Fusarium oxysporum f. sp. *mori*, a New Forma Specialis Causing Fusarium Wilt of Blackberry

A. M. Pastrana and S. C. Kirkpatrick, Department of Plant Pathology, University of California, Davis 95616; M. Kong and J. C. Broome, Driscoll's Inc., Watsonville, CA 95076; and T. R. Gordon,[†] Department of Plant Pathology, University of California, Davis 95616

Abstract

Fusarium oxysporum has recently been identified as the cause of a wilt disease affecting blackberry in California and Mexico. Thirty-six isolates of *F. oxysporum* obtained from symptomatic blackberry plants in California and Mexico were comprised of nine distinct somatic compatibility groups (SCGs). Phylogenetic analysis of a concatenated data set, consisting of sequences of the translation elongation factor 1- α and β -tubulin genes and the intergenic spacer of the ribosomal DNA, identified nine three-locus sequence types, each of which corresponded to an SCG. Six SCGs were present only in California, two only in Mexico, and one in both California and

Mexico. An isolate associated with the most common SCG in California was tested for pathogenicity on blueberry, raspberry, strawberry, and lettuce. All blueberry, raspberry, and lettuce plants that were inoculated remained healthy, but two of the five strawberry cultivars tested developed symptoms. The three strawberry cultivars that were resistant to the blackberry pathogen were also resistant to *F. oxysporum* f. sp. *fragariae*, the cause of Fusarium wilt of strawberry. We propose to designate strains of *F. oxysporum* that are pathogenic to blackberry as *Fusarium oxysporum* f. sp. *mori forma specialis* nov.

In 2007, wilting of a few blackberry (*Rubus* L. subgenus *Rubus*) transplants that originated from Merced Co., CA, was observed in a small planting in Watsonville, CA, and *Fusarium oxysporum* Schlechtendahl emend. Snyder & Hansen was identified as the cause (Gordon et al. 2016). Since that time, this wilt disease has become more widespread in California and is now severely reducing fruit production in susceptible cultivars, with disease incidence ranging from 10 to 40% in affected fields. Fusarium wilt of blackberry is also reported to occur in Mexico (Hernández-Cruz et al. 2015). The earliest symptom of disease is wilting of older leaves, followed by external discoloration of the stem (Fig. 1) and more extensive wilting. Orange spore masses are commonly observed on stems of dead and dying plants.

Fusarium wilt represents a significant threat to the blackberry industry in the United States and Mexico. In 2015, total blackberry production in the United States was estimated at 51.3 million pounds, up 13% from 2014 (USDA-NASS 2015). In 2015, the United States imported fresh blackberries valued at almost \$270 million and frozen blackberries valued at \$30.1 million. Mexico provided nearly all imported fresh blackberry, with a four-year annual average market share of 97.6% from 2012 to 2015 (<https://www.ers.usda.gov>).

Management of Fusarium wilt diseases has been most effectively achieved through deployment of cultivars that are resistant to the disease. Genetic resistance can be improved through plant breeding, which requires identification of appropriate pathogen strains to include in a screening program. A useful step in the strain selection process is to assess the extent of diversity in the pathogen population to which the crop will be exposed. Accordingly, it was an objective of this study to sample the population of *F. oxysporum* strains capable of causing disease on blackberry, and characterize the range of variation based on somatic compatibility and DNA sequences of conserved genomic regions.

Another important element in disease management is suppression of pathogen inoculum levels in soil. Whereas this can be accomplished very effectively through preplant fumigation, cost and regulatory constraints have made this option less available to growers. In the absence of fumigation, crop rotation is the primary means by

which inoculum levels can be maintained below a damaging threshold. However, the benefits of this practice will be diminished if rotation crops serve as reproductive hosts for the pathogen. For this reason, we have tested crops commonly grown in rotation with blackberry in California for susceptibility to an isolate of *F. oxysporum* that causes Fusarium wilt of blackberry.

Materials and Methods

***Fusarium* spp. isolates.** Isolates of *F. oxysporum* were obtained from symptomatic blackberry plants in production fields in Monterey and Santa Cruz counties, CA, and Mexico (Table 1). To recover fungi from plants, stem and petiole cross sections were rinsed with 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO), submersed in 70% ethanol for 20 s followed by 60 s in 1% NaOCl, and then placed on Komada's selective medium (KM) (Komada 1975). After 5 to 7 days at room temperature under continuous fluorescent light, colonies tentatively identified as *F. oxysporum* based on colony morphology were subcultured as single spores and stored on dried filter paper (Gordon and Okamoto 1991). Each isolate was grown on carnation leaf agar (CLA) (Nelson et al. 1983) for identification based on morphological criteria. CLA plates were incubated under continuous fluorescent light for 7 days at room temperature prior to examination.

DNA isolation. Isolates were cultured on potato dextrose agar (PDA) for 5 to 7 days at room temperature. Mycelium was scraped from the surface of each culture with a sterile scalpel blade. DNA was isolated by Isolate II Plant DNA Kit (Bioline, London UK) following the manufacturer's instructions with some modifications: Mycelium was placed in a centrifuge tube containing lysis buffer and homogenized with three glass beads (diameter 3 mm) by vortexing for 15 min. DNA concentration was determined on a ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE) and genomic DNA was stored at -20°C .

TEF, TUB and IGS amplification and sequence analysis. The polymerase chain reaction (PCR) was used to amplify and sequence the translation elongation factor 1- α (TEF), the β -tubulin gene (TUB), and the intergenic spacer (IGS), using primers EF1 and EF2 (O'Donnell et al. 1998), T1 and T22 (O'Donnell and Cigelnik 1997), and primer pairs iNL11/CNSa and iCNS11/NLa (O'Donnell et al. 2009), respectively. Amplifications were carried out in an Applied Biosystems 2720 thermal cycler and PCR products were sized by electrophoresis in 1% agarose gels run in 1 $\times$ TAE buffer, stained with EZ-Vision (AMRESCO LLC., Solon, OH) and visualized over a UV transilluminator. Negative controls, in which water was added to the reaction mixture instead of DNA template, were included in every assay. PCR products were purified with the AccuPrep PCR Purification Kit

[†]Corresponding author: T. R. Gordon; E-mail: trgordon@ucdavis.edu

(Bioneer, Smith's Parish, Bermuda) according to manufacturer's instructions and sequenced by Quintara Bioscience (San Francisco, CA). Trace files were visualized and sequences were edited in MEGA 6 (Tamura et al. 2013). Sequences were compared using BLAST analyses in the GenBank database, where only published, peer-reviewed sequences were considered for reliable species identifications. The edited sequences were deposited in GenBank (Table 1).

Phylogenetic analysis. Sequences were aligned using Muscle software following the default settings displayed in MEGA 6. Phylograms were constructed for TEF, TUB, and IGS sequences singly, and the three combined datasets for 34 blackberry isolates. Analyses were conducted using maximum likelihood based on the Hasegawa-Kishino-Yano model with 1,000 bootstrap replicates. The rate of variation among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions included were first + second + third + noncoding. All ambiguous positions were removed for each sequence pair. A total of 3,796 positions were included in the final dataset. Evolutionary analyses were conducted in MEGA 6. The TEF-TUB-IGS phylogenetic tree was deposited in TreeBASE under accession no. S20516.

Another phylogram was constructed, as described above, using IGS sequences for the 34 blackberry isolates and IGS sequences

available in GenBank for the following formae speciales: *F. oxysporum* f. sp. *cepa* (DQ831891), *F. oxysporum* f. sp. *fabae* (DQ831902), *F. oxysporum* f. sp. *heliotropii* (DQ831903), *F. oxysporum* f. sp. *lactuca* (DQ831874), *F. oxysporum* f. sp. *lycopersici* (DQ831894), *F. oxysporum* f. sp. *medicaginis* (DQ831901), *F. oxysporum* f. sp. *phaseoli* (DQ831900), *F. oxysporum* f. sp. *spinaciae* (DQ831888), *F. oxysporum* f. sp. *tracheiphilum* (FJ883651), *F. oxysporum* f. sp. *vasinfectum* (DQ831896) (Mbofung et al. 2007; Troisi et al. 2013), and three isolates of *F. oxysporum* f. sp. *fragariae* corresponding to the three SCGs known to occur in California (Henry et al. 2017). A total of 1,975 positions were included in the final dataset. The IGS phylogenetic tree was deposited in TreeBASE under accession no. S21206.

Somatic compatibility. Isolates of *F. oxysporum* were cultured on plates of PDA supplemented with 4.5% potassium chlorate to obtain nitrate nonutilizing (nit) mutants (Jacobson and Gordon 1988). Complementary nit phenotypes, differentiated as described by Correll et al. (1987), were obtained for individual isolates of *F. oxysporum* to serve as tester strains. Compatibility between two isolates was recognized as a line of aerial mycelium (wild-type growth) where two complementing nit mutants grew together on Czapek solution agar, which is indicative of heterokaryon formation. Isolates capable of forming a

Fig. 1. External discoloration on the stem of a blackberry plant infected by *Fusarium oxysporum* f. sp. *mori*.

heterokaryon were assigned to the same somatic compatibility group (SCG).

Pathogenicity test. Twelve isolates obtained from symptomatic blackberry plants identified as *F. oxysporum* were tested for pathogenicity to blackberry. Each isolate was included in two independent inoculation trials, each of which included six isolates. In each trial, five plants of the known susceptible blackberry cultivar BJ110.2 (~8 month old) were inoculated using a root-dip procedure. Plants to be inoculated were removed from pots, and their roots were washed under water before immersion in a spore suspension for 10 min. The spore suspension was prepared from fully colonized plates of PDA, as described by Schmale and Gordon (2003), and adjusted to a density of 10^6 spores/ml in 0.1% water agar. Inoculated plants were placed in 15-cm-diameter pots filled with Sunshine Mix 1 (Sun Gro Horticulture, Vancouver, Canada). Control plants were immersed in sterile 0.1% water agar instead of a spore suspension. Inoculated and control plants were arranged in randomly assigned positions in a greenhouse with temperatures ranging from 15 to 30°C and relative humidity between 15 and 75%. Four to nine weeks after inoculation, plants were rated on a semicontinuous 1 to 5 scale, where: 1 = no symptoms; 2 = drying and death of older leaves; 3 = symptomatic older leaves accompanied by wilting of younger leaves and/or stunting; 4 = severe foliar symptoms and stem

blackening; and 5 = dead plant. Intermediate ratings in increments of 0.5 were given to plants according to the extent of symptom development. Stem and petiole tissues of blackberry plants were cultured as described above to confirm colonization by *F. oxysporum*.

Host range. Strawberry, raspberry, blueberry, and lettuce were tested for susceptibility to an isolate of *F. oxysporum* confirmed to be pathogenic to blackberry (Table 2). A single cultivar of each crop was tested, except for strawberry, for which five cultivars were tested. The isolate with which plants were inoculated (GL1804) was representative of the most common SCG identified in California. As positive controls, strawberry plants were inoculated with an isolate of *F. oxysporum* f. sp. *fragariae* (GL1080) and lettuce was inoculated with an isolate *F. oxysporum* f. sp. *lactucae* (GL1515). Plants used for inoculations were either transplants (raspberry, blueberry, and blackberry), dormant crowns (strawberry), or two-week-old seedlings (lettuce). Spore suspensions were prepared and inoculations performed as described above. In each experiment, five plants of each host crop/cultivar were subjected to each treatment in a completely randomized design (Table 2). The experiment was conducted twice. Plants were assessed for wilting, chlorosis, and stunting weekly after the first symptoms were observed. Reisolation of the pathogen was attempted from different tissues according to the host: stems and petioles for blackberry; crowns and petioles for strawberry;

Table 1. Isolates of *Fusarium oxysporum* recovered from symptomatic blackberry plants

Isolate	Year ^s	Location ^t	Cultivar	SCG ^u	Pathogenicity ^v	TEF ^w	TUB ^x	IGS ^y
GL1311	2007	Merced	BD467.1	0490	+	KT265338	KY498497	KY522817
GL1787	2015	Santa Cruz	BJ110.2	0490	N/A	KY498493	KY498496	KY522820
GL1794	2015	Santa Cruz	BJ110.2	0490	N/A	KY498492	KY498495	KY522819
GL1803	2015	Santa Cruz	BJ110.2	0490	+	KY498491	KY498494	KY522818
GL1312 ^z	2011	Tehama	BD467.1	0491	+	KT265339	KY508658	KY515229
GL1313 ^z	2011	Tehama	BD467.1	0491	+	KT265340	KY508659	KY515230
GL1314 ^z	2011	Ventura	BD467.1	Unique	+	KT265341	KY508660	KY522802
GL1300 ^z	2011	Mexico	Tupi	Unique	+	KT265335	KY508655	KY515227
GL1301 ^z	2011	Mexico	BY48.1	Unique	+	KT265336	KY498526	KY515228
GL1302 ^z	2011	Mexico	BY48.1	0492	+	KT265337	KY498490	KY553157
GL1784	2015	Santa Cruz	BJ110.2	0492	N/A	KY488194	KY498483	KY553158
GL1785	2015	Santa Cruz	BJ110.2	0492	N/A	KY488195	KY498484	KY553159
GL1786	2015	Santa Cruz	BJ110.2	0492	N/A	KY488196	KY498485	KY553160
GL1789	2015	Santa Cruz	BJ110.2	0492	+	KY488197	KY498486	KY553161
GL1804	2015	Santa Cruz	BJ110.2	0492	+	KY488198	KY498487	KY553162
GL1805	2015	Monterey	BJ110.2	0492	+	KY488199	KY498488	KY553163
GL1808	2015	Santa Cruz	BJ110.2	0492	+	KY488200	KY498489	KY553164
GL1516	2015	Santa Cruz	BJ110.2	0493	+	KY508653	KY508656	KY522800
GL1518	2015	Santa Cruz	BJ110.2	0493	+	KY508654	KY508657	KY522801
GL1783	2015	Santa Cruz	BJ110.2	0494	N/A	KY498509	KY498523	KY522814
GL1788	2015	Santa Cruz	BJ110.2	0494	N/A	KY498510	KY498524	KY522815
GL1790	2015	Santa Cruz	BJ110.2	0494	N/A	KY498508	KY498522	KY522813
GL1791	2015	Santa Cruz	BJ110.2	0494	N/A	KY498511	KY498525	KY522816
GL1792	2015	Santa Cruz	BJ110.2	0494	N/A	KY498507	KY498521	KY522812
GL1793	2015	Santa Cruz	BJ110.2	0494	N/A	KY498506	KY498520	KY522811
GL1795	2015	Santa Cruz	BJ110.2	0494	N/A	KY498505	KY498519	KY522810
GL1796	2015	Santa Cruz	BJ110.2	0494	+	KY498504	KY498518	KY522809
GL1798	2015	Santa Cruz	BJ110.2	0494	N/A	KY498503	KY498517	KY522808
GL1799	2015	Santa Cruz	BJ110.2	0494	N/A	KY498502	KY498516	KY522807
GL1800	2015	Santa Cruz	BJ110.2	0494	+	KY498501	KY498515	KY522806
GL1801	2015	Santa Cruz	BJ110.2	0494	N/A	KY498500	KY498514	KY522805
GL1802	2015	Santa Cruz	BJ110.2	0494	N/A	KY498498	KY498512	KY522803
GL1809	2015	Santa Cruz	BJ110.2	0494	+	KY498499	KY498513	KY522804
GL1806	2015	Santa Cruz	BJ110.2	Unique	+	KY553165	KY553166	KY553156

^s Year isolate was collected.

^t The country (Mexico) or county in California where the isolate was obtained. Each isolate came from a different field with the following exceptions: isolates GL1783 to GL1804 were obtained from different plants in the same field; and each of the following pairs of isolates came from different plants in one field: GL1808 and GL1809, GL1516 and GL1517, GL1312 and GL1313, and GL1301 and GL1302.

^u Somatic compatibility group; "Unique" indicates that the isolate is somatically compatible with itself but with none of the other isolates listed.

^v '+' = pathogenic to blackberry; N/A = not tested.

^w GenBank accession nos. for sequences of the translation elongation factor -1 α .

^x GenBank accession nos. for sequences of the β -tubulin gene.

^y GenBank accession nos. for sequences of the intergenic spacer.

^z Pathogenicity test results previously reported by Gordon et al. (2016).

roots and stems for raspberry and blueberry; and tap root cortex and vascular stele for lettuce. Tissue samples were cultured on KM as described above and colonies of *F. oxysporum* were identified based on morphological criteria.

Statistical analysis. Statistical tests were performed using the R programming language (version 3.0.3). The Mann-Whitney *U* test was used to determine if experiment had a significant effect on disease severity in the host range trials. Because this effect was not significant ($P > 0.05$), data were pooled across experiments, and a Kruskal Wallis test was used to determine if the effect of isolate on disease severity was significant.

Results

Isolate characterization. Thirty-four isolates recovered from symptomatic blackberry plants in California and Mexico were identified as *F. oxysporum* based on morphological criteria and sequence data (Table 1). On CLA, all isolates produced microconidia in false heads on short monophialides and macroconidia with elongated, bent, apical cells and notched basal cells, consistent with identification as *F. oxysporum* (Gordon et al. 2016; Leslie et al. 2006). All sequence comparisons revealed a 99 to 100% match with *F. oxysporum* sequences in GenBank. Blackberry plants inoculated with pathogenic isolates developed symptoms that included wilting, chlorosis, and stunting. Symptoms were evident as soon as 4 weeks after inoculation in some cases, and all symptomatic plants died in less than 8 weeks. Pathogenic isolates were recovered from stems and petioles of all symptomatic plants. No symptoms developed in negative control plants. Similar results were obtained in both experiments (*data not shown*).

Nitrate non-utilizing (Nit) mutants were successfully generated for all the isolates. Pairing isolates pathogenic to blackberry in all combinations revealed nine different SCGs. Six SCGs were present only in California, two only in Mexico, and one in both California and Mexico. Somatic compatibility groups 0490 and 0492 were found in more than one location, whereas all others were identified at only one location (Table 1). Numbers were assigned to SCGs in accordance with the classification proposed by Puhalla (1985), Kistler (1997), and Katan (1999). Somatic compatibility groups represented by a single isolate were not given numbers.

Phylogenetic analysis. Amplification of the TEF, TUB, and IGS regions resulted in PCR products approximately 650, 1,400, and 2,100 bp long, respectively. The numbers of parsimony informative sites were 3, 2, and 73 for TEF, TUB, and IGS, respectively. Isolates associated with the same SCG shared identical sequences for all three regions. Trees based on TEF and TUB sequences had the lowest resolution, resolving six and four sequence types (STs), respectively, with low bootstrap support (*trees not shown*). Phylogenetic analysis based on IGS revealed eight different STs with well supported branches. However, using the 3-gene concatenated data set, consisting of 3796 bp, phylogenetic analysis generated a tree with nine three-locus STs (1 to 9), each of which corresponded to an SCG (Fig. 2).

The alignment of IGS sequences of the blackberry isolates and other formae speciales revealed 102 of 1,975 sites to be parsimony informative. The resulting tree is composed of three main clades. Clade 1 includes all blackberry isolates associated with SCG0494 and GL1301, along with eight other formae speciales, but with only weak support from bootstrap (Fig. 3). Clade 2 is comprised of blackberry isolates associated with SCGs 0490, 0491, 0493, and isolates GL1300, GL1314, GL1806, along with isolates representing *F. oxysporum* f. sp. *fragariae* and *F. oxysporum* f. sp. *tracheiphilum*. Clade 3 includes blackberry isolates associated with SCG0492, and isolates of *F. oxysporum* f. sp. *fragariae* and *F. oxysporum* f. sp. *spinaceae*.

Host range. All negative controls remained healthy in both experiments. The *F. oxysporum* isolate pathogenic to blackberry (GL1804) did not induce symptoms on blueberry, raspberry, or lettuce. The fungus was isolated from roots of all inoculated blueberry plants but from stems of only two of the 10 inoculated plants. GL1804 was isolated from roots of five of six raspberry plants (two of the five plants in each experiment died shortly after transplanting and so were not among the plants from which isolations were attempted) but not from

stems of any plants. The blackberry pathogen was isolated from the root cortex of all inoculated lettuce plants and from the vascular stele of six plants. Lettuce plants inoculated with *F. oxysporum* f. sp. *lactucae* developed typical Fusarium wilt symptoms.

Strawberry cultivars San Andreas and Portola inoculated with GL1804 showed no symptoms of disease. These cultivars also remained healthy when inoculated with an isolate of *F. oxysporum* f. sp. *fragariae* (GL1080). Plants of the strawberry cultivar Petaluma did not develop symptoms when inoculated with GL1804 but were killed when inoculated with GL1080 (Table 2). Strawberry cultivars Albion and Monterey were both susceptible to the blackberry pathogen. A Kruskal Wallis test showed the isolate of *F. oxysporum* f. sp. *fragariae* (GL1080) to be significantly more virulent than the blackberry pathogen on both Albion and Monterey cultivars (Table 2). The blackberry pathogen was isolated from petioles of all symptomatic strawberry plants.

Discussion

The origin of the pathogen causing Fusarium wilt of blackberry is unknown, and because the disease has not been reported from anywhere else in the world, there is no obvious source for an introduction. It is possible the pathogen emerged locally (in California or Mexico) through a chance encounter between roots of a blackberry plant and a genotype of *F. oxysporum* with the capacity to infect and cause disease. A similar process presumably explains the origin of most pathogenic strains of *F. oxysporum*, with subsequent movement of plant materials and soil resulting in a wider distribution of the pathogen (Gordon and Martyn 1997). It would be consistent with that scenario for the new pathogen to be associated with a single clonal lineage. In contrast, our limited sampling of the pathogen population in California has revealed considerable diversity based on somatic compatibility and sequence variation in the TEF and TUB genes and the IGS.

There is no simple explanation for this pattern but it could reflect selection from a population associated with Himalayan blackberry (*Rubus armeniacus*), an adventive species that is widely distributed in California, and/or other *Rubus* species found in California (Baldwin et al. 2012). In support of this conjecture is the close proximity of *Rubus* spp. to some blackberry nurseries and fruit production fields. If

Table 2. Disease severity in crop cultivars inoculated with an isolate of *Fusarium oxysporum* f. sp. *fragariae* (GL1080), *Fusarium oxysporum* f. sp. *lactucae* (GL1515), or *F. oxysporum* pathogenic to blackberry (GL1804).

Cultivar	GL1080 ^v	GL1515 ^w	GL1804 ^x	Control
Strawberry cv. Albion ^y	5.0 ± 0.0 a	N/A	3.0 ± 0.002 b	1.0 ± 0.0 c
Strawberry cv. Monterey ^y	5.0 ± 0.0 a	N/A	2.1 ± 0.002 b	1.0 ± 0.0 c
Strawberry cv. Petaluma ^y	5.0 ± 0.0 a	N/A	1.0 ± 0.0 b	1.0 ± 0.0 b
Strawberry cv. Portola	1.0 ± 0.0	N/A	1.0 ± 0.0	1.0 ± 0.0
Strawberry cv. San Andreas	1.0 ± 0.0	N/A	1.0 ± 0.0	1.0 ± 0.0
Lettuce cv. Bondi	1.0 ± 0.0	5.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Blueberry cv. O'Neal	1.0 ± 0.0	N/A	1.0 ± 0.0	1.0 ± 0.0
Raspberry cv. Lupita ^z	1.0 ± 0.0	N/A	1.0 ± 0.0	1.0 ± 0.0
Blackberry cv. BJ110.2 ^y	1.0 ± 0.0 a	N/A	5.0 ± 0.0 b	1.0 ± 0.0 a

^v Mean disease severity on 1 to 5 scale (± standard error) induced by a known pathogenic isolate of *Fusarium oxysporum* f. sp. *fragariae*; n = 10 for all cultivars except raspberry for which n = 6.

^w Mean disease severity on 1 to 5 scale (± standard error) induced by a known pathogenic isolate of *Fusarium oxysporum* f. sp. *lactucae*; N/A = not tested (n = 10).

^x Mean disease severity on 1 to 5 scale (± standard error) induced by an isolate of *Fusarium oxysporum* pathogenic to blackberry; n = 10 for all cultivars except raspberry for which n = 6.

^y Mean disease severity ratings in the same row followed by different letters are significantly different according to Tukey's Honestly Significant Difference (HSD) test at $P = 0.05$.

^z In both experiments, two raspberry plants died shortly after transplanting so means are based on a total of six plants.

one or more of these *Rubus* species are a source of inoculum, they must be symptomless hosts of strains that are pathogenic to blackberry, or the occurrence of symptoms has escaped notice. An examination of isolates associated with Himalayan blackberry and other *Rubus* species is needed to evaluate the merits of this hypothetical explanation for the origin of the blackberry pathogen.

Our study included three isolates of *F. oxysporum* from Mexico, each of which was associated with a different SCG. One SCG was found also in California, whereas the other two were unique to Mexico. A common SCG between the two locations suggests movement of the pathogen, but there is no basis for inferring the direction in which this may have occurred.

Because there are no prior reports of *Fusarium oxysporum* causing a wilt disease of blackberry, we propose that these strains be recognized as a new forma specialis, to be designated as *Fusarium oxysporum* f. sp. *mori*. Formae speciales of *F. oxysporum* are groupings of strains unified by pathogenicity to a particular crop, as proposed by Snyder and Hansen (1940). Often formae speciales appear to be host-specific, but this is not always the case. For example, some isolates of

F. oxysporum f. sp. *cucumerinum* are pathogenic not only to cucumber but also to muskmelon (Cafri et al. 2005), and isolates of *F. oxysporum* f. sp. *raphani* have been shown to cause disease on numerous crops in the mustard family (Brassicaceae). Likewise, the host range of *Fusarium oxysporum* f. sp. *mori* is not limited to blackberry, as some cultivars of strawberry were shown to be susceptible. Although mean disease severity ratings induced by the blackberry pathogen on strawberry were lower than for the strawberry pathogen (Table 2), some strawberry plants inoculated with *F. oxysporum* f. sp. *mori* were killed. Thus, this pathogen is clearly a threat to strawberries, which can be grown in rotation with blackberry in California.

From our assessment of relationships between blackberry isolates and isolates associated with other formae speciales (Fig. 3), it is clear that blackberry pathogens do not constitute a monophyletic group. The lack of alignment between host range and affinities based on conserved genomic regions such as IGS and TEF has also been reported for other formae speciales (Enya et al. 2008; Lievens et al. 2009; Mbofung et al. 2007). The association of virulence to a particular crop with two or more discrete lineages could result from horizontal

Fig. 2. Maximum likelihood tree of pathogenic *Fusarium oxysporum* strains isolated from blackberry, inferred from concatenated translation elongation factor 1- α (TEF), β -tubulin (TUB), and intergenic spacer (IGS) sequences. Strains were grouped into three-locus sequence types (ST1-ST9). Bootstrap values are noted at the internodes. Bootstrap values are based on 1,000 resamplings. Somatic compatibility groups are indicated in parentheses. Scale bar indicates 0.1% sequence dissimilarity.

gene transfer. Horizontal gene transfer has been demonstrated to occur between strains of *F. oxysporum* under experimental conditions (Ma et al. 2010), and genetic evidence suggests it has occurred in a number of *F. oxysporum* lineages (Henry et al. 2017; Laurence et al. 2015).

For many crops, management of Fusarium wilt diseases has been achieved through deployment of genetically resistant varieties. The root-dip inoculation procedure we have used to confirm pathogenicity can be employed to screen germplasm and thereby identify differences in susceptibility to Fusarium wilt. Similar procedures have been used in breeding for resistance to wilt diseases in many other crops (Gordon and Koike 2015; Koike and Gordon 2015; McGovern 2015). However, in the case of blackberry, it will be necessary to determine if the multitude of genetically distinct strains interact differentially with host genotypes. If so, screening against a single isolate may not be sufficient to ensure that selections will manifest resistance that is broadly effective under field conditions.

Even where resistant cultivars are available, it is desirable to maintain pathogen inoculum levels in soil as low as possible. It is therefore useful to know to what extent crops grown in rotation with blackberry

may serve as reproductive hosts for the Fusarium wilt pathogen. Our findings indicate that neither raspberry, blueberry, nor lettuce are susceptible to disease caused by *F. oxysporum* f. sp. *mori*. Although the pathogen was recovered from inoculated plants of each of the aforementioned crops, this may be an artifact of the inoculation method. Further study will be required to determine if any of these crops allow for extensive, symptomless colonization.

The exception to the pattern noted above is strawberry, which was clearly susceptible to disease caused by *F. oxysporum* f. sp. *mori*. Because previous studies had revealed this possibility (T. R. Gordon, *unpublished*), we elected to include several cultivars in the present study. It was of interest to see that cultivars resistant to *F. oxysporum* f. sp. *fragariae* were also resistant to the blackberry pathogen. However, it remains to be determined how extensively resistant strawberry cultivars are colonized by *F. oxysporum* f. sp. *mori*.

In summary, Fusarium wilt of blackberry is caused by a diverse array of strains, which we propose be recognized as a new forma specialis of *F. oxysporum*. Further research is needed to determine if the nine SCGs identified to date manifest quantitative or qualitative

Fig. 3. Maximum likelihood tree of pathogenic *Fusarium oxysporum* strains isolated from blackberry and other *Fusarium oxysporum* formae speciales inferred from intergenic spacer (IGS) sequences. Strains were grouped into three main clades. Bootstrap values are noted at the internodes. Bootstrap values are based on 1,000 resamplings. Scale bar indicates 0.1% sequence dissimilarity.

differences in virulence to blackberry. This information will be critical to implementation of screening procedures to identify resistance that will be broadly effective against disease caused by *F. oxysporum* f. sp. *mori*.

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Literature Cited

- Baldwin, B. G., Goldman, D. H., Keil, D. J., Patterson, R., Rosatti, T. J., and Wilken, D. H. 2012. The Jepson Manual: Vascular plants of California, 2nd Ed. Univ. of California Press, Berkeley, CA.
- Cafri, D., Katan, J., and Katan, T. 2005. Cross-pathogenicity between formae speciales of *Fusarium oxysporum*, the pathogens of cucumber and melon. *J. Phytopathol.* 153:615-622.
- Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77:1640-1646.
- Enya, J., Togawa, M., Takeuchi, T., Yoshida, S., Tsushima, S., Arie, T., and Sakai, T. 2008. Biological and phylogenetic characterization of *Fusarium oxysporum* complex, which causes yellows on *Brassica* spp., and proposal of *F. oxysporum* f. sp. *rapae*, a novel forma specialis pathogenic on *B. rapa* in Japan. *Phytopathol.* 98:475-483.
- Gordon, T. R., Kirkpatrick, S. C., Henry, P. M., Kong, M., and Broome, J. C. 2016. First report of a wilt disease of blackberry caused by *Fusarium oxysporum* in California. *Plant Dis.* 100:1018.
- Gordon, T. R., and Koike, S. T. 2015. Management of Fusarium wilt of lettuce. *Crop Prot.* 73:45-49.
- Gordon, T. R., and Martyn, R. D. 1997. The evolutionary biology of *Fusarium oxysporum*. *Annu. Rev. Phytopathol.* 35:111-128.
- Gordon, T. R., and Okamoto, D. 1991. Vegetative compatibility groupings in a local population of *Fusarium oxysporum*. *Can. J. Bot.* 69:168-172.
- Henry, P. M., Kirkpatrick, S. C., Islas, C. M., Pastrana, A. M., Yoshisato, J. A., Koike, S. T., Daugovish, O., and Gordon, T. R. 2017. The population of *Fusarium oxysporum* f. sp. *fragariae*, cause of Fusarium wilt of strawberry, in California. *Plant Dis.* 101:550-556.
- Hernández-Cruz, A., Rebollar-Alviter, A., Silva-Rojas, H.V., and Urbina-Cortés, T.B. 2015. Identification and pathogenicity of *Fusarium* sp. associated with yellowing and wilting of blackberry (*Rubus* sp.) in Michoacán, México. *Phytopathology* 105:S4.59.
- Jacobson, D. J., and Gordon, T. R. 1988. Vegetative compatibility and self-incompatibility within *Fusarium oxysporum* f. sp. *melonis*. *Phytopathol.* 78:668-672.
- Katan, T. 1999. Current status of vegetative compatibility groups in *Fusarium oxysporum*. *Phytoparasitica* 27:51-64.
- Kistler, H. C. 1997. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. *Phytopathol.* 87:474-479.
- Koike, S. T., and Gordon, T. R. 2015. Management of Fusarium wilt of strawberry. *Crop Prot.* 73:67-72.
- Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soils. *Rev. Plant Prot. Res.* 8:114-125.
- Laurence, M. H., Summerell, B. A., and Liew, E. C. Y. 2015. *Fusarium oxysporum* f. sp. *canariensis*: Evidence for horizontal gene transfer of putative pathogenicity genes. *Plant Pathol.* 64:1068-1075.
- Leslie, J. F., Summerell, B. A., and Bullock, S., eds. 2006. The *Fusarium* Lab Manual. Blackwell, Ames, IA.
- Lievens, B., Van Baaren, P., Verreth, C., Van Kerckhove, S., Rep, M., and Thomma, B. P. H. J. 2009. Evolutionary relationships between *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* isolates inferred from mating type, elongation factor-1 α and exopolysaccharuronase sequences. *Mycol. Res.* 113:1181-1191.
- Ma, L.-J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M.-J., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P. M., Kang, S., Shim, W.-B., Woloshuk, C., Xie, X., Xu, J.-R., Antoniw, J., Baker, S. E., Bluhm, B. H., Breakspear, A., Brown, D. W., Butchko, R. A. E., Chapman, S., Coulson, R., Coutinho, P. M., Danchin, E. G. J., Diener, A., Gale, L. R., Gardiner, D. M., Goff, S., Hammond-Kosack, K. E., Hilburn, K., Hua-Van, A., Jonkers, W., Kazan, K., Kodira, C. D., Koehrsen, M., Kumar, L., Lee, Y.-H., Li, L., Manners, J. M., Miranda-Saavedra, D., Mukherjee, M., Park, G., Park, J., Park, S.-Y., Proctor, R. H., Regev, A., Ruiz-Roldan, M. C., Sain, D., Sakthikumar, S., Sykes, S., Schwartz, D. C., Turgeon, B. G., Wapinski, I., Yoder, O., Young, S., Zeng, Q., Zhou, S., Galagan, J., Cuomo, C. A., Kistler, H. C., and Rep, M. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature* 464:367-373.
- Mbofung, G. Y., Hong, S. G., and Pryor, B. M. 2007. Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* inferred from mitochondrial small subunit, elongation factor 1- α , and nuclear ribosomal intergenic spacer sequence data. *Phytopathol.* 97:87-98.
- McGovern, R. J. 2015. Management of tomato diseases caused by *Fusarium oxysporum*. *Crop Prot.* 73:78-92.
- Nelson, P. E., Toussoun, T. A., and Marasas, W. F. O. 1983. *Fusarium* Species: An Illustrated Manual for Identification. Pennsylvania State Univ. Press, University Park.
- O'Donnell, K., and Cigelnik, E. 1997. Two divergent Intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol. Phylogenet. Evol.* 7:103-116.
- O'Donnell, K., Gueidan, C., Sink, S., Johnston, P. R., Crous, P. W., Glenn, A., Riley, R., Zitomer, N. C., Colyer, P., Waalwijk, C., Van der Lee, T., Moretti, A., Kang, S., Kim, H. S., Geiser, D. M., Juba, J. H., and Baayen, R. P. 2009. A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal Genet. Biol.* 46:936-948.
- O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* 95:2044-2049.
- Puhalla, J. E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Can. J. Bot.* 63:179-183.
- Schmale, D. G., and Gordon, T. R. 2003. Variation in susceptibility to pitch canker disease, caused by *Fusarium circinatum*, in native stands of *Pinus muricata*. *Plant Pathol.* 52:720-725.
- Snyder, W. C., and Hansen, H. N. 1940. The species concept in *Fusarium*. *Am. J. Bot.* 27:64-67.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30:2725-2729.
- Troisi, M., Bertetti, D., Gullino, M. L., and Garibaldi, A. 2013. Race differentiation in *Fusarium oxysporum* f. sp. *chrysanthemi*. *J. Phytopathol.* 161:675-688.