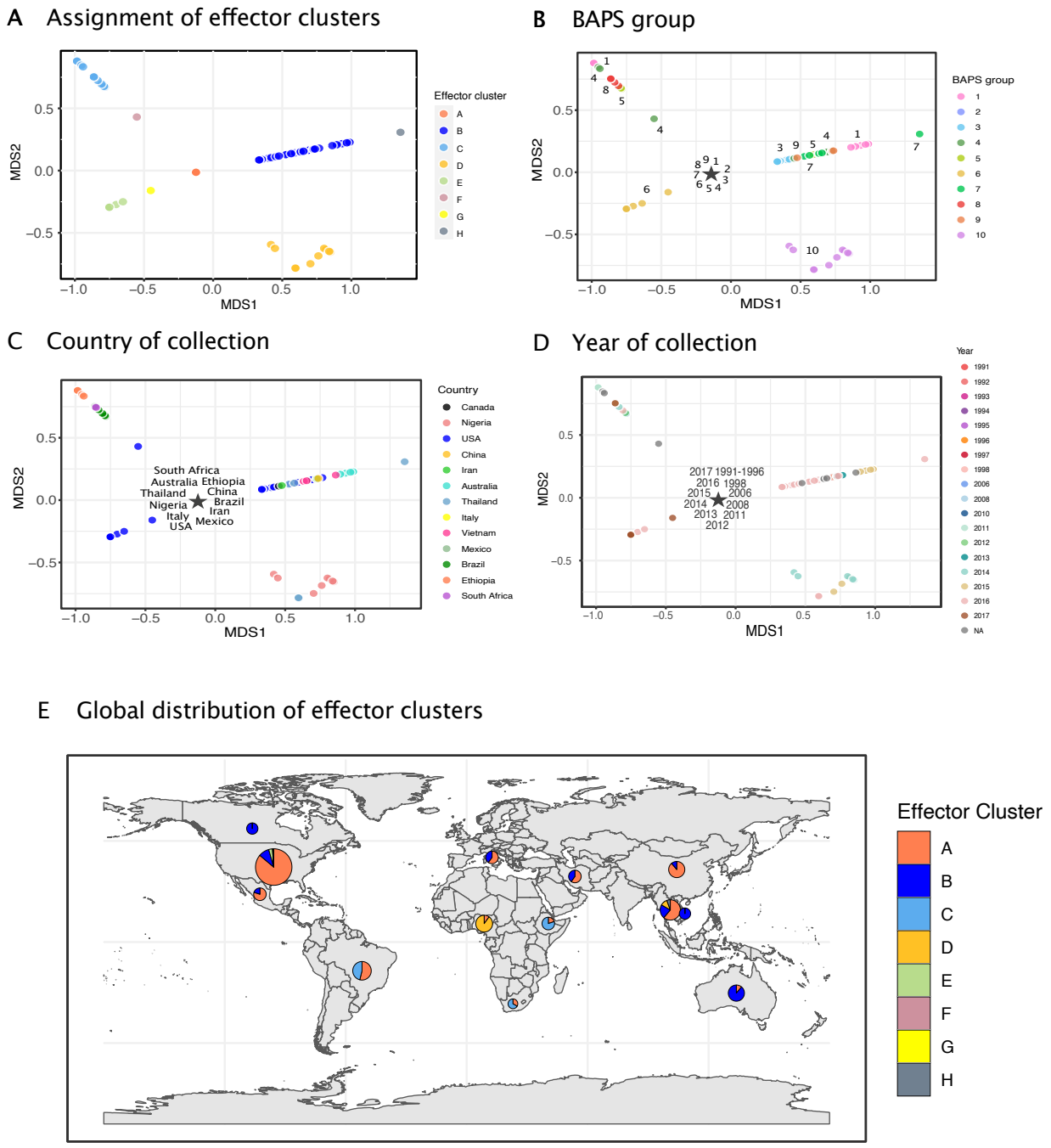
S2 Figure. Accessory genome variation in *Xanthomonas euvesicatoria* pv. *perforans*. (A) Visualization of pangenome variation by non-metric multidimensional scaling of gene presence-absence for all 270 *X. perforans* strains by BAPS cluster. Ellipses assume a multivariate t-distribution. (B) Increase in gene count with increasing number of strains sampled. Clusters 1 and 2 were represented by the most strains, but other clusters showed similar rates of increase in the pangenome of the cluster.



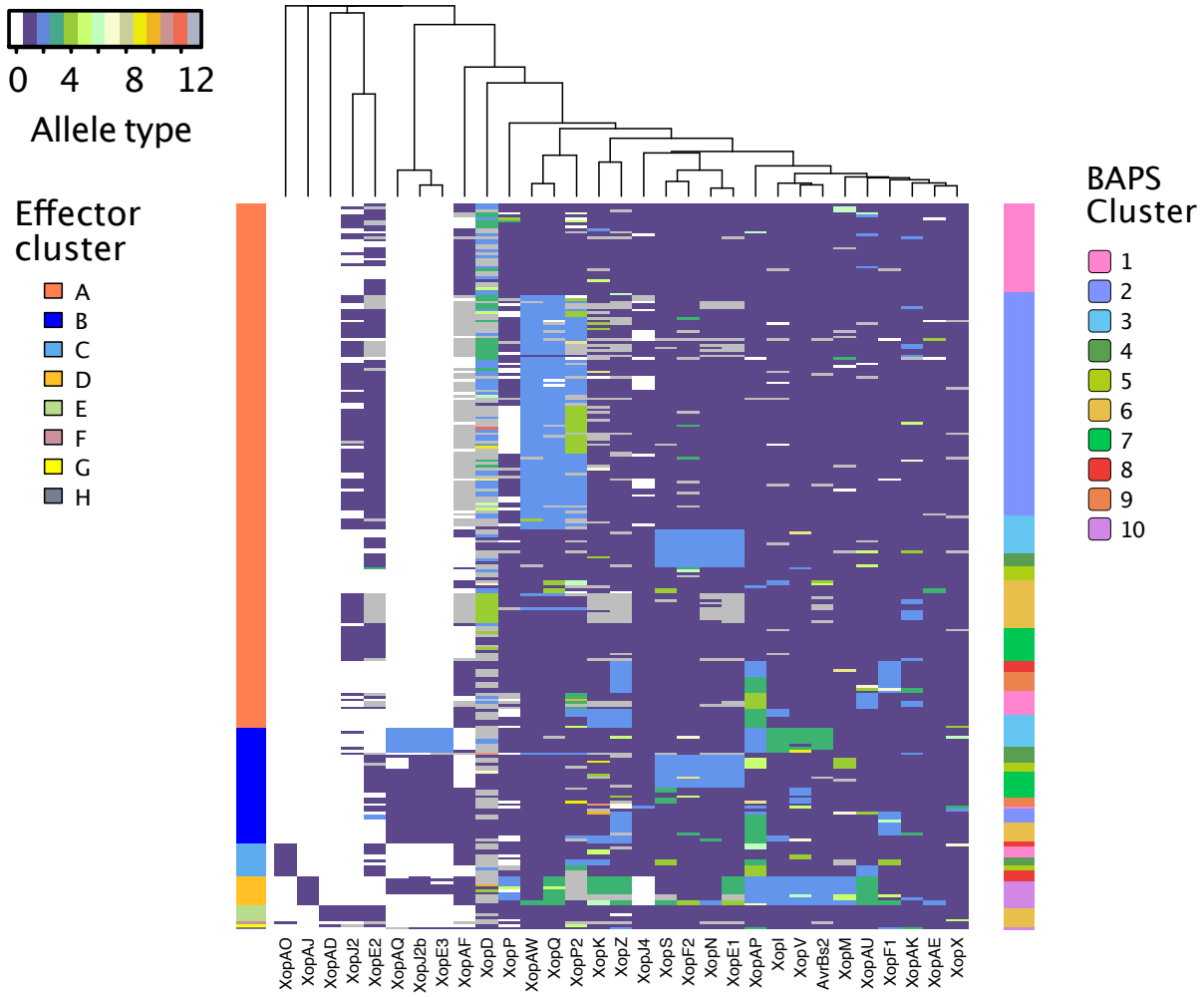
S3 Figure. Temporal signal in phylogenetic tree of 259 *X. euvesicatoria* pv. *perforans* strains. (A) Correlation between sampling year and root-to-tip distance in maximum likelihood phylogenetic tree inferred from alignment of whole genome sequences. Output was generated from BactDating R package. (B) Temporal signal within the phylogenetic determined using Phylostems tool. Tree is rooted as in Figure 2. Nodes with statistically significant temporal signals are indicated with colored circles. Adjusted R-squared values by color: dark green 0–0.2; light green 0.2–0.4; yellow 0.4–0.6; orange 0.6–0.8; red 0.8–1.

S4 Figure. Dated phylogenies of 259 *X. euvesicatoria* pv. *perforans* strains. (A) Dating using BactDating relaxed clock analysis on RAxML-generated phylogeny. This is the tree shown in Figure 2, shown here without collapsed nodes. (B) Dating of same dataset using BEAST with coalescent Bayesian skyline priors and an uncorrelated relaxed clock.

S5 Figure. Frequency of Xop effectors among 270 *Xanthomonas euvesicatoria* pv. *perforans* strains. The most common allele observed was assigned to allele type 1, second most frequent allele to allele type 2, and so on. Note that alleles classified as pseudogenes included contig breaks, which include assembly errors. For example, all strains appear to have *xopD*, but a repeat caused a contig break in the gene in nearly half of the genomes.



S6 Figure. Clustering of 270 *Xanthomonas perforans* effector profiles by non-metric multidimensional scaling and distribution of resulting clusters among geographic regions. Analysis did not include TAL effectors. (A) The most frequently observed group of effector profiles form cluster A. This cluster of 188 strains is represented as a star in plots B-C, as it is represented in most BAPS core gene clusters (B), most of the sampled tomato production regions (C), and in collections from 1991 to 2017 (D). Clusters were largely defined by low frequency effectors (S7 Figure). (E) Distribution of strains by effector clusters among sampled countries.



S7 Figure. Variation in Type III secreted effector profiles in 270 *Xanthomonas euvesicatoria* pv. *perforans* strains ordered according to NMDS of effector profiles. Analysis did not include TAL effectors. Type III secreted effectors are in columns and *Xep* strains in rows. Effector status is shown by allele type: absence is indicated by allele type 0 (white), while the most frequent allele observed when the effector is present is allele type 1 (purple), second most frequent is allele type 2 (blue), and so on. Putative pseudogenized effectors are shown as allele 13 (gray). The order of columns was determined by hierarchical clustering analysis, placing similarly distributed effectors adjacent to each other. Order of rows is based on NMDS clustering analysis of effector profiles (see S6 Figure, part A).