

## Races of the Celery Pathogen *Fusarium oxysporum* f. sp. *apii* Are Polyphyletic

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Accepted for publication 3 December 2016.

### ABSTRACT

*Fusarium oxysporum* species complex (FOSC) isolates were obtained from celery with symptoms of Fusarium yellows between 1993 and 2013 primarily in California. Virulence tests and a two-gene dataset from 174 isolates indicated that virulent isolates collected before 2013 were a highly clonal population of *F. oxysporum* f. sp. *apii* race 2. In 2013, new highly virulent clonal isolates, designated race 4, were discovered in production fields in Camarillo, California. Long-read Illumina data were used to analyze 16 isolates: six race 2, one of each from races 1, 3, and 4, and seven genetically diverse FOSC that were isolated from symptomatic celery but are nonpathogenic on this host. Analyses of a 10-gene dataset

comprising 38 kb indicated that *F. oxysporum* f. sp. *apii* is polyphyletic; race 2 is nested within clade 3, whereas the evolutionary origins of races 1, 3, and 4 are within clade 2. Based on 6,898 single nucleotide polymorphisms from the core FOSC genome, race 3 and the new highly virulent race 4 are highly similar with Nei's  $D_a = 0.0019$ , suggesting that *F. oxysporum* f. sp. *apii* race 4 evolved from race 3. Next generation sequences were used to develop PCR primers that allow rapid diagnosis of races 2 and 4 in planta.

*Additional keywords:* *Apium graveolens*, Fusarium yellows, phylogeny.

Celery (*Apium graveolens* L. var. *dulce* (Miller) Pers.) is a major vegetable crop within the United States, with 12,000 ha planted with a 458-million-dollar value in 2013 (USDA National Agricultural Statistics Service 2014). Approximately 95% of celery production in the United States occurs in California and Michigan. Historically, Fusarium yellows caused by *F. oxysporum* f. sp. *apii* has been the most important disease in celery production (Correll et al. 1986a).

Fusarium yellows was first reported on the self-blanching celery cultivar Golden Self Blanching in 1914 in Michigan where it was responsible for severe outbreaks (Nelson et al. 1937). *F. oxysporum* f. sp. *apii* race 1 apparently spread on infected or infested seed or seedlings to other Midwestern states, the eastern United States, and eventually to California where it was first reported as a serious problem near Lodi in the Central Valley in 1936 (Nelson et al. 1937). *F. oxysporum* f. sp. *apii* race 1 was managed with *F. oxysporum* f. sp. *apii*-resistant cultivars, particularly green cultivars, such as Tall Utah 52-70, first introduced in 1952. Few reports of Fusarium yellows on celery between 1952 and 1976 are available. However, in 1976 a race 2 of *F. oxysporum* f. sp. *apii* was first identified in California as being virulent on Tall Utah 52-70 and Golden Self Blanching (Hart and Endo 1978; Otto et al. 1976). Since then, *F. oxysporum* f. sp. *apii* race 2 has been a production threat in California and in the other areas where it has been identified: Michigan in 1982 (Toth and Lacy 1991), New York in 1984 (Awuah et al. 1986), Texas in 1987 (Martyn et al. 1987), and Ontario, Canada in 1989 (Cerkaskas and McDonald 1989). In 1984, Puhalla (1984) reported that there were two *F. oxysporum* f. sp. *apii* races in California: race 2 and race 3, with the latter being virulent on Tall Utah cultivars but

avirulent on Golden Self Blanching. All *F. oxysporum* f. sp. *apii* race 2 isolates belong to vegetative compatibility group 0010 (Correll et al. 1986a; Puhalla 1985; Toth and Lacy 1991), which is distinct from *F. oxysporum* f. sp. *apii* races 1 (vegetative compatibility group [VCG] 0011 and 0012) and 3 (VCG code not designated) (Puhalla 1984), nonpathogenic isolates from celery, and other *F. oxysporum* formae speciales (Correll et al. 1986b; Puhalla 1985).

Following an extensive screening of celery germplasm for resistance against *F. oxysporum* f. sp. *apii* race 2, a major resistance gene *Ful* from celeriac (*A. graveolens* var. *rapaceum* PI169001) was identified (Orton et al. 1984a, b; Quiros et al. 1993). *Ful* and minor genes were introduced into commercial cultivars including Challenger, Stix, Sabroso, and Command. This source of resistance has been the mainstay of Fusarium yellows management for nearly three decades. In 2013, celery production in California was confronted with a new challenge with the discovery of the novel *F. oxysporum* f. sp. *apii* race 4 in Camarillo, CA. This race is highly virulent on race 2-resistant Challenger, the older cultivar Tall Utah 52-70 R Improved, and a variety of current cultivars in California.

Races are defined based on virulence on differential cultivars: race 1, virulent on Golden Self-Blanching and avirulent on Tall Utah 52-70 R Improved; race 2, virulent on Golden Self Blanching and Tall Utah 52-70 R Improved, and avirulent on Challenger; race 3, avirulent on Golden Self Blanching and Challenger, and virulent on Tall Utah 52-70 R Improved; and race 4, virulent on Golden Self Blanching, Tall Utah 52-70 R Improved, and Challenger. The objectives of the current study were to (i) determine the diversity of *F. oxysporum* f. sp. *apii* races in California by conducting standard greenhouse virulence assays on differential celery cultivars for race evaluation; (ii) determine the phylogenetic and phylogenomic diversity of *F. oxysporum* f. sp. *apii* races and putatively nonpathogenic FOSC isolates recovered from celery crown and root tissues with symptoms of Fusarium yellows; and (iii) use the comparative genomic data to develop a PCR assay for the rapid detection and identification of the economically important *F. oxysporum* f. sp. *apii* races in planta.

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\*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary figure, three supplementary tables, and two supplementary material files are published online.

## MATERIALS AND METHODS

**Culture and storage conditions.** Celery plants symptomatic for Fusarium yellows were collected and brought to the laboratory. Symptomatic plants with visible rotting were not sampled to avoid isolation of secondary organisms. After crown and root tissue of the symptomatic plants were washed in tap water for 2 min, 1 to 1.5 cm segments of either roots or crown with symptoms consistent with Fusarium yellows were surface-disinfested with 0.62% sodium hypochlorite for 2 min, and blotted dry; elimination of the standard post hypochlorite aqueous rinse reduced contamination without having any apparent effect on pathogen recovery. Segments that included a small discolored portion and the adjacent nondiscolored tissue were plated onto potato dextrose agar (PDA) amended with 100 µg of chloramphenicol/ml. After incubation under approximately 5,000 lux cool-white fluorescent lights for 5 to 7 days at 22°C, colonies with appearance and morphology consistent with members of the FOSC were transferred onto new PDA plates without chloramphenicol and grown for 3 to 5 days. Colonies were then hyphal-tip purified and cultures were archived. For axenic storage, 0.5 cm<sup>2</sup> pieces of sterile filter paper were placed on PDA. After dishes were inoculated and incubated for 8 to 10 days under lights as indicated above, filter paper discs were transferred to a sterile dish, dried in a laminar flow hood for 24 h, transferred to sterile 3-ml tubes, capped, and stored at 5°C.

To assess whether archived isolates from celery were *F. oxysporum* f. sp. *apii*, 31 isolates from California that were primarily collected between 1993 and 1996, and six isolates from Ohio that were collected in 1996 were tested for virulence. From 2010 to 2014, we collected an additional 121, 8, 4, and 4 FOSC isolates from celery in California, Wisconsin, Michigan, and Ontario, Canada, respectively. After early results with Koch's postulates indicated that *F. oxysporum* f. sp. *apii* race 2 was the cause of Fusarium yellows in California, our collections between 2010 and 2014 were biased toward the diversity of isolates emerging from the margin of symptomatic tissue. We also tested one archived *F. oxysporum* from celery (NRRL 38295, =FRC O-1120), which was deposited in 1981 by California researcher S. N. Smith, as the "T" strain; we now refer to this strain as FoaR3.

**Pathogenicity tests.** Isolates were grown on PDA for 7 days under continuous fluorescent light at 22°C before harvesting conidia in 0.1% water agar. Before inoculation, 3 to 4 cm of the root tips of 8-week-old transplants were excised. Freshly cut root tips were dipped in a suspension of 10<sup>7</sup> conidia per ml in 0.1% water agar for 5 min and then transplanted in a steam-sterilized perlite/soil (3:1) mix in planting tubes. Water agar (0.1%) was used for a mock-inoculated control. All isolates were tested with either 8 or 10 replicates per cultivar on Tall Utah 52-70 R Improved and Challenger. All potentially nonpathogenic isolates and representatives of each race were also inoculated onto cultivar Golden Self Blanching. To establish reproducibility of results, each experimental trial included two race 2 isolates with demonstrated virulence.

After 8 weeks incubation, plants were harvested, washed, and scored for typical Fusarium yellows symptoms on a 0 to 5 severity scale: 0, asymptomatic; 1, lateral roots discolored; 2, main root discolored; 3, crown discolored; 4, crown extensively discolored; and 5, plant dead. Based on the mock-inoculated controls, interactions between race and cultivar that were >0.0 and <1.0 were rated as nonpathogenic. To fulfill Koch's postulates, reisolations and inoculations were performed with representatives of each race.

**Two-locus DNA sequences.** FOSC isolates from celery are listed in Supplementary Table S1. To obtain material for a genomic DNA miniprep, 40 to 50 mg of mycelium was scraped off the surface of 3- to 4-day-old cultures on PDA (MP Biomedicals). DNA was extracted and purified as described previously (Daugovish et al. 2012)

and the concentration was determined with a NanoDrop ND-1000 spectrophotometer. An amplicon of the translation elongation factor 1- $\alpha$  (*TEF1*) was PCR-amplified as previously described (O'Donnell et al. 1998). Briefly, a 700-bp portion of *TEF1* $\alpha$  was amplified in a total volume of 20 µl containing 1× Go Taq Green Master Mix, 0.25 µM EF-1 and EF-2 primers, and 8 to 10 ng of template DNA. PCR conditions were 94°C for 10 min, 40 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. A 970-bp portion of the IGS rDNA was PCR-amplified in a total volume of 25 µl containing 1× Go Taq Green Master Mix, 0.1 µM of the iNL11 (O'Donnell et al. 2009) and FoIGS-R primer (5'GCCGACACCGCGCTCTTAA3') and 8 to 10 ng of template DNA. PCR conditions were 94°C for 90 s, 35 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 60 s, and a final extension at 72°C for 10 min.

After PCR, the amplified products were purified with an AccuPrep PCR Kit (Bioneer Corporation, Alameda, CA) and then sequenced at the Department of Biological Sciences sequencing facility at the University of California at Davis. All sequences were manually inspected for quality, aligned with either ClustalW2 or Muscle ([www.ebi.ac.uk/Tools/msa/muscle/](http://www.ebi.ac.uk/Tools/msa/muscle/)), and trimmed. All polymorphisms were checked manually. NCBI GenBank accession numbers for the 174 isolates for *TEF1* are KX619056 to KX619229 and for IGS are KX619230 to KX619403.

**Next generation sequencing (NGS).** Sixteen FOSC isolates from celery were selected for NGS (Table 1). Total genomic DNA was purified using a protocol modified from M. Rep, University of Amsterdam. Briefly, FOSC were cultured on PDA as indicated above. Conidia from six to eight 100-mm-diameter Petri dishes were harvested in water, filtered through Miracloth to remove mycelia, washed in water, and then pelleted by centrifugation. The spore pellet was frozen, lyophilized, and ground in liquid N<sub>2</sub> with ≤106-µm-diameter-glass beads (Sigma-Aldrich, St. Louis, MO). After DNA was extracted in 225 mM sodium 4-aminosalicylate dehydrate, 22 mM triisopropylmethylsulfonic acid sodium salt, 200 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 50 mM EDTA, contaminants were removed using the phenol/chloroform/isoamyl alcohol method and a Qiagen genomic DNA (Germantown, MD) kit. Dual indexed libraries were prepared with a Nextera kit (Illumina, San Diego, CA), except for Foa\_R3 and one of two preparations of 274.AC, which were prepared with a Kapa Hyper Prep kit (Kapa Biosystems, Wilmington, MA). When indicated in Table 1, libraries were whole genome-amplified for 16 h at 30°C with a REPLI-g Mini kit (Qiagen, Germantown, MD). Insert size and library quality was confirmed before sequencing using the Agilent 2100 Bioanalyzer (Agilent Technologies). DNA from the 16 strains were sequenced on an Illumina MiSeq platform as 2 × 250 bp paired end reads at the University of California at Davis Genome Center; Illumina reads from 12 additional FOSC strains sequenced by the Broad Institute were downloaded from the NCBI Short Read Archives (Ma et al. 2010, 2014). All Illumina reads were mapped to the NRRL 34936 *F. oxysporum* f. sp. *lycopersici* 4287 (FO2) reference using BWA MEM (Li and Durbin 2010). Polymorphisms were called using the GATK pipeline (McKenna et al. 2010), which considers indel realignment and base quality score recalibration, and calls variants across all samples simultaneously through the HaplotypeCaller program. Variants were filtered using standard hard filtering parameters according to GATK Best Practices recommendations (DePristo et al. 2011; Van der Auwera et al. 2013). A total of 472,629 single nucleotide polymorphisms (SNP) were called across all 28 samples. Of these SNPs, for phylogenetic and diversity analysis, a subset of 6,898 SNPs was selected with each SNP at a minimum of 5 kb apart. The Illumina data from the 16 strains are deposited in the NCBI Short Read Archive under BioProject PRJNA318926.

**Phylogenetic analyses.** We first examined an aligned, 182 taxon two-locus dataset that contained partial *TEF1* + IGS rDNA

sequences (1.3 kb alignment); the study included the 174 FOSC that we isolated from celery symptomatic for Fusarium yellows and one *F. oxysporum* f. sp. *apii* that we obtained from the ARS Culture Collection as NRRL 38295 (GenBank FJ985371.1 and FJ985604.1) that we characterized as *F. oxysporum* f. sp. *apii* race 3. We also included five previously well-characterized FOSC in order to place all of the isolates within the previously defined FOSC clades 1 to 3 (NRRL 32931 from human; NRRL 34936 f. sp. *lycopersici* race 2; NRRL 37622, =HDV247, =NRRL54007 f. sp. *pisi*; NRRL 26035 (=CBS 130183) f. sp. *canariensis*; and NRRL 36111 (=CBS 102022) f. sp. *cubense*) (O'Donnell et al. 1998, 2009), and the sequences of two *F. foetens* to root the phylogeny (O'Donnell et al. 2013). Before conducting the phylogenetic analyses, the 182 taxon dataset was collapsed using Collapser ver. 1.1 (Posada 1999) to a set of 31 unique two-locus haplotypes plus a 32nd haplotype so that both f. sp. *apii* race 4 (11 isolates) and NRRL 38295 f. sp. *apii* race 3 were included in the phylogenetic analysis, even though races 3 and 4 shared the same two-locus haplotype. Maximum likelihood (ML) analyses were conducted with GARLI 1.0 (Zwickl 2006) employing the GTR + I +  $\Gamma$  model of evolution on the CIPRES Science Gateway TeraGrid (<http://www.phylo.org>); maximum parsimony (MP) analyses were implemented with PAUP\* 4.0b10 (Swofford 2003), employing 10 random addition sequences per replicate, TBR branch swapping, and 1,000 pseudoreplicates of the data. ML and MP clade support was assessed by 1,000 bootstrapped pseudoreplicates of the data.

We obtained full genomic sequence data of 12 phylogenetically diverse FOSC reference strains from the Broad Institute: NRRL 34936 f. sp. *lycopersici* race 2; NRRL 26406 f. sp. *melonis*; NRRL 54003 (=MN25) f. sp. *lycopersici* race 3; NRRL 54002 (=Fo47) biocontrol strain from soil; NRRL 26381 (CL57) f. sp.

*radicis-lycopersici*; NRRL 25433 f. sp. *vasinfectum*; NRRL 37622 (=HDV247, =NRRL54007) f. sp. *pisi*; NRRL 32931 from human; NRRL 54005 (=PHW815) f. sp. *raphani*; NRRL 54008 (=PHW808) f. sp. *conglutinans* race 2; NRRL 66176 (=Fo5176) from *Arabidopsis*; and NRRL 54006 (IL5) f. sp. *cubense* tropical race 4. Ten phylogenetically informative loci that were mined from the genomes of the FOSC reference strains were kindly provided by L.-J. Ma, University of Massachusetts at Amherst. Loci and their chromosomal locations in the reference genome of NRRL 34936 *F. oxysporum* f. sp. *lycopersici* race 2 (=4287) are as follows: chromosome 1, DNA-directed RNA polymerase III subunit *RPC2* and *RPB1*; chromosome 4, minichromosome maintenance protein 3 and anaphase-promoting complex subunit 1; chromosome 5, two hypothetical proteins FOXG\_1751.3 and 2073.3; chromosome 7, DNA-directed RNA polymerase II subunit *RPB2*; chromosome 8, hypothetical protein FOXG 3560.3; and chromosome 9, clathrin heavy chain and DNA polymerase gamma (Supplementary Table S2 provides additional details). Sequences of these 10 genes were mined from our 16 NGS strains and from the genomes of the 12 FOSC reference strains. From these data, we obtained a 10-gene dataset totaling 38 kb for 28 taxa. The data were analyzed phylogenetically by MP and ML as indicated above. The Kishino-Hasegawa likelihood test was used to assess whether the 10 genes could be analyzed as a combined dataset; most parsimonious trees (MPTs) were inferred from the individual partitions and then each dataset was constrained to the topology of a MPT inferred from the 10-gene combined dataset. The Kishino-Hasegawa likelihood test also was used to assess the monophyly of the four *F. oxysporum* f. sp. *apii* races and to determine if any of the partitions within the 10-gene dataset were genealogically discordant.

TABLE 1. Origin, race, and disease severity ratings of 16 *Fusarium oxysporum* species complex (FOSC) isolates from celery with symptoms of Fusarium yellows that were subjected to next generation sequencing with Illumina MiSeq

Sample ID	Year isolated	Collection location <sup>o</sup>	<i>F. oxysporum</i> f. sp. <i>apii</i> race	Celery cultivars <sup>a</sup>			NGS analysis		
				Tall Utah	Challenger	Golden	FOSC clade <sup>d</sup>	WGA <sup>r</sup>	Coverage depth <sup>s</sup>
				Disease ratings <sup>p</sup>					
268.2	2012	Davis, CA	Race 1	0.1 d	0.3 bc	1.6 bc	1	WGA	8.3
003	1993	Oxnard, CA	Race 2	2.9 b	0.6 bc	1.2 cd	3	No	8.6
226.2A	2011	Oxnard, CA	Race 2	4.1 a	0.4 bc	NT <sup>t</sup>	3	WGA	10.1
247.1A	2011	Oxnard, CA	Race 2	2.8 b	0.6 bc	2.8 ab	3	No	10.2
067	1994	Santa Maria, CA	Race 2	3.2 b	0.8 bc	1.5 bcd	3	WGA	8.3
207.A1A2 <sup>u</sup>	2010	Santa Maria, CA	Race 2	3.4 ab	0.4 bc	NT	3	WGA	14.7
258.1B	2011	Santa Maria, CA	Race 2	3.2 b	1.0 b	NT	3	WGA	16.1
Foa_R3 <sup>v</sup>	Before 1981	CA (presumed)	Race 3	1.8 c	0.3 bc	0.8 cd	2	WGA	26.0
274.AC	2013	Camarillo, CA <sup>w</sup>	Race 4	3.0 b	3.2 a	3.4 a	2	WGA	24.0
270.A	2012	Berlin, WI	NP <sup>x,y</sup>	0.0 d	0.0 c	0.2 cd	2	No	11.0
223.1A	2010	Oxnard, CA	NP <sup>y</sup>	0.1 d	0.0 c	0.0 d	3	No	12.2
226.1A	2011	Oxnard, CA	NP	0.0 d	0.0 c	0.0 d	3	WGA	22.6
226.1B	2011	Oxnard, CA	NP	0.2 d	0.0 c	0.0 d	3	No	11.3
241.1A	2011	Oxnard, CA	NP	0.0 d	0.2 bc	0.0 d	3	No	9.7
221-B	2010	Santa Maria, CA	NP	0.0 d	0.1 bc	0.5 cd	3	No	14.1
261.1A	2011	Santa Maria, CA	NP	0.0 d	0.0 c	0.8 cd	3	WGA	10.4
Mock	—	—	—	0.05 d	0.05 d	0.2 d	NA <sup>z</sup>	NA	NA

<sup>a</sup> Tall Utah, Tall Utah 52-70 R Improved; Golden, Golden Self Blanching.

<sup>o</sup> CA, California; WI, Wisconsin.

<sup>p</sup> For Tall Utah and Challenger, 8 weeks postincubation, either 8 or 10 plants/cultivar in a completely randomized design for each of two trials were scored for typical Fusarium yellow symptoms on a 0 to 5 severity scale: 0, asymptomatic; 1, lateral roots discolored; 2, main root discolored; 3, crown discolored; 4, crown extensively discolored; and 5, plant dead. Strains were tested on Golden Self Blanching in one trial with 10 replicates, except for race 1, which was tested in two trials. Within a column, means followed by the same letter are not significantly different ( $\alpha = 0.05$ ) according to Tukey's honest significant difference test.

<sup>d</sup> *F. oxysporum* species complex, based on the next generation sequence analysis shown in Table 2 and Figure 2.

<sup>r</sup> WGA, whole genome amplification.

<sup>s</sup> Estimates of coverage are based on the analysis of the 6,898 polymorphic single nucleotide polymorphisms as shown in Table 3.

<sup>t</sup> NT, not tested.

<sup>u</sup> Equivalent IDs 207.A, 207A1A2.

<sup>v</sup> Equivalent IDs Foa-R3: NRRL 38295; O-1120 = FRC O-1120.

<sup>w</sup> Camarillo, CA is adjacent to Oxnard, CA.

<sup>x</sup> NP, nonpathogenic.

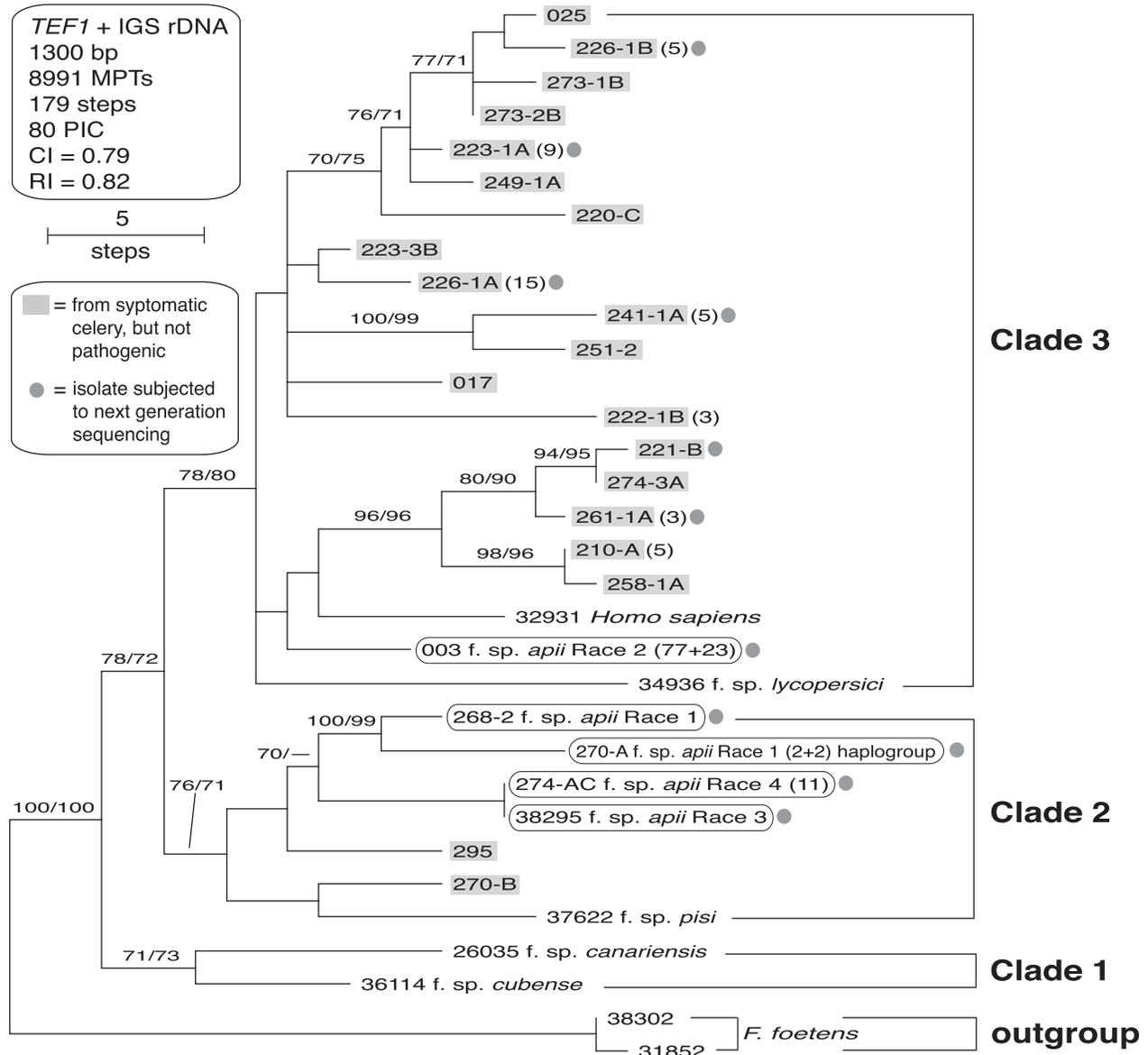
<sup>y</sup> Nonpathogenic, but in a two-locus haplogroup with *F. oxysporum* f. sp. *apii* race 1 from Michigan.

<sup>z</sup> NA, not applicable.

***F. oxysporum* f. sp. *apii* race 2 haplogroup and race 4 PCR assays.** To develop primers that differentiate between the race 2 haplogroup and race 4, Illumina sequences were assembled de novo for each of the 28 strains. Reads were filtered with Trimmomatic (Bolger et al. 2014) and assembled using Velvet (Zerbino and Birney 2008) with a kmer size of 35. Assembled sequences from all strains were combined using CAP3 (Huang and Madan 1999) to produce a common reference. All programs were run with default parameters. Illumina reads were then mapped using BWA and the reference was cut into 1 kb segments. The number of reads mapped in each segment was counted separately for each strain. To reduce issues associated with

sequence duplication and paralogy, for each respective strain, segments were considered present only when the read count was within 2 standard deviations of the median count for that strain. Candidate segments were identified as those present in either *F. oxysporum* f. sp. *apii* race 2 or race 4, with a 0 read count in all of the other 26 strains, and no homology in either the *Fusarium* Comparative Genomics Platform (FCGP, <http://genomics.fusariumdb.org/intro.php>) or NCBI GenBank.

Primers for PCR were designed with the Primer3Plus software (<http://primer3plus.com/>). We again confirmed that the selected *F. oxysporum* f. sp. *apii* race 2 haplogroup and race 4 primers were not in either FCGP or NCBI GenBank. The efficiency of candidate



**Fig. 1.** Maximum parsimony phylogram inferred from portions of *TEF1* and IGS rDNA totaling 1.3 kb from 175 *Fusarium oxysporum* species complex (FOSC) isolates from celery with symptoms of Fusarium yellows. Celery isolates with identical sequences were collapsed to 25 two-locus haplotypes using Collapse 1.1 (Posada 1999) to facilitate the phylogenetic analysis. Note that f. sp. *apii* races 3 and 4 were both included in the analysis even though they share an identical two-locus haplotype. Eleven strains that were analyzed by next generation sequencing are identified by a gray circle and described in Table 1. The number of strains in each haplogroup is indicated in parentheses; in cases in which some of the isolates were pathogenic and some were nonpathogenic, the number of isolates is shown as the number of pathogenic + the number of nonpathogenic isolates. For example, for the isolates in the haplogroup with f. sp. *apii* race 2-type strains, (77 + 23) indicates that 77 were virulent on Tall Utah 52-70 R Improved and 23 were nonpathogenic. Similarly, in the case of the 270-A haplogroup, two of the isolates were f. sp. *apii* race 1 and two were nonpathogenic on the three cultivars; a third f. sp. *apii* race 1 (268-2) was in another haplogroup. The tree is rooted on sequences of *F. foetens* as the outgroup. Selected non-celery isolates, identified by a five-digit NRRL strain number followed by their host, were included in the analysis to identify FOSC clades. Numbers at the nodes indicate bootstrap support based on 1,000 maximum likelihood (ML) and maximum parsimony (MP) bootstrap (BS) pseudoreplicates (i.e., ML-BS/MP-BS). The NEXUS file of the collapsed dataset is provided as Supplementary Material S1.

TABLE 2. Summary of marker loci and tree statistics for the 10 individual partitions and combined dataset (Fig. 2)

Gene name	Chromosome no. <sup>†</sup>	bp <sup>‡</sup>	MPTs <sup>‡</sup>	Tree length	CI <sup>‡</sup>	RI <sup>‡</sup>	AUT <sup>‡</sup>	PIC <sup>‡</sup>
Hypothetical protein	5	2,883	13	15	1	1	1	14
Hypothetical protein	5	5,668	18	32	0.91	0.98	3	26
Hypothetical protein	8	1,902	616	20	0.85	0.97	0	17
Minichromosome maintenance protein 3	4	2,640	1	20	0.95	0.99	1	18
Anaphase-promoting complex subunit 1	4	5,357	42	46	0.93	0.99	2	40
Clathrin heavy chain	9	3,630	1	15	0.93	0.99	0	14
DNA directed polymerase	9	3,420	1	22	0.91	0.98	0	20
RNA polymerase II subunit ( <i>RPB2</i> )	7	3,813	17	31	0.87	0.97	4	23
RNA polymerase III subunit	1	3,474	2	22	1	1	0	22
RNA polymerase II subunit ( <i>RPB1</i> )	1	5,253	36	20	0.85	0.97	0	17
Combined	–	38,060	18	295	0.76	0.94	11	211

<sup>†</sup> Chromosome number is based on the whole genome sequence of NRRL 34936 (*Fusarium oxysporum* f. sp. *lycopersici* 4287) (Ma et al. 2010).

<sup>‡</sup> bp, base pairs.

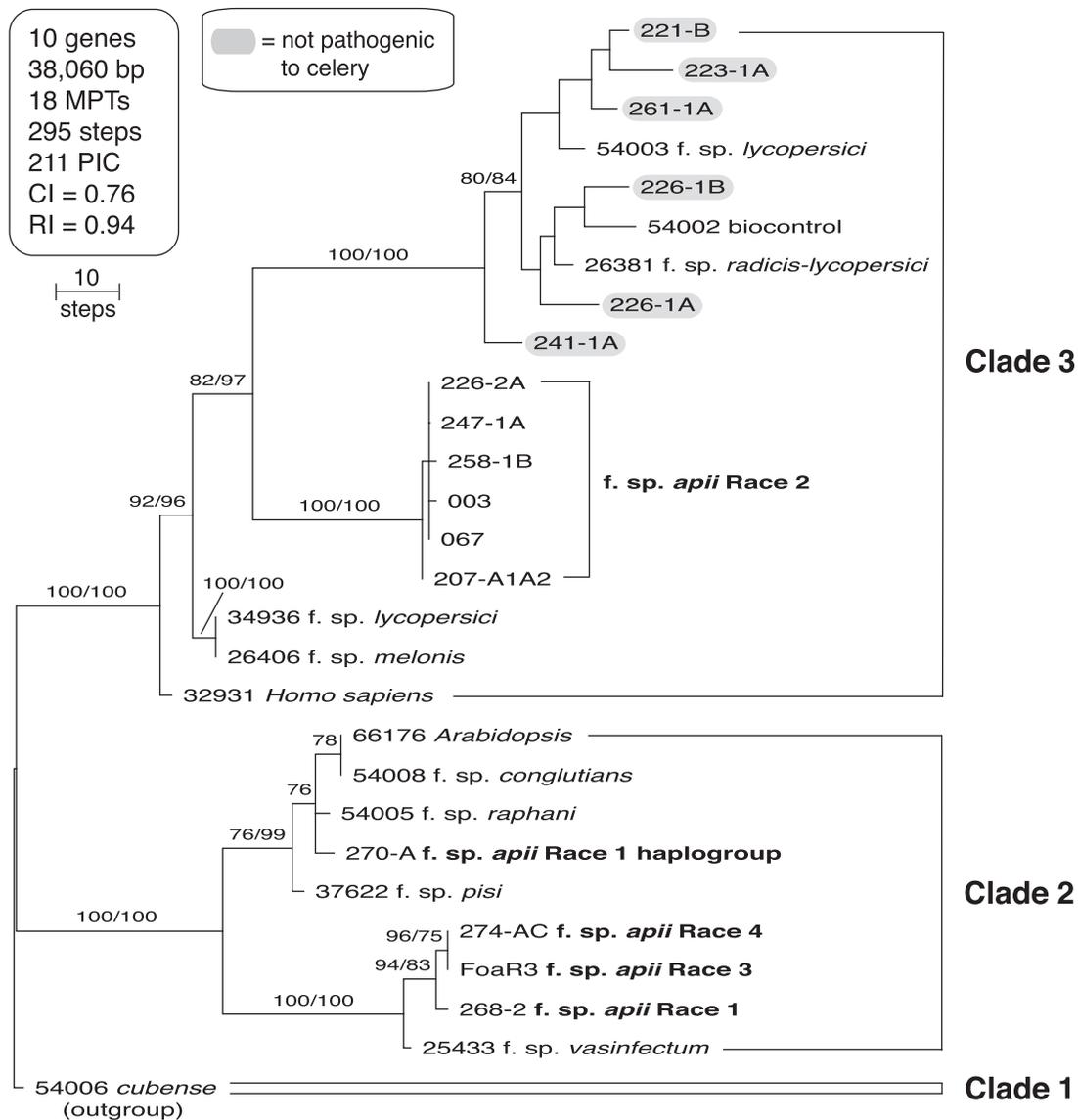
<sup>‡</sup> MPTs, most-parsimonious trees.

<sup>‡</sup> CI, consistency index, which is determined by dividing the minimum possible number of steps by those observed.

<sup>‡</sup> RI, retention index.

<sup>‡</sup> AUT, autapomorphy or a derived character unique to a particular taxon (i.e., not parsimony-informative).

<sup>‡</sup> PIC, parsimony-informative or shared derived character.



**Fig. 2.** Maximum parsimony phylogram inferred from 10 genes from 28 FO SC isolates, including 16 strains from celery with symptoms of *Fusarium* yellows that were analyzed by next generation sequencing and 12 deeply sequenced strains at the Broad Institute. The latter are identified by the five-digit NRRL accession number. The tree was rooted on sequences of NRRL 54006 *Fusarium oxysporum* f. sp. *cubense* as the outgroup based on more inclusive analyses (O'Donnell et al. 2009). Numbers at the nodes indicate bootstrap support based on 1,000 maximum likelihood (ML) and maximum parsimony (MP) bootstrap (BS) pseudoreplicates (i.e., ML-BS/MP-BS). The NEXUS file of the collapsed dataset is provided as Supplementary Material S2.

TABLE 3. Nei's *D<sub>a</sub>* of pairwise comparisons of 6,898 single nucleotide polymorphisms (SNPs) in the 16 next generation sequenced *Fusarium oxysporum* species complex (FOSC) strains from symptomatic celery and the 12 FOSC reference strains at the Broad Institute<sup>w</sup>

Isolate <sup>z</sup>	FOSC clade 3 <sup>x</sup>													
	<i>lyco</i> <sup>y</sup>	<i>melo</i> <sup>y</sup>	<i>Foa</i> race 2						Nonpathogens (NP) in celery					
	34936	26406	207.A	258.1B	226.2A	067	003	247.1A	226.1A	241.1A	223.1A	221.B	261.1A	226.1B
34936	0.000	0.025	0.085	0.085	0.085	0.086	0.085	0.085	0.139	0.144	0.153	0.155	0.156	0.161
26406		0.000	0.088	0.088	0.089	0.089	0.089	0.089	0.143	0.148	0.157	0.159	0.160	0.165
<b>207.A</b>			0.000	0.001	0.001	0.002	0.001	0.002	0.093	0.098	0.104	0.107	0.110	0.112
<b>258.1B</b>				0.000	0.001	0.001	0.002	0.002	0.092	0.097	0.103	0.106	0.109	0.111
<b>226.2A</b>					0.000	0.002	0.002	0.002	0.093	0.097	0.103	0.107	0.110	0.112
<b>067</b>						0.000	0.002	0.002	0.093	0.098	0.103	0.107	0.110	0.113
<b>003</b>							0.000	0.002	0.093	0.097	0.104	0.107	0.110	0.113
<b>247.1A</b>								0.000	0.093	0.097	0.104	0.107	0.109	0.113
<b>226.1A</b>									0.000	0.067	0.053	0.065	0.070	0.068
<b>241.1A</b>										0.000	0.072	0.077	0.077	0.080
<b>223.1A</b>											0.000	0.053	0.055	0.064
<b>221.B</b>												0.000	0.043	0.061
<b>261.1A</b>													0.000	0.064
<b>226.1B</b>														0.000
54003														
54002														
26381														
<b>268.2</b>														
<b>274.AC</b>														
<b>FoaR3</b>														
25433														
270.A														
54007														
32931														
54005														
54008														
66176														
54006														

(continued on next page)

<sup>w</sup>The NRRL 34936 (=4287) *F. oxysporum* f. sp. *lycopersici* assembly was used as the reference strain (Ma et al. 2010). After mapping sequences of the 11 FOSC strains from the Broad Institute and our 16 FOSC strains from celery onto the reference strain, 6,898 SNPs were identified. Strains from celery are in bold type.

<sup>x</sup> FOSC clades, guided by results in Figure 2, are added for reference. Based on the 10-gene phylogeny in Figure 2, the human pathogenic FOSC NRRL 32931 is at the base of clade 3; in the structure analysis here, it is within FOSC clade 2.

<sup>y</sup> Reference strains abbreviations: *lyco*, f. sp. *lycopersici*; *melo*, f. sp. *melonis*; Bioc, biocontrol strain Fo47; *radi*, f. sp. *radici-lycopersici*; *vasi*, f. sp. *vasinfectum*; *Homo*, human pathogen; *raph*, f. sp. *raphani*; *cong*, f. sp. *conglutinans*; *Arab*, *Arabidopsis*-infecting; and *cube*, f. sp. *cubensis*.

<sup>z</sup> FOSC reference strains are indicated by the NRRL five-digit numbers.

primers with the appropriate race-specificity was checked using real-time quantitative PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA) with a dilution series of purified and Qubit-quantified *F. oxysporum* f. sp. *apii* DNA; efficiency was >90%. DNA was extracted from cultures or naturally infected celery crowns as described previously (Daugovish et al. 2012) except that the DNA concentration from crown tissue was determined using Qubit 2.0 (Invitrogen, Life Technologies, Carlsbad, CA). The 20- $\mu$ l PCR reaction mixture contained 1 $\times$  Go Taq Green Master Mix, 200 nM of the indicated primers, 0.2  $\mu$ g of BSA, and either 2 or 4 ng of DNA from a culture, or 10 ng of DNA from a celery crown. PCR conditions were 94°C for 10 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min.

The candidate *F. oxysporum* f. sp. *apii* race 2 haplogroup and race 4 primers were first tested on DNA extracts from strains that had been used to select the primers: the 16 NGS isolates; and 11 of the full genome-sequenced reference strains (NRRL 25433, 26381, 26406, 32931, 34936, 54002, 54003, 54005, 54006, 54007, and 54008). Candidate primers were further tested on field-grown celery that was infected with either *F. oxysporum* f. sp. *apii* race 2 or race 4, the 10 additional *F. oxysporum* f. sp. *apii* race 4 isolates, nonpathogenic isolates from six different fields that were in the race 2 haplogroup (205C, 222-2A, 235-A, 244-2B, 251-4, and 260-1), and representatives from the haplotypes in our collection of *Fusarium* spp. from celery that were not included in the NGS isolates: nonpathogens 017, 210-A, 220-C, 221-B,

223-3B, 249-1A, 251-2, 258-1A, 273-1B, 273-2B, 274-3A, and 295; and race 1 250-7 from Michigan. We also tested the primers on two strains of the sister taxa of FOSC, (*F. foetens* NRRL 31852 and 38302) and 12 additional FOSC: two additional FOSC used in our phylogenetic analyses (NRRL 26035 and 36114), two additional FOSC f. sp. *radices-lycopersici* (NRRL 26379 and 26380), three additional FOSC f. sp. *lycopersici* (NRRL 36379, 6423, 36423 and 36425), *F. oxysporum* f. sp. *fragariae* GL1080, and four archived FOSC sp. *apii* (NRRL 22534, 36312, 36287, and 36316) that were reported in O'Donnell et al. (2009). Each extract was tested with three primer pairs: *TEF1* as a positive control to verify that each extract had amplifiable DNA; FoaRace2 N4851, and FoaRace4 N3875. All samples tested amplified with *TEF1*.

## RESULTS

One hundred seventy-four FOSC isolates from the crown and root tissue of celery symptomatic for Fusarium yellows were collected in Californian commercial fields from 1993 to 2014. The 174 isolates were derived from 135 plants and, based on the virulence tests, included isolates from *F. oxysporum* f. sp. *apii* race 1 (*N* = 3), *F. oxysporum* f. sp. *apii* race 2 (*N* = 77), *F. oxysporum* f. sp. *apii* race 4 (*N* = 11) and 83 that were nonpathogenic on celery (Fig. 1). Symptomatic plants had a distinctive orangish-brown discoloration in the vasculature in the crown and/or roots, the leaves were often chlorotic and the plants were generally stunted. Virulence tests revealed that the archived isolate from celery (NRRL 38295) is

TABLE 3. (continued from preceding page)

FOSC clade 2 <sup>x</sup>													Clade 1 <sup>x</sup>
<i>lyco</i> <sup>y</sup>	<i>Bioc</i> <sup>y</sup>	<i>radi</i> <sup>y</sup>	CA <i>Foa</i> race 1	<i>Foa</i> race 4	<i>Foa</i> race 3	<i>vasi</i> <sup>y</sup>	<i>Foa</i> race 1 haplotype	<i>pisi</i> <sup>y</sup>	<i>Homo</i> <sup>y</sup>	<i>raph</i> <sup>y</sup>	<i>cong</i> <sup>y</sup>	<i>Arab</i> <sup>y</sup>	<i>cube</i> <sup>y</sup>
54003	54002	26381	<b>268.2</b>	<b>274. AC</b>	<b>FoR3</b>	25433	<b>270.A</b>	54007	32931	54005	54008	66176	54006
0.171	0.174	0.174	0.219	0.211	0.212	0.239	0.233	0.261	0.263	0.269	0.264	0.270	0.353
0.175	0.178	0.178	0.224	0.215	0.216	0.243	0.236	0.264	0.266	0.272	0.268	0.275	0.357
0.122	0.122	0.125	0.187	0.179	0.181	0.207	0.200	0.229	0.230	0.235	0.231	0.237	0.308
0.121	0.122	0.124	0.187	0.179	0.181	0.207	0.200	0.229	0.231	0.236	0.231	0.237	0.307
0.122	0.122	0.124	0.186	0.179	0.181	0.206	0.200	0.228	0.230	0.235	0.231	0.237	0.307
0.122	0.122	0.125	0.187	0.180	0.181	0.207	0.201	0.229	0.231	0.236	0.231	0.237	0.308
0.122	0.123	0.125	0.187	0.180	0.181	0.207	0.201	0.229	0.231	0.235	0.231	0.237	0.308
0.122	0.122	0.125	0.186	0.178	0.180	0.206	0.200	0.228	0.230	0.235	0.230	0.236	0.307
0.080	0.079	0.077	0.239	0.232	0.233	0.261	0.253	0.283	0.284	0.290	0.286	0.292	0.368
0.086	0.093	0.092	0.244	0.236	0.238	0.266	0.259	0.288	0.289	0.296	0.292	0.297	0.373
0.051	0.073	0.079	0.250	0.242	0.243	0.271	0.265	0.293	0.295	0.302	0.298	0.303	0.378
0.059	0.077	0.076	0.254	0.246	0.248	0.277	0.268	0.298	0.300	0.307	0.302	0.308	0.386
0.053	0.079	0.083	0.253	0.246	0.247	0.277	0.269	0.299	0.301	0.307	0.302	0.307	0.385
0.066	0.076	0.059	0.259	0.252	0.253	0.282	0.274	0.306	0.307	0.313	0.309	0.314	0.392
0.000	0.088	0.091	0.272	0.264	0.265	0.294	0.288	0.318	0.320	0.327	0.322	0.327	0.405
	0.000	0.085	0.276	0.268	0.269	0.298	0.291	0.322	0.323	0.330	0.326	0.331	0.408
		0.000	0.275	0.267	0.269	0.298	0.291	0.322	0.324	0.330	0.324	0.330	0.409
			0.000	0.023	0.023	0.032	0.125	0.145	0.147	0.153	0.150	0.153	0.351
				0.000	0.002	0.032	0.122	0.141	0.143	0.149	0.145	0.149	0.344
						0.032	0.123	0.142	0.144	0.150	0.146	0.150	0.345
						0.000	0.141	0.160	0.162	0.169	0.166	0.169	0.376
							0.000	0.072	0.073	0.083	0.116	0.118	0.370
								0.000	0.002	0.086	0.139	0.140	0.399
									0.000	0.086	0.140	0.141	0.401
										0.000	0.148	0.150	0.409
											0.000	0.005	0.411
												0.000	0.413
													0.000

*F. oxysporum* f. sp. *apii* race 3 (Table 1); however, isolates of this race were not recovered from symptomatic celery in this study. Instead, all of the isolates collected between 1993 and 2012 that were virulent on Tall Utah 52-70 R Improved were from race 2. During this period, three isolates that were characterized as race 1 were collected from Michigan (250-12B and 250-7) and from a celery germplasm plot in Davis, California (268-2). However, isolates forming a new *F. oxysporum* f. sp. *apii* race designated as race 4 were recovered in the Camarillo/Oxnard area, CA from 2013 onwards (Fig. 1).

A 1.3-kb *TEF1* + IGS rDNA two-locus dataset for the 174 isolates and *F. oxysporum* f. sp. *apii* race 3 was generated. ML and MP phylogenetic analyses of the collapsed (Posada 1999), 32-taxon dataset (with 25 taxa from celery, two taxa for rooting the tree, and five reference taxa for assigning FOSC clades) revealed that *F. oxysporum* f. sp. *apii* race 2 is nested with FOSC clade 3, while *F. oxysporum* f. sp. *apii* races 1, 3, and 4 are within clade 2. Our single archaic *F. oxysporum* f. sp. *apii* race 3 and the current *F. oxysporum* f. sp. *apii* race 4 share an identical two-locus haplotype (Fig. 1). Results of the phylogenetic analyses also indicate that among the 83 isolates scored as nonpathogenic to celery, 23 were in the race 2 haplogroup and 2 were in one of two race 1 haplogroups. The remaining 58 nonpathogenic isolates represent 20 phylogenetically diverse two-locus haplotypes, with 18 lineages nested within FOSC clade 3 and two within clade 2 (Fig. 1). Thirteen of the putatively nonpathogenic lineages were represented by singletons. By way of contrast, no allelic variation was detected within either the 100 isolates in the *F. oxysporum* f. sp. *apii* race 2 haplogroup or the 11 isolates of *F. oxysporum* f. sp. *apii* race 4, which suggests they are clonal lineages. Lastly, it is worth noting that ML and MP bootstrap (BS) analyses of the *TEF1* + IGS rDNA dataset recovered only modest support for the monophyly of FOSC clade 1 (ML-BS/MP-BS = 71/73%), clade 2 (ML-BS/MP-BS = 76/71%), clade 3 (ML-BS/MP-BS = 78/80%), and a sister group relationship of clades 2 and 3 (ML-BS/MP-BS = 78/72%).

To further elucidate evolutionary relationships of the four *F. oxysporum* f. sp. *apii* races and nonpathogens from celery, 16 strains were selected for NGS to >8.3× coverage using a MiSeq platform (Table 1). The NRRL 34936 *F. oxysporum* f. sp. *lycopersici* race 2 (4287) strain was used as a reference genome (Ma et al. 2010 and 2014). A 10-gene dataset of phylogenetically informative genes from the 12 reference FOSC was generously provided by L.-J. Ma. After sequences from the 10 genes from the 16 celery strains were mined from their genomes, we conducted ML and MP phylogenetic analyses of the 10 genes, and the 38 kb combined dataset (Table 2). The 10 marker loci were mapped to six chromosomes (i.e., 1, 4, 5, 7, 8, and 9) in the core genome of NRRL 34936 (=4287) *F. oxysporum* f. sp. *lycopersici* (Ma et al. 2010). The Kishino-Hasegawa likelihood test implemented in PAUP\* indicated that trees inferred from the following three partitions, when constrained to a MPT inferred from the combined dataset, were significantly different at  $P < 0.05$ : anaphase-promoting complex subunit 1 (from 46 to 60 steps), DNA directed polymerase (from 22 to 29 steps), and *RPB2* (from 31 to 42 steps). However, when the seven-gene dataset was analyzed without these putatively discordant partitions, the topology of the MPTs and bootstrap support was essentially identical to that of the 10-gene dataset (data not shown). Thus, the MP analysis of the 10-gene dataset is presented as our strongest hypothesis of evolutionary relationships of the *F. oxysporum* f. sp. *apii* races and related FOSC (Fig. 2). In analyses of the 10-gene dataset, FOSC clades 2 and 3 were strongly supported as monophyletic (ML-BS/MP-BS = 100/100%), as were *F. oxysporum* f. sp. *apii* races 1, 3, and 4 (ML-BS/MP-BS = 94/83%). To assess whether *F. oxysporum* f. sp. *apii* race 2, which was nested within clade 3, and races 1, 3, and 4 within clade 2 evolved monophyletically, the *F. oxysporum* f. sp. *apii* races were constrained in PAUP\* to form a monophyletic group. The *F. oxysporum* f. sp. *apii* monophyly constraint was significantly worse than an unconstrained MPT inferred from the 10-gene dataset (i.e., 93 steps longer) based on

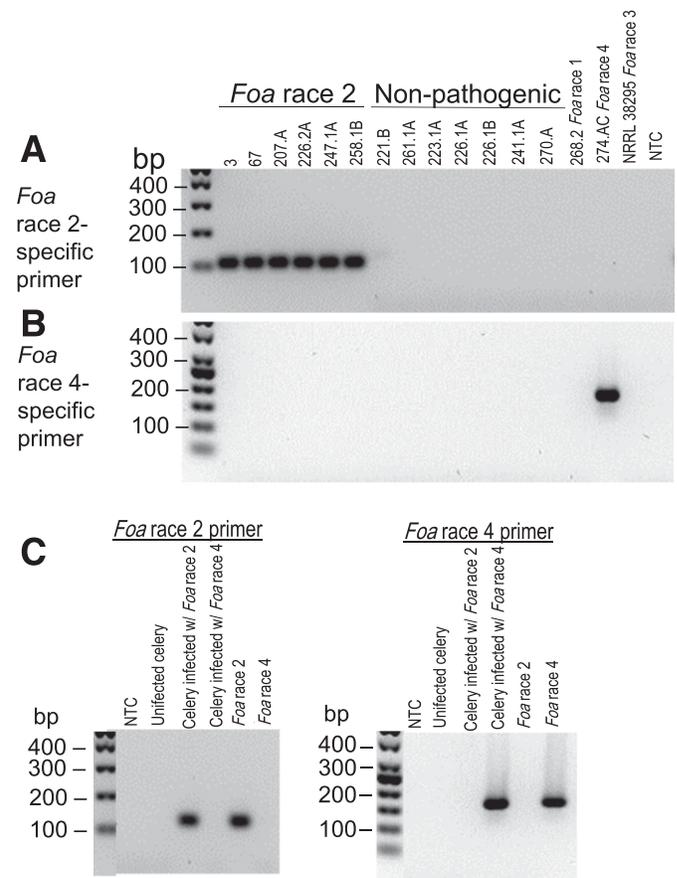
results of the Kishino-Hasegawa likelihood test ( $P < 0.05$ ) implemented in PAUP\* (data not shown).

To further assess the genetic diversity of the selected FOOSC, we mapped Illumina sequences from the 11 other FOOSC reference strains and the 16 NGS strains from celery onto the assembled NRRL 34936 *F. oxysporum* f. sp. *lycopersici* reference genome. SNPs that could be scored for all isolates were selected, and a subset of 6,898 SNPs were used to quantify pairwise diversity (Table 3). Nei's Da provides a measure of SNP genetic diversity with a scale of 0 for identical isolates to 1 for isolates that are divergent at all loci. Races 3 and 4 are highly similar ( $Da = 0.0019$ ), which is within the range of the six *F. oxysporum* f. sp. *apii* race 2 isolates to each other. The latter vary from  $0.0012 \leq Da \leq 0.0020$ . The California *F. oxysporum* f. sp. *apii* race 1 isolate is very similar to *F. oxysporum* f. sp. *apii* races 3 and 4 (average  $Da = 0.023$ ). Of the 12 reference strains, *F. oxysporum* f. sp. *apii* races 1, 3, and 4 are most closely related to NRRL 25433 *F. oxysporum* f. sp. *vasinfectum* race 7 (average  $Da = 0.032$ ). Of the 12 reference strains, *F. oxysporum* f. sp. *apii* race 2 is most closely related to NRRL 34936 *F. oxysporum* f. sp. *lycopersici* race 2 ( $Da = 0.085$ ) and to NRRL 26406 *F. oxysporum* f. sp. *melonis* race 1 (Ma et al. 2014) ( $Da = 0.089$ ). Assuming that the two locus haplogroups shown in Figure 1 represent clonal lineages, the six presumably nonpathogenic isolates from celery in clade 3 represent 64% of our nonpathogenic isolates; these isolates are somewhat related to *F. oxysporum* f. sp. *apii* race 2 ( $0.093 \leq Da \leq 0.113$ ). As a group, the six nonpathogenic strains are closely related to each other ( $0.053 \leq Da \leq 0.080$ ) and to NRRL 54003 *F. oxysporum* f. sp. *lycopersici* race 3 ( $0.051 \leq Da \leq 0.086$ ) and to NRRL 26381 *F. oxysporum* f. sp. *radicilycopersici* ( $0.059 \leq Da \leq 0.092$ ). The six nonpathogenic strains from celery are also closely related to NRRL 54002 (=Fo47) *F. oxysporum* biocontrol strain ( $0.051 \leq Da \leq 0.086$ ).

Nei's Da mirrors the clade structure. In clade 3, *F. oxysporum* f. sp. *apii* race 2 ( $n = 6$ ), the six nonpathogenic isolates from celery, and five FOOSC reference strains have  $0.001 \leq Da \leq 0.178$ . In clade 2, *F. oxysporum* f. sp. *apii* races 1, 3, and 4, one nonpathogenic isolate from celery which is in a two-locus haplotype with two *F. oxysporum* f. sp. *apii* race 1 isolates from Michigan, and nine FOOSC reference strains have  $0.002 \leq Da \leq 0.169$ . In comparison with the single FOOSC reference strain in clade 1, all of the clade 2 and 3 FOOSC from celery have a  $0.307 \leq Da \leq 0.413$ . Lastly, divergence between a clade 2 and clade 3 strain ranged from  $0.180 \leq Da \leq 0.327$ . A structure analysis of the SNP data is shown in Supplementary Fig. S1.

We used the NGS to select PCR primers (Table 4) for rapid diagnosis of the *F. oxysporum* f. sp. *apii* race 2 (Fig. 3A) haplogroup and race 4 (Fig. 3B) from cultures and directly from field-infected crown tissue (Fig. 3C). As potential amplicons, we selected 1-kb segments that appeared to be present in only *F. oxysporum* f. sp. *apii* race 2 or race 4, and not in any of the other celery NGS, the 12 reference FOOSC genomes, GenBank or FCGP. The similarity of *F. oxysporum* f. sp. *apii* races 3 and 4 was further indicated by the fact that we ultimately only identified one amplicon for *F. oxysporum* f. sp. *apii* race 4 primer design, in contrast to multiple options for *F. oxysporum* f. sp. *apii* race 2 haplogroup primers. We tested the specificity of the selected primers using 28 isolates from celery that were not in the NGS pool, including 12 nonpathogenic isolates, each of which were from a distinct haplogroup. We also tested four archived FOOSC f. sp. *apii* that were deposited into culture collections in either 1935 or 1938, a *F. oxysporum* f. sp. *fragariae* from California,

23 diverse FOOSC and two *F. foetens* (Supplementary Table S3). As expected, all five of the nonpathogenic isolates in the *F. oxysporum* f. sp. *apii* race 2 two-locus haplogroup amplified a single 100-bp product with the *F. oxysporum* f. sp. *apii* race 2 haplogroup primers and had no product with the race 4 primer pair. Only one of the 59 isolates that was not in the *F. oxysporum* f. sp. *apii* race 2 haplogroup (nonpathogenic isolate 249-1A from celery in FOOSC clade 3) produced a false positive with the *F. oxysporum* f. sp. *apii* race 2 haplogroup primer pair. All eleven *F. oxysporum* f. sp. *apii* race 4 isolates amplified a single 187 bp product with the *F. oxysporum* f. sp. *apii* race 4 primer pair, and had no product with the *F. oxysporum* f. sp. *apii* race 2 haplogroup primer pair. In total, 2 of 59 isolates that were not *F. oxysporum* f. sp. *apii* race 4 (nonpathogenic isolates from celery 273-2B and 273-1B in FOOSC clade 3) produced a false positive with a single band of approximately 190 bp with the *F. oxysporum* f. sp. *apii* race 4 primer pair. However, 15 of the 59 non-*F. oxysporum* f. sp. *apii* race 4 isolates produced an extremely faint band of either  $<100$  bp or  $>1,000$  bp; these would be easily distinguishable from a *F. oxysporum* f. sp. *apii* race 4 positive. Thus, there can be false positives with these primer pairs, but apparently relatively few.



**Fig. 3.** *Fusarium oxysporum* f. sp. *apii* **A**, race 2 and **B**, race 4 PCR primers. DNA was isolated from either **A** or **B**, axenic cultures or **C**, either crowns of field-grown, naturally infected celery plants or cultures. **A** and **B**, 2 ng of DNA per lane was loaded; and **C**, 10 ng of total DNA from celery extracts and 2 ng of DNA from cultures. Primers are described in Table 4. NTC, no template control.

TABLE 4. Putative diagnostic PCR primers for *Fusarium oxysporum* f. sp. *apii* race 2 haplogroup and race 4<sup>z</sup>

Specificity	Name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size, bp
<i>F. oxysporum</i> f. sp. <i>apii</i> race 2	N4851	TGTTTCGAGTACATTGGAGACTTTGG	CAGCATAGATCGTAGGTGGATATAGAG	100
<i>F. oxysporum</i> f. sp. <i>apii</i> race 4	N3875-2	AGGACTTGAATCACGGCTCG	CCTGCCACTCGCTTTTGTAG	187

<sup>z</sup> Annealing temperature, 60°C.

## DISCUSSION

Results of the present study show that Fusarium yellows in celery in California between 1993 and 2013 was caused by *F. oxysporum* f. sp. *apii* race 2, consistent with prior vegetative compatibility group analyses (Correll et al. 1986a; Toth and Lacy 1991). Race 2 is monomorphic, with no evidence from our next generation sequencing of a change over time or location. In addition, race 2 is genetically distinct from the 12 reference FOSC strains and to the five previously reported *F. oxysporum* f. sp. *apii* in O'Donnell et al. (2009); the latter FOSC strains from celery were from archived collections, with four from European collections with two isolates from Germany that were deposited by H. W. Wollenweber in 1935 (NRRL 22534, =CBS 175.35 and NRRL 36287, =CBS 176.35) and two isolates from an unidentified location that were deposited by R.R. Nelson in 1937 (NRRL 36312, =CBS 183.38 and NRRL 36316, =CBS 184.38), and NRRL 38295, which we characterized here as *F. oxysporum* f. sp. *apii* race 3.

*F. oxysporum* f. sp. *apii* race 4 appeared in Camarillo, CA in an intensive celery production area in 2013; as of March 2016, we confirmed it in 12 fields in Camarillo and one in neighboring Santa Paula, CA. We note that Camarillo is adjacent to Oxnard and that the Camarillo area is often called Oxnard. *F. oxysporum* f. sp. *apii* race 4 is highly virulent against all currently tested celery cultivars in California (data not shown), and has caused greater crop loss in infested fields than we have observed in *F. oxysporum* f. sp. *apii* race 2-infested fields. Consequently, it is reasonable to assume that *F. oxysporum* f. sp. *apii* race 4 has only been present in the Camarillo area for a relatively few years, since such a virulent strain would have been noticed earlier. The disease in a new field often starts as an isolated focus of infection, and we speculate that after the first plants were infected in the field where it initially appeared, the pathogen may have been moved to new fields by farm equipment or vehicles.

Although *F. oxysporum* f. sp. *apii* races 1, 3, and 4 are similar in sequence, they have significant differences in virulence (Table 1). Our sequence comparison of the comparatively weakly virulent *F. oxysporum* f. sp. *apii* race 3 with the highly virulent *F. oxysporum* f. sp. *apii* race 4 suggests that a relatively small change in DNA sequence may have resulted in a large change in virulence, consistent with the hypothesis by Rep and Kistler (2010) and many others (Kashiwa et al. 2016) that *F. oxysporum* isolates can evolve into new races. We note that we never isolated *F. oxysporum* f. sp. *apii* race 3 from field-grown celery and that our single race 3 (Foa\_R3, =NRRL38295) was retrieved from an archived collection. Consequently, while it is conceivable that Foa\_R3 lost virulence during storage, we also note that this isolate was collected during the time period and presumably the region where *F. oxysporum* f. sp. *apii* race 3 was identified (Puhalla 1984), and that the historical *F. oxysporum* f. sp. *apii* race 3 was not highly virulent, in contrast to *F. oxysporum* f. sp. *apii* race 4. Regardless, partly because a sexual cycle has not been observed within the FOSC, the great diversity of host and cultivar specificity has been attributed to nonsexual mechanisms, especially transposon movement and chromosomal rearrangements induced by transposon recombination (Kashiwa et al. 2016; Vlaardingerbroek et al. 2016). As one example, Ma et al. (2010) presented experimental evidence that a small, transposon-rich accessory chromosome with multiple virulence genes from NRRL 34936 *F. oxysporum* f. sp. *lycopersici* race 2 transformed a nonpathogenic isolate of *F. oxysporum* into a tomato pathogen by lateral transfer of an accessory chromosome.

We show here that *F. oxysporum* f. sp. *apii* race 2 is in a different FOSC clade than *F. oxysporum* f. sp. *apii* races 1, 3, and 4. Current phylogenetic analysis supports division of the FOSC into four clades (O'Donnell et al. 2009). Together, the four FOSC clades contain hundreds of putatively clonal lineages that are plant pathogens, saprophytes, and some opportunistic pathogens of humans and other animals (Aoki et al. 2014; Laurence et al. 2014). The economically important plant pathogenic strains are the best

characterized, and most are thought to have a narrow host range (Ma et al. 2013). Historically, the plant-pathogenic FOSC were categorized based on host (forma specialis, f. sp.). However, multiple formae speciales have been shown to be polyphyletic: the melon pathogen f. sp. *melonis* (Gordon and Martyn 1997); the banana pathogen f. sp. *cubense* (Fourie et al. 2009; O'Donnell et al. 1998); the cotton pathogen f. sp. *vasinfectum* (Skovgaard et al. 2001); and f. spp. *asparagi*, *dianthi*, *gladioli*, *lini*, and *opuntiarum* (Baayen et al. 2000). Here, we show that *F. oxysporum* f. sp. *apii* is another example of a polyphyletic forma specialis within the FOSC. Based on two of the FOSC reference strains, we also show that *F. oxysporum* f. sp. *lycopersici* is polyphyletic.

The NGS allowed us to develop PCR primers for diagnosis directly from crown tissue of the agronomically important races of *F. oxysporum* f. sp. *apii* from symptomatic celery (Fig. 3C); DNA isolation from symptomatic crown tissue, PCR, and gel electrophoresis can be completed in 1 day. However, although we were able to use the NGS to develop primers that only amplified DNA from either the *F. oxysporum* f. sp. *apii* race 2 haplogroup or race 4 and not DNA from the other NGS isolates, we had amplification from 3 of 12 nonpathogenic isolates. We note however, that assuming that our two-locus haplotypes represent clonal lineages, all three false positives were singletons in their haplotype, and consequently each only represents 0.6% of a collection that was designed to sample the diversity of FOSC isolates in symptomatic celery. In addition, we do not know the relative quantities of pathogenic and nonpathogenic isolates in the margin of symptomatic tissue. In our experience, symptomatic tissue has one *F. oxysporum* f. sp. *apii* race and zero to two nonpathogenic strains.

We isolated nearly equal numbers of *F. oxysporum* f. sp. *apii* race 2 ( $n = 77$ ) and nonpathogenic isolates ( $n = 83$ ) that did not induce vascular discoloration in greenhouse virulence tests. However, 23 and 2 of the isolates were in the same two-locus haplotypes as *F. oxysporum* f. sp. *apii* races race 2 and 1, respectively. Nonetheless, 58 of our 174 isolates (33%) were from either other formae speciales of *F. oxysporum* or other saprophytic FOSC lineages. Isolation of nonpathogenic isolates from celery with Fusarium yellows has been reported previously (Correll et al. 1986b; Schneider 1984). The majority of the nonpathogenic isolates that were colocalized in symptomatic tissue are nested in FOSC clade 3. In contrast to *F. oxysporum* f. sp. *apii* races 2 and 4, the nonpathogenic isolates are phylogenetically diverse, consistent with earlier vegetative compatibility group analysis (Correll et al. 1986b). Interestingly, the subclade that includes most of our diverse nonpathogenic isolates also includes the biocontrol strain Fo47/NRRL 54002 (Alabouvette et al. 2007; Edel-Hermann et al. 2009; Fuchs et al. 1999). Data in Schneider (1984) suggest that the nonpathogenic FOSC may contribute specifically to suppression of celery Fusarium yellows. In other *Fusarium* diseases, nonpathogenic FOSC isolates have also been implicated as agents of suppressiveness (Bolwerk et al. 2005; Larkin et al. 1996), although other biotic agents (Mazurier et al. 2009) and abiotic factors have also been postulated. It is unknown whether the nonpathogenic isolates that share the two-locus sequence type with races 1 and 2 are either *bona fide* saprophytes that have lost virulence in the field (as they often do in the laboratory) or that the standard vascular-discoloration-based disease assay for Fusarium yellows has a relatively high false negative detection rate. Regardless, there is a diverse community of putatively nonpathogenic FOSC in symptomatic tissue; in preliminary greenhouse assays, we did not detect a role for these isolates in either increasing or decreasing the severity of disease (data not shown).

Although many FOSC are highly specialized plant pathogens, presumably either *F. oxysporum* f. sp. *apii* race 1 or 3 was considered to be a primary pathogen in both celery, which is in the Apiaceae, and in *Tithonia rotundifolia* in the Asteraceae (Armstrong and Armstrong 1966, 1975). Furthermore, *F. oxysporum* f. sp. *apii* has been reported to be a secondary pathogen in a broad diversity of hosts including cotton (*Gossypium barbadense* and *G. arboreum*)

in the Malvaceae, garden pea (*Pisum sativum*) and sickle pod (*Cassia tora*) in the Fabaceae, and eggplant (*Solanum melongena*) in the Solanaceae (Armstrong and Armstrong 1967, 1975). Although *F. oxysporum* f. sp. *apii* races do not appear to be identical to any of the currently published races of other formae speciales based on the available molecular phylogenetic data, *F. oxysporum* f. sp. *apii* races 1, 3 and 4 are in FOsc clade 2, which includes strains that are pathogenic on cotton (NRRL 25433), pea (HDV247 = NRRL 37622), and other hosts.

*Fusarium oxysporum* f. sp. *apii* race 1 sensu lato appears to include a variety of strains. In his 1937 monograph, Nelson et al. (1937) concluded there were two “forms” of *F. oxysporum* f. sp. *apii* in Michigan and possibly a third form in California. Subsequently, Puhalla (1985) determined VCGs for isolates of *F. oxysporum* f. sp. *apii* race 1 from New York and France, along with one *F. oxysporum* f. sp. *apii* race 2 from California, and concluded that all three were in separate VCGs. These and other data led him to conclude that race 1 is polymorphic, in contrast to monomorphic race 2. In 2009, O’Donnell et al. (2009) included five relatively old isolates reported as *F. oxysporum* f. sp. *apii* in a two-locus sequence analysis of the FOsc; however, the race of four of them is unknown and it is unclear whether Koch’s postulates were ever completed on these four isolates. Although these isolates are nested within FOsc clade 2, they are genetically distinct from our isolates. Consistent with Puhalla (1984), we never isolated *F. oxysporum* f. sp. *apii* race 1 from a celery-producing region in California; we isolated *F. oxysporum* f. sp. *apii* race 1 (268.2) from a University of California at Davis celery germplasm plot and two *F. oxysporum* f. sp. *apii* race 1 (presumably clones) from celery in Michigan; our *F. oxysporum* f. sp. *apii* race 1 from California and Michigan are genetically distinct but both are in FOsc clade 2.

Nelson et al. (1937) describe the repeated planting of celery without rotation in Michigan between 1913 and his 1937 publication and the dispersal of what we now call *F. oxysporum* f. sp. *apii* race 1, at least partly by infected seedlings. Similarly, *F. oxysporum* f. sp. *apii* race 2 spread from its original focal point in California in 1976 (Otto et al. 1976) to other celery production areas (Lacy et al. 1996). Ultimately, *F. oxysporum* f. sp. *apii* race 1 and race 2 were controlled by the introduction of resistant cultivars. In contrast, fumigation (Otto et al. 1976), fungicides (Awuah et al. 1986), and rotation have not been effective in controlling *Fusarium* yellows. We are currently trying to identify germplasm with either resistance or tolerance to *F. oxysporum* f. sp. *apii* race 4. Ultimately, understanding the genetic diversity and evolutionary origins of new races will be critical for the sustainable control of *Fusarium* yellows.

## ACKNOWLEDGMENTS

We thank the following: California Celery Research Advisory Board, USDA NIFA (CA-D-PPA-2165-H), and the Program of Study Abroad for Young Teachers by the Agricultural University of Hebei, China for funding; L.-J. Ma, University of Massachusetts at Amherst for providing unpublished results on the *Fusarium* reference genomes; M. Rep for his DNA purification protocol for NGS; S. Koike for collecting some of the older isolates; T. Gordon and P. Henry for the *F. oxysporum* f. sp. *fragariae* isolate; L. Pierce, D. Pereira, S. Donovan, C. Quiros, V. D’Antonio, and M. Govindarajulu Elmore for helpful conversations; and Q. Pham, R. Kim, A. Aquino, N. Villalpando, G. Doehring, and R. Nandety for assistance. The twelve assembled FOsc genomes were obtained from the *Fusarium* Comparative Sequencing Project, Broad Institute of Harvard and MIT (<https://www.broadinstitute.org/>). The raw Illumina reads from Broad Project PRJNA38293 were downloaded from NCBI. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned. The USDA is an equal opportunity provider and employer.

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