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Techniques

A Systematic Approach for Identifying Unique Genomic Sequences for *Fusarium oxysporum* f. sp. *lactucae* Race 1 and Development of Molecular Diagnostic Tools

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Abstract

Fusarium oxysporum f. sp. *lactucae* (FOLac) is a soil- and seedborne fungal pathogen that causes Fusarium wilt of lettuce, an important disease threatening global lettuce production. Based on pathogenicity on differential lettuce cultivars, four races (1 to 4) have been identified, with race 1 being the only race detected in the United States and the closely related, emerging race 4 known only in Europe. The development of race-specific diagnostic tools is hindered by insufficient genomic data to distinguish between the two races and FOLac from other *F. oxysporum formae speciales* and nonpathogenic isolates. Here, we describe a systematic approach for developing diagnostic markers for FOLac race 1 that utilized a comprehensive sequence database of *F. oxysporum* to identify 15 unique genomic sequences. Marker specificity was validated through an exhaustive screening process against genomic data from 797 *F. oxysporum* isolates representing 64 *formae speciales* and various plants and non-plant substrates. One of the unique sequences was used to develop a TaqMan quantitative PCR assay and a recombinase polymerase amplification assay, both exhibiting 100% sensitivity and specificity when tested against purified DNA from 171 *F. oxysporum* isolates and 69 lettuce samples. The relationship between quantitative PCR cycle threshold values and CFU/g values was also determined. This study not only introduces a new marker for FOLac race 1 diagnostics and soil quantitation but also underscores the value of an extensive genomic database and screening software pipeline for developing molecular diagnostics for *F. oxysporum formae speciales* and other fungal taxa.

Keywords: Fusarium wilt, genome sequencing, *Lactuca sativa*, molecular diagnostics, recombinase polymerase amplification, soil quantification, TaqMan quantitative polymerase chain reaction

Lettuce (*Lactuca sativa* L.) is one of the most consumed vegetables in the world and was produced in 108 countries in 2022 (FAOSTAT 2022). The United States ranked second in lettuce production, with head lettuce being the most widely grown lettuce type, followed by romaine (Davis et al. 2023). According to the U.S. Department of Agriculture National Agricultural Statistics Service (NASS 2022), in 2021, lettuce production in the United States totaled 74.8 million cwt with a gross value of nearly $2.7 billion, making lettuce the second most valuable vegetable crop. California and Arizona account for 98% of the total lettuce production in the United States through a sequence of production in Southern California's imperial county and the Yuma area of Arizona (harvested from November to April) and Central California (harvested from April to November). Global lettuce production is threatened by an emerging disease, Fusarium wilt, which is caused by *Fusarium oxysporum* (Schlechtend.:Fr.) f. sp. *lactucae* (FOLac). The pathogen is soil- and seedborne (Garibaldi et al. 2004) and colonizes the vascular tissues, disrupting water movement. Typical disease symptoms include reddish-brown discoloration of the cortex of taproots and crown, leaf chlorosis, vascular browning, wilting, and stunting. As the disease progresses, the whole plant may collapse. Up to 70% yield losses have been reported in contaminated lettuce fields (Pasquali et al. 2007).

FOLac was first described in Japan in 1955 (Motohashi 1960) and now occurs in most of the world where lettuce is cultivated. Due to their differential pathogenicity on specific lettuce cultivars, FOLac is differentiated into four pathogenic races (Fujinaga et al. 2003; Gilardi et al. 2017). Race 1 is the prevalent race worldwide, which has been reported in Japan (Fujinaga et al. 2001; Matuo and Motohashi 1967), the United States (Hubbard and Gerik 1993), Taiwan (Lin et al. 2014), Iran (Millani et al. 1999), Brazil (Cabral et al. 2014), Argentina (Malbrán et al. 2014), and Europe (Guerrero et al. 2020; Herrero et al. 2021; Pasquali et al. 2007; Tziros and Karaoglanidis 2023). In contrast, races 2 and 3 have restricted geographic distributions, with race 2 reported only in Japan (Fujinaga et al. 2001) and race 3 in Japan (Fujinaga et al. 2003) and Taiwan (Lin et al. 2014). In 2015, a new race of FOLac (race 4) was

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reported in the Netherlands and has spread rapidly across Europe. Phylogenetic analyses of the four FOLac races using the translation elongation factor gene and in combination with a few other DNA markers revealed that races 1 and 4 are closely related to the extent that they cannot be distinguished (Claerbout et al. 2023; Gilardi et al. 2017). Similarly, a more recent phylogenetic analysis of the *F. oxysporum* species complex using 41 full-length, single-copy, protein-coding gene orthologs, totaling 69.4 kb, showed that FOLac races 1 and 4 represent a monophyletic clade in Lineage 3F with no distinguishing sequence divergence between the two races (D. M. Geiser, *unpublished data*). In contrast, races 2 and 3, falling in Lineages 1 and 2G, respectively, are genetically distinct from races 1 and 4, consistent with findings in other studies (Fujinaga et al. 2005; Gilardi et al. 2017; Mbofung et al. 2007). Although the separation of FOLac races 1 and 4 can be achieved using genotyping-by-sequencing (Claerbout et al. 2023), the lack of accompanying genomic information prevents us from fully understanding the genetic diversity between the two races and their relationship with nonpathogenic isolates of lettuce. As genome sequencing is becoming easier, faster, and more affordable, whole-genome sequencing can be used to address this question more precisely and efficiently.

Early detection of FOLac race 1 from lettuce fields, including infested soils, is crucial for rapid response to the disease with proper control measures and for evaluating the efficacy of soil fumigation, crop rotation, and various other management strategies in reducing the pathogen pressure in soil. However, field diagnosis is hindered by the diversity of pathogens that cause Fusarium wilt-like symptoms, including *Verticillium dahliae* (Verticillium wilt), *Sclerotinia sclerotiorum* and *S. minor* (lettuce drop), *Botrytis cinerea* (gray mold), and *Rhizorhapis suberifaciens* (corky root) (Gordon and Koike 2015). Physiological disorders, such as ammonium toxicity, can also lead to reddish-brown vascular discoloration of the taproot and wilting of lettuce plants, which can be confused with Fusarium wilt (Koike et al. 2007). Traditional laboratory diagnosis of FOLac using morphology-based methods can sometimes be useful. According to Gordon and Koike (2015), when grown on Komada's selective medium, 99% of FOLac isolates surveyed formed a pink pigmentation on the underside of the colony with white aerial mycelia present as tufts. However, this morphology alone cannot be used to accurately identify FOLac because nonpathogenic *F. oxysporum* isolates that can grow endophytically in lettuce produce similar morphological characteristics. Pathogenicity assays using a defined set of lettuce cultivars that show race-specific reactions continue to be the standard method for accurate identification of FOLac and differentiation of FOLac races (Fujinaga et al. 2003; Gilardi et al. 2017). However, pathogenicity assays are cumbersome and often lack consistency as disease symptoms can vary depending on inoculation methods and environmental conditions (Claerbout et al. 2023; Gordon and Koike 2015). Franco Ortega et al. (2018) developed a loop-mediated isothermal amplification assay that allowed for qualitative but not quantitative detection of FOLac in soil, with limited capacity in the amount of soil to be tested.

Due to these challenges, selective and specific detection methods that are based on unique, pathogen-specific genomic sequences are highly desirable for precise identification of the pathogen because these sequences are absent outside of the specific *forma specialis* (f. sp.) or race of interest. Several DNA-based diagnostic assays for FOLac race 1 identification have been developed (Mestdagh et al. 2023; Pasquali et al. 2007; Shimazu et al. 2005), with the capacity for pathogen detection and quantification from plant and soil samples (Mestdagh et al. 2023; Pasquali et al. 2007; Sanna et al. 2022). However, query of these markers against the sequence database that was developed as part of another project (F. N. Martin, N. Li, and D. M. Geiser, *unpublished data*) revealed that they appeared to be not race 1 specific (Supplementary Table S1; Supplementary Fig. S1). Gilardi et al. (2017) reported that the marker developed by Pasquali et al. (2007) was unable to detect the Dutch isolates belonging to race 1. An increase in diagnostic assay accuracy might be attained by expanding the genomic resources to include a wider diversity of *F. oxysporum*, encompassing both pathogenic and apparently nonpathogenic isolates, which can be used in specificity screening.

With hundreds of publicly available *F. oxysporum* genome sequences, and genome sequencing becoming less expensive and producing better genome assemblies, development and validation of highly specific diagnostic markers for *F. oxysporum* pathogens, potentially to the race level, are now accessible. In support of this, we generated whole-genome sequencing data from a total of 351 *F. oxysporum* isolates (referred to as Reference Panel 1), including 44 FOLac isolates collected from diverse geographical regions worldwide, 12 *F. oxysporum* isolates not pathogenic on lettuce, 179 *F. oxysporum* isolates representing 47 other *F. oxysporum formae speciales* and races, and 116 *F. oxysporum* isolates recovered from other plants and non-plant substrates. The resulting sequence database was used to achieve the following objectives: (i) to understand genetic variability between the very closely related FOLac races 1 and 4; (ii) to develop a systematic approach for identifying unique diagnostic regions for FOLac race 1; and (iii) to develop TaqMan real-time PCR and recombinase polymerase amplification (RPA) assays for specific detection of FOLac race 1 from purified fungal cultures, lettuce tissue, and pathogen quantification in soil. Previously, our lab developed a large-scale method to extract high-quality DNA from 10 to 15 g of soil, which allowed for accurate quantification of *F. oxysporum* f. sp. *fragariae* below 10 CFU/g using the TaqMan qPCR assay developed for the specific pathogen (Matson et al. 2024). This study was aimed to achieve a similarly high level of sensitivity for FOLac race 1. Furthermore, both diagnostic assays developed in this study can be run with an internal control marker designed to reveal false negatives, a common but often ignored quality issue in PCR-based diagnostics (Bilodeau et al. 2012; Geiser et al. 2023; Haudenshield and Hartman 2011).

Materials and Methods

F. oxysporum isolate collection

The FOLac isolates ($N = 44$) used for whole-genome sequencing, which were generously provided by collaborators, included 28 FOLac race 1 isolates from diverse geographic locations, 13 FOLac race 4 isolates obtained from several European countries, and three Japanese isolates, SB1-1 (=MAFF244120, race 1; Fujinaga et al. 2001), F9501 (=MAFF244121, race 2; Fujinaga et al. 2001), and FLK1001 (=MAFF24412, race 3) (Table 1). We also sequenced 12 nonpathogenic isolates of lettuce that were originally recovered from symptomatic lettuce plants but unable to cause disease on lettuce (Table 1). Ten race 1 isolates (GL1546, GL1692, GL1693, GL1815, JCP024, JCP043, JCP053, JCP293, JCP360, and JCP381) originally isolated from California were obtained from two previous studies (Hubbard and Gerik 1993; Paugh and Gordon 2020). The three Japanese cultures were deposited to the Genetic Resources Center at National Agriculture and Food Research Organization in Japan.

Culture growth and DNA extraction for sequencing

Cultures were started on potato dextrose agar (Difco Laboratories, Detroit, MI) under dark incubation for 5 days at 25°C. Six agar plugs from colony edges were transferred to Petri plates containing 25 ml of potato dextrose broth (Difco Laboratories) and incubated unagitated for 2 days at 25°C. The resulting mycelia were harvested on filter paper, washed twice with sterile distilled water, blotted dry, and flash frozen in liquid nitrogen prior to freeze drying. Genomic DNA was extracted from 20 mg of freeze-dried mycelia using a DNeasy Plant Mini Kit (Qiagen, Germantown, MD), according to the manufacturer's instructions, with the following modifications: For the lysis step, incubation of the mixture at 65°C lasted 1 h instead of 10 min, and the tube was inverted every 10 min; at the final step, DNA was eluted in 50 µl of AE buffer instead of 100 µl.

Genomic DNA was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA).

Genome sequencing and assembly

Genomic DNA of the 56 lettuce isolates and one *F. commune* isolate (Table 1), which served as an outgroup taxon, was submitted to the Genomics Core of Michigan State University for library preparation and Illumina sequencing. Library preparation was performed using the Illumina TruSeq nano DNA library prep kit, according to the manufacturer's protocol (Illumina, San Diego, CA). The sequencing of the libraries was then performed using the Illumina HiSeq 4000 platform with 150-bp paired-end reads. The HiSeq reads were processed with CLC Genomics Workbench (version 22; Qiagen) for de novo genome assembly (mapping options: mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.5, and similarity fraction of 0.95). All raw sequence data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under BioProject accession number PRJNA1098703 with the associated short read accession numbers SRR28734888 to SRR28734944.

TABLE 1. *Fusarium oxysporum* f. sp. *lactucae* isolates and other *F. oxysporum* isolates recovered from lettuce but unable to cause disease on lettuce that were sequenced in this study

Isolate name	Species name and race (subgroup)[a]	Host plant/substrate	Origin	Lineage[b]
2a	*F. oxysporum* f. sp. *lactucae* race 1	*Lactuca sativa*	USA: Florida	3F
20a	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	USA: Florida	3F
45a	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	USA: Florida	3F
50a	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	USA: Florida	3F
52a	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	USA: Florida	3F
53a	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	USA: Florida	3F
58a	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	USA: Florida	3F
74a	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	USA: Florida	3F
6-14	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Italy	3F
7-14	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Italy	3F
GL1546	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup B)	*L. sativa*	USA: Santa Maria, California	3F
GL1692	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup A)	*L. sativa*	USA: Watsonville, California	3F
GL1693	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup A)	*L. sativa*	USA: Salinas, California	3F
GL1815	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup A)	*L. sativa*	USA: Salinas, California	3F
JCP024	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup C)	*L. sativa*	USA: Wellton, Arizona	3F
JCP043	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup A)	*L. sativa*	USA: Huron, California	3F
JCP053	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup C)	Soil associated with *L. sativa*	USA: Yuma, Arizona	3F
JCP293	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup A)	*L. sativa*	USA: Huron, California	3F
JCP360	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup A)	*L. sativa*	USA: King City, California	3F
JCP381	*F. oxysporum* f. sp. *lactucae* race 1 (subgroup A)	*L. sativa*	USA: Gonzales, California	3F
SB1-1	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Japan	3F
Mya	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Italy	3F
AL010	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Portugal	3F
AM163	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Chile	3F
AP068	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Poland	3F
AP057	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Spain	3F
AC013	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Mexico	3F
AS147	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Mexico	3F
AT142	*F. oxysporum* f. sp. *lactucae* race 1	*L. sativa*	Spain	3F
F9501	*F. oxysporum* f. sp. *lactucae* race 2	*L. sativa*	Japan	1
FLK1001	*F. oxysporum* f. sp. *lactucae* race 3	*L. sativa*	Japan	2G
888	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Italy	3F
R4	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Italy	3F
AT141	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Spain	3F
AL088	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Netherlands	3F
AL185	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	United Kingdom	3F
AU069	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	United Kingdom	3F
AU122	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Italy	3F
AM020	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	United Kingdom	3F
AL127	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Ireland	3F
AP114	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Denmark	3F
AP004	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Italy	3F
AN072	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	Ireland	3F
AU078	*F. oxysporum* f. sp. *lactucae* race 4	*L. sativa*	France	3F
AU068	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	Spain	3G
AT115	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	France	2E
AJ194	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	Brazil	2E
AJ14	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	USA	2G
AU074	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	France	3G
AJ195	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	Brazil	2E
AG108	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	Chile	3G
63a	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	USA: Florida	3G
18-9	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	USA: California	3G
18-10	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	USA: California	3G
18-12	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	USA: California	3G
18-15	*F. oxysporum*, nonpathogenic on lettuce	*L. sativa*	USA: California	3G
NRRL36408	*F. commune*	*Lupinus* sp.	–	Outgroup

[a] Somatic compatibility subgroups as reported in Paugh and Gordon (2020).
[b] Lineage designation was obtained from a phylogenetic study (D. M. Geiser, *unpublished data*).

A broader Illumina genomic sequence database and corresponding assembled genome database generated as part of another project (F. N. Martin, N. Li, and D. M. Geiser, *unpublished data*), which included data from all the isolates listed in Reference Panel 1 (Supplementary Table S2), was used for evaluating marker specificity. Illumina sequencing data and genome assemblies were generated as noted above.

Mash analysis

We utilized Mash, version 2.3 (Ondov et al. 2016), a program that approximates genetic distances between genomes using a *k*-mer-based algorithm, to assess genomic differences among the 44 FOLac and 12 nonpathogenic isolates of lettuce. Raw paired-end Illumina reads were trimmed using the software Trimmomatic, version 0.38 (Bolger et al. 2014), to remove low-quality reads and adaptor sequences, with the following parameters: "ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:8:TRUE LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36". The trimmed reads were then randomly subsampled to 50× coverage with an estimated genome size of 55 Mb using the software program Rasusa, version 0.8.0 (Hall 2022), to normalize the amount of input reads for the mash analysis. Illumina data from *F. commune* strain NRRL36408 were included as an outgroup in the analysis. Genetic distances between all 57 genomes were calculated using "mash dist" with a *k*-mer size of 21 and a sampling size of 100,000. The resulting output was a distance matrix, which is an approximation of the mutation rate that can also be used for phylogenetic reconstruction (Ondov et al. 2016). The distance output was then converted into a Newick format Mash tree file (Supplementary File S1) using the R package *ape*, version 5.6.2 (Paradis and Schliep 2019). The resulting neighbor-joining tree was visualized in FigTree, version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Identification of unique diagnostic regions for FOLac race 1

An overview of the workflow is illustrated in Figure 1. Using a *k*-mer based approach called "hitting associations with *k*-mers" (HAWK; version 1.5.0; Rahman et al. 2018), which finds genomic regions associated with a categorical trait using sequencing reads without the need to map reads to the reference genome, Illumina data generated from JCP043 (FOLac race 1) and R4 (FOLac race 4) were compared with default settings (kmerLength = 31). The *k*-mers that were found only in JCP043 were extracted and de novo assembled using SeqMan NGen (LaserGene, version 16; DNASTAR, Madison, WI) with a stringency of 90% sequence identity and *k*-mer size of 21 nucleotides (nt). The assembled contigs were size selected (≥150 bp) because the length of the shortest sequence suitable to designing an RPA assay is about 150 bp. Subsequently, Illumina reads from 322 nontarget *F. oxysporum* isolates, which included all the isolates in Reference Panel 1 except FOLac race 1, were mapped to the unique contigs using SeqMan NGen with 85% sequence identity and a *k*-mer size of 30 nt. Regions that had zero read coverage were extracted using a customized script, followed by a second round of read mapping using the same Illumina dataset to remove additional non-unique regions. To ensure the unique sequences were present among all the race 1 isolates and to avoid creating chimeric sequences that did not belong to the race 1 genome, the resulting unique regions were BLASTed against 29 race 1 genome assemblies using Geneious, version 9.0.5 (http://www.geneious.com/). Additional evaluation of the specificity of the unique sequences was performed through MegaBLAST against the 322 nontarget *F. oxysporum* genomes that were assembled in-house and 446 selected *F. oxysporum* genomes from GenBank (referred to as Reference Panel 2; Supplementary Table S3), using Geneious. Finally, the unique sequences were searched against the NCBI nucleotide database using BLASTn to ensure they had no homology to any sequences deposited in the database.

In silico specificity assessment of previously developed FOLac race 1 markers

To determine if the FOLac race 1 markers developed in previous studies were present in any nontarget *F. oxysporum* isolates, and if those markers were always present in FOLac race 1, we used the BLAST function in Geneious to query the full-length DNA sequences of three previously developed markers (Mestdagh et al. 2023; Pasquali et al. 2007; Shimazu et al. 2005) against our *F. oxysporum* assembled genome database encompassing 351 in-house *F. oxysporum* genomes and 446 GenBank accessions. For the FLA0101 marker (Shimazu et al. 2005), raw Illumina reads of four representative FOLac isolates—SB1-1 (race 1), F9501 (race 2), FLK1001 (race 3), and R4 (race 4)—were mapped to the reference sequence using SeqMan NGen (30 million reads, 90% sequence identity, *k*-mer size of 30 nt) because the BLAST analysis resulted in too many fragments to assess the specificity. Two regions of about 500 bp flanking the FLA0101 marker were included in the read mapping analysis to ensure reads could be aligned to the entire marker region.

Marker development for FOLac race 1

The unique sequences (Supplementary Table S4; Supplementary File S2) were first examined for their feasibility for a TaqMan assay with the following primer and probe parameters: GC content of 30 to 80%; optimal melting temperatures of 62 and 70°C for primers and probes, respectively; and a lack of homopolymeric repeats or predicted secondary structure. The specificity of the developed primers was checked in silico using Primer-BLAST at NCBI against the nucleotide database. The qualified regions were then examined to ensure primers and probes could be designed for an RPA assay, in which specific requirements for primers (size: 30 to 36 nt; GC content: 20 to 70%; Tm: 50 to 100°C; fewer than five mononucleotide repeats) and probes (size: 46 to 52 nt, at least 30 of which are placed 5′ to the tetrahydrofuran residue, and at least a further 15 are placed 3′ to it) had to be satisfied, according to the TwistDx assay design manual. As a result, five candidate markers eligible for both TaqMan qPCR and RPA assays were chosen for further evaluation. All primers and probes used in this study were obtained from Integrated DNA Technologies (Coralville, IA) and LGC Biosearch Technologies (Petaluma, CA), respectively.

Initial screening of the candidate markers for specificity via conventional PCR

Primer pairs for the five candidate markers were tested for specificity on a small panel of DNA, including two FOLac race 1 isolates, two FOLac race 4 isolates, and two nonpathogenic isolates of lettuce. Negative controls without DNA templates were included to rule out contamination and monitor background nonspecific amplification. For this testing, amplifications were carried out in a 25-µl reaction volume with 2 ng of genomic DNA, 400 nM each primer, and 1× Perfecta Multiplex qPCR ToughMix (Quantabio, Beverly, MA) in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) under the following conditions: 95°C for 3 min and 45 cycles of 95°C for 15 s and 62°C for 30 s. The PCR products were checked by 1.5% agarose gel electrophoresis. One marker, Folac_R1_uniq-13, which produced a single distinct band for the FOLac race 1 isolates and no band for the other isolates, was selected as the final marker to develop TaqMan qPCR and RPA assays.

Testing of the TaqMan qPCR assay for specificity and sensitivity

TaqMan primers and the probe for detection of FOLac race 1 are shown in Table 2 and Figure 2. Genomic DNA of 142 nontarget *F. oxysporum* isolates from Reference Panel 1 were used to determine the specificity of the TaqMan qPCR assay (Supplementary Table S5). Two technical replicates were tested per isolate. The TaqMan assay was also run in duplicate on the 29 FOLac race 1 isolates, and the average of the two values was used in calculations

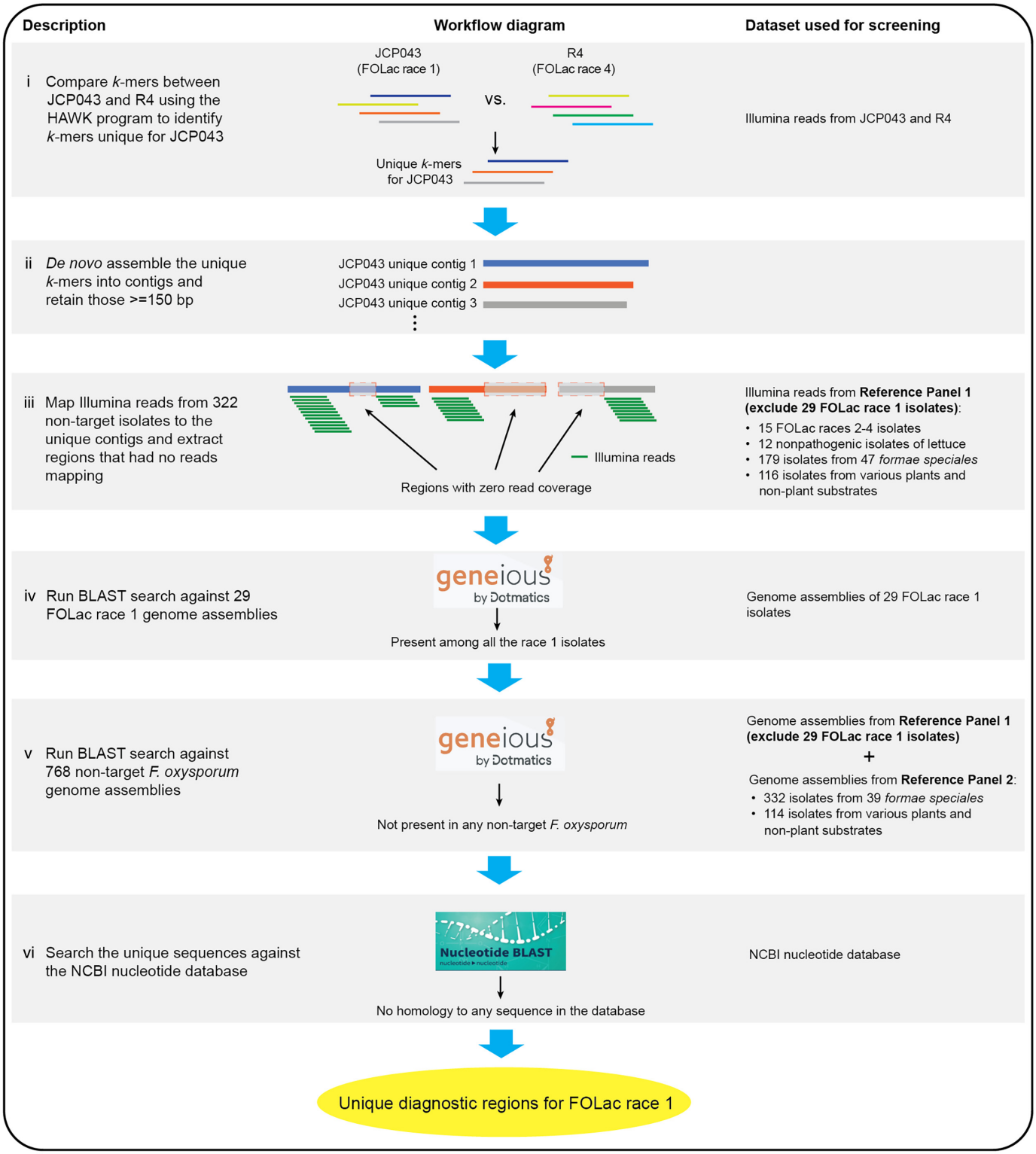

Fig. 1. Workflow of the in-silico analysis to identify unique genomic sequences as potential diagnostic regions for *Fusarium oxysporum* f. sp. *lactucae* (FOLac) race 1. The analysis started with (i) comparing *k*-mers in sequence reads between two FOLac isolates JCP043 and R4, representing races 1 and 4, respectively, using the HAWK program (Rahman et al. 2018). (ii) The *k*-mers that were found only in JCP043 were then assembled into contigs, and those above 150 bp were retained for the downstream screening process. (iii) The unique contigs were assessed by mapping sequence reads from 322 nontarget *F. oxysporum* isolates (refer to Supplementary Table S2 for the isolates in Reference Panel 1), and the regions where no reads mapped were extracted. This step was repeated once to remove additional mapped regions. (iv) The resulting unique sequences were queried against 29 FOLac race 1 genome assemblies to ensure they were present among all the race 1 isolates. Additional evaluation of the specificity was carried out by BLASTing the unique sequences (v) against 768 nontarget *F. oxysporum* genome assemblies, including 446 GenBank accessions (refer to Supplementary Table S3 for the isolates in Reference Panel 2), and (vi) against the NCBI nucleotide database.

for each sample. Amplifications were carried out in a 25-µl reaction volume with 2 ng of genomic DNA, 400 nM Folac_TaqMan_F and Folac_TaqMan_R primers, 200 nM Folac_TaqMan_probe, and a 1× Perfecta Multiplex qPCR ToughMix in a CFX96 Real-Time PCR Detection System with the same parameters as described above. The baseline threshold for the fluorophore signal was set at 50 RFU. Standard curves for this assay were generated using serially diluted genomic DNA from race 1 isolate JCP024, ranging from 200 fg (2×10^{-4} ng) to 2 ng. The average and standard deviation were calculated from three replicates. The cycle threshold (C_t) value of the FOLac race 1 marker was then plotted against the log of the template amount to determine the sensitivity and amplification efficiency (e = 10 – 1/slope) for this assay. The lowest concentration that consistently amplified and kept the relationship linear was defined as the detection limit.

To determine whether this assay could detect FOLac race 1 from diseased lettuce samples, one set of the standard curve samples was run in the presence of 1 µl of plant DNA, which was extracted from a healthy lettuce taproot (as described below) and quantified using a Qubit Fluorometer. The TaqMan probe and primers for the *cox1* gene-based plant internal control (IC) (Table 2) developed by Bilodeau et al. (2014) were also used in this assay to confirm the extracted plant DNA is amplifiable. All amplifications were performed using 1 µl of plant DNA (70 ng/µl), the standard curve samples (JCP024; ranging from 200 fg to 2 ng), 400 nM Folac_TaqMan_F and Folac_TaqMan_R primers, 200 nM Folac_TaqMan_probe, 12.5 nM FMPl2b and FMPl3b primers, 10 nM Plant CAL-Red probe, and 1× Perfecta Multiplex qPCR ToughMix, in a reaction volume of 25 µl. The negative control included everything except DNA of JCP024. One set of the standard curve samples without plant DNA was used as the positive control. Three technical replicates were tested per sample.

The ability of this assay to detect FOLac race 1 from infested soils was evaluated as previously reported (Bilodeau et al. 2012). One set of the standard curve samples was run in the presence of 1 µl of soil DNA, which was extracted using the large-scale soil DNA extraction method as described by Matson et al. (2024). The soil sample was collected from a strawberry production field with no known history of Fusarium wilt of lettuce. An exogenous IC template developed by Bilodeau et al. (2012) was used in this assay at a concentration optimized to yield a C_t of about 32 to monitor for the presence of amplification inhibitors in the qPCR. All amplifications were performed using 1 µl of soil DNA, the standard curve samples (JCP024; ranging from 200 fg to 2 ng), 1 µl of the exogenous IC template, 400 nM Folac_TaqMan_F and Folac_TaqMan_R primers, 200 nM Folac_TaqMan_probe, 400 nM Vd-F929-947 and Vd-R1076-1094 primers (Table 2), 40 nM PPF_Probe_543 probe (Table 2), and 1× Perfecta Multiplex qPCR ToughMix, in a reaction volume of 25 µl. The negative control included everything except DNA of JCP024. One set of the standard curve samples without soil DNA was used as the positive control. Three technical replicates were tested per sample.

DNA extraction and fungal isolation from symptomatic lettuce plants

Symptomatic lettuce plants were collected from commercial lettuce production fields in California and Arizona and processed in the TriCal Diagnostics lab. Root samples were surface sterilized by agitating in 1% bleach for 5 min and then were rinsed three times in sterile distilled water. The roots were cut open lengthwise to expose vascular tissue, and the lesion margins were cut into 0.5 cm^3 segments. Total DNA from 20 mg of freeze-dried root segments was extracted using a DNeasy Plant mini kit (Qiagen), according to the manufacturer's instructions. To determine the presence of FOLac

TABLE 2. Primer and probe sequences used in this study

Primer/probe name	Sequence 5′-3′	Length (nt)	Target	Reference
Primers				
Folac_TaqMan_F	CCACATAATAGACAGTGAAC	20	FOLac race 1	This study
Folac_TaqMan_R	CAATGAGATGGGAGATTT	18	FOLac race 1	This study
FMPl2b	GCGTGGACCTGGAATGACTA	20	Plant Cox1	Bilodeau et al. 2014
FMPl3b	AGGTTGTATTAAAGTTTCGATCG	23	Plant Cox1	Bilodeau et al. 2014
Vd-F929-947	CGTTTCCCGTTACTCTTCT	19	Internal control	Bilodeau et al. 2012
Vd-R1076-1094	GGATTTCGGCCCAGAAACT	19	Internal control	Bilodeau et al. 2012
Folac_RPA_F	TCTTGGGATGGTTTTACTCAATGATGTCACCAC	33	FOLac race 1	This study
Folac_RPA_R	GCCACAAGCCGTACCCAGGACTCTTCGAGGCAA	33	FOLac race 1	This study
Cox1-IPC-F	CATGCGTGGACCTGGAATGACTATGCATAGA	31	Plant Cox1	Miles et al. 2015
Cox1-IPC-R	GGTTGTATTAAAGTTTCGATCGGTTAATAACA	32	Plant Cox1	Miles et al. 2015
Probes				
Folac-TaqMan_probe	[FAM]CGCTGTCTGTAACCTTCTTCCGCA[BHQ1]	24	FOLac race 1	This study
Plant CAL-Red probe	[CALFluorRed610]CTTTTATTATCACTTCCGGTACTGGCAGG[BHQ2]	29	Plant Cox1	Bilodeau et al. 2014
PPF_Probe_543	[CALFluorRed610]AAAGTAAGCTTATCGATACCGTCGACCT[BHQ2]	28	Internal control	Bilodeau et al. 2012
Folac_RPA_probe	GTTCTAAGTCCTGTCGCTGTCTGTAACCT[T(FAM)][dSpacer][T(BHQ-1)]TCCGCAGGTCTTGA[3′-C3SPACER]	45	FOLac race 1	This study
Cox1-IPC-P	GGTCCGTTCTAGTGACAGCATTCCYACTTTTATTA[T(CAL Fluor Orange 560)]C[dSpacer]C[T(BHQ1)]YCCGGTACTGGC[3′-C3SPACER]	51	Plant Cox1	Miles et al. 2015

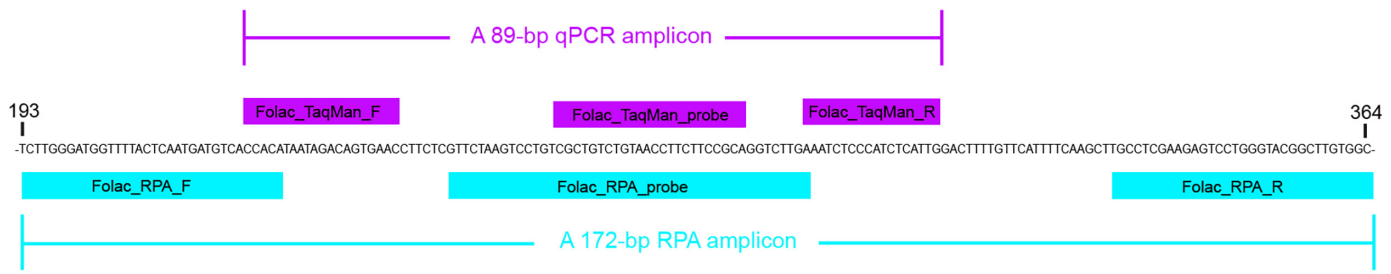

Fig. 2. Diagram of the unique sequence Folac_R1_uniq-13 from which the TaqMan quantitative polymerase chain reaction (qPCR) and recombinase polymerase amplification (RPA) assays specific for *Fusarium oxysporum* f. sp. *lactucae* race 1 were derived. Magenta and cyan boxes indicate the primer and probe binding sites for the TaqMan and RPA assays, respectively.

race 1 in each lettuce sample, three root segments were randomly selected and transferred at equidistant positions to Komada's selective medium (Komada 1975). The Petri plates were incubated for 7 to 10 days at room temperature under continuous fluorescent light (24 h/day). *Fusarium*-like colonies emerging from the root segments were transferred onto a potato dextrose agar medium via hyphal tip transfer and sub-cultured twice to obtain pure cultures. DNA from the purified fungal cultures was extracted using a rapid DNA extraction protocol as described previously (Abd-Elsalam et al. 2003). As negative controls, three healthy lettuce samples were processed following the same procedure.

Validation of the TaqMan qPCR assay using field samples

DNA extracted from symptomatic lettuce samples (as described above) was tested using the multiplexed TaqMan assay as follows. The reaction was carried out in a volume of 25 µl, with 2 µl of DNA from field samples, 400 nM Folac_TaqMan_F and Folac_TaqMan_R primers, 200 nM Folac_TaqMan_probe, 12.5 nM FMPl2b and FMPl3b primers, 10 nM Plant CAL-Red probe, and 1× Perfecta Multiplex qPCR ToughMix. Each plant sample was tested in duplicate. TaqMan qPCR cycling conditions were set at 95°C for 3 min and 45 cycles of 95°C for 15 s and 60°C (2°C lower than the temperature set for testing purified fungal cultures) for 30 s. The sample was quality checked by monitoring the amplification curve using the Texas Red channel (for detection of the plant endogenous IC probe), which is expected to enter the exponential phase shortly after the C_t reaches 22 or sooner and quickly plateau with the RFU level around 100. In addition, DNA extracted from purified fungal cultures that were isolated from the same lettuce sample was also tested using the TaqMan qPCR assay to confirm the presence/absence of FOLac race 1.

Development of an RPA assay

Four staggered (overlapping in a few bases) primer pairs were examined to identify the best pair that gave the earliest onset of amplification, which was determined by the time (in seconds) at which the fluorescence value crossed 500, according to the TwistDx assay design manual. The optimal primer pair and the probe for the RPA assay are shown in Table 2 and Figure 2. The sensitivity of the RPA assay was evaluated using serially diluted DNA from three FOLac race 1 isolates, ranging from 200 fg to 2 ng. Two technical replicates were tested per isolate. Amplification was carried out in a 50-µl reaction volume, with 2 ng of genomic DNA (ranging from 200 fg to 2 ng), 300 nM Folac_RPA_F and Folac_RPA_R primers, 120 nM Folac_RPA_probe, 14 mM magnesium acetate, and 1× TwistAmp exo rehydration buffer (TwistDx, Cambridge, U.K.). The reaction was prepared and run as described previously (Burkhardt et al. 2018; Pastrana et al. 2019). The RPA assay was also evaluated with the presence of a crude lettuce taproot extract to determine whether the crude extract affected the onset of amplification. This was achieved by running one set of the standard curve samples in the presence of 2 µl of a crude lettuce taproot extract that did not contain any FOLac race 1. See the section "Validation of the RPA assay using field samples" for the extraction method. The log-transformed value of the onset of amplification was then plotted against the log of template concentration to determine the sensitivity for this assay.

The specificity of the RPA assay was evaluated using purified DNA from the same set of *F. oxysporum* isolates used for the TaqMan assay evaluation. For the nontarget isolates, their DNA was bulked in batches of five (1 µl from each of the five isolates bulked) to save reagents. If the bulked sample tested positive, the assay was repeated with each isolate individually. Two technical replicates were tested per isolate.

Validation of the RPA assay using field samples

The same lettuce plants, which were tested with the TaqMan qPCR assay, were processed for RPA by macerating 0.5 g of diseased lettuce tissue with 5 ml of 1× general extraction buffer 2 (Agdia, Elkhart, IN) using techniques previously reported (Miles et al. 2015). The extract was used immediately for the assay or flash frozen and stored at −80°C until it was thawed on ice before use. To determine whether inhibitors in the crude extract interfered with the amplification, the probe and primers for the *cox1* gene-based plant endogenous IC (Table 2) were used for the multiplexed RPA assay as previously reported (Miles et al. 2015). The reaction was carried out under the following conditions: 2 ng of crude extract, 300 nM Folac_RPA_F and Folac_RPA_R primers, 120 nM Folac_RPA_probe, 250 nM Cox1-IPC-F and Cox1-IPC-R primers, 120 nM Cox1-IPC-P, 14 mM magnesium acetate, and 1× TwistAmp exo rehydration buffer. Each plant sample was tested in duplicate. Results were considered positive when the fluorescence value crossed 500 within 15 min. If a negative result was obtained from the RPA assay but the same field sample produced a positive result from the TaqMan qPCR assay, DNA from the lettuce sample used for the TaqMan assay was then tested via the RPA assay to ensure the RPA assay had the same level of diagnostic sensitivity as the TaqMan assay and vice versa.

Soil quantification assay for FOLac race 1

To assess the feasibility of the TaqMan qPCR assay to detect and quantify FOLac race 1 in soil, we evaluated the correlation between colony forming units (CFUs) of infested soils and results from the TaqMan qPCR assays. Soil infestation of autoclaved soil was done with isolate JCP043, a wild-type FOLac race 1 strain, using the procedure of G. E. Vallad (*unpublished data*). The race 1 isolate was started on potato dextrose agar medium for 7 days before transferring to 100 ml of potato dextrose broth medium. The culture was incubated for 7 days at 25°C in the dark with agitation (250 rpm) before blending with a Waring laboratory blender (Conair LLC, Stamford, CT) for 10 s on low speed. The slurry was added to a sterile soil substrate composed of 100 ml of fine sand, 300 ml of finely sieved soil (loamy texture), and 120 g of Oxoid corn meal agar (Thermo Fisher Scientific) in an autoclave bag, which was then sealed with a foam plug wrapped in autoclave tape to allow ventilation; the bag was massaged daily to ensure robust fungal growth. After 2 weeks of incubation, the soil was dried in a laminar flow hood for 7 days to prevent contamination and to promote chlamydospore formation. Once dry, a sample of this "original" soil was assayed immediately to determine the approximate CFU/g soil. A dilution series of soil was prepared by mixing the "original" soil in a 1:1 ratio with autoclaved inert soil, down to the level where the soil is supposed to carry no pathogen. Plating assays were conducted by adding 10 g of soil to 100 ml of 1% (wt/vol) sodium hexametaphosphate buffer and resuspending while stirring for 5 min. Depending on the estimated pathogen load in each dilution, 200 and 300 µl of the suspended mixture was plated on 5 and 15 Petri plates containing Komada's medium. CFU/g of soil was calculated based on the number of *Fusarium* colonies emerging after 7 days of incubation under continuous fluorescent light at room temperature. Three replicates per soil dilution were used. The inert soil used in the dilution series was also plated on Komada's medium to confirm its sterility. DNA from 15 g of soil was extracted using the large-scale DNA extraction method as described by Matson et al. (2024), followed by quantification using a Qubit fluorometer. The time interval from the initial drying of the soil and when it was used for DNA extraction was approximately 12 weeks. The TaqMan qPCR assay developed in this study was used to quantify FOLac race 1 in each diluted soil sample. All amplifications were carried out using 1 µl of soil DNA. See the section "Testing of the TaqMan qPCR assay for specificity and sensitivity" for the assay setup. TaqMan qPCR cycling conditions were set at 95°C for 3 min and 45 cycles of 95°C for 15 s and 60°C (2°C lower than the temperature set for testing purified fungal cultures) for 30 s. For soil DNA samples that had C_t value of 32 or above, a second qPCR assay was conducted in the absence of IC to accurately quantify the pathogen. A C_t value of 36 was chosen as the upper C_t limit for what is considered a valid quantification

estimate. In cases where soil DNA tested positive but yielded a C_t value higher than 36, 4 µl of soil DNA was used to rerun the qPCR assay.

Data analysis

The relationship between the serial dilution for the standard curve and C_t (for TaqMan assays) or log of onset of amplification (for RPA assays) was determined by linear regression on data using the statistical software R, version 4.2.2 (R Core Team 2022). ggplot2 (Wickham 2016) was used for graphic plotting.

Results

FOLac races 1 and 4 resolved as sister lineages

Whole-genome sequence data were generated for 56 *F. oxysporum* isolates associated with lettuce, including 44 FOLac isolates representing four different races and 12 nonpathogenic isolates recovered from symptomatic lettuce samples that did not cause disease on lettuce (Table 1). To help understand the genetic diversity among these isolates, we utilized the Mash program to estimate genetic distances at the *k*-mer level and built a neighbor-joining phylogram to reveal the relationships among the lettuce isolates (Fig. 3). Based on the Mash analysis, the four FOLac races resolved as four distinct lineages, with races 1 and 4 resolving as sister lineages and quite distant from races 2 and 3. All the race 1 and race 4 isolates showed greater intra-race similarity than they did to other races or lineages in the dataset. Nonpathogenic isolates of lettuce displayed a high level of genetic diversity and were distinctly different from FOLac.

Unique diagnostic sequences for FOLac race 1 were identified using a systematic approach

We employed a systematic approach to identify unique genomic regions for FOLac race 1 diagnostics, as illustrated in Figure 1.

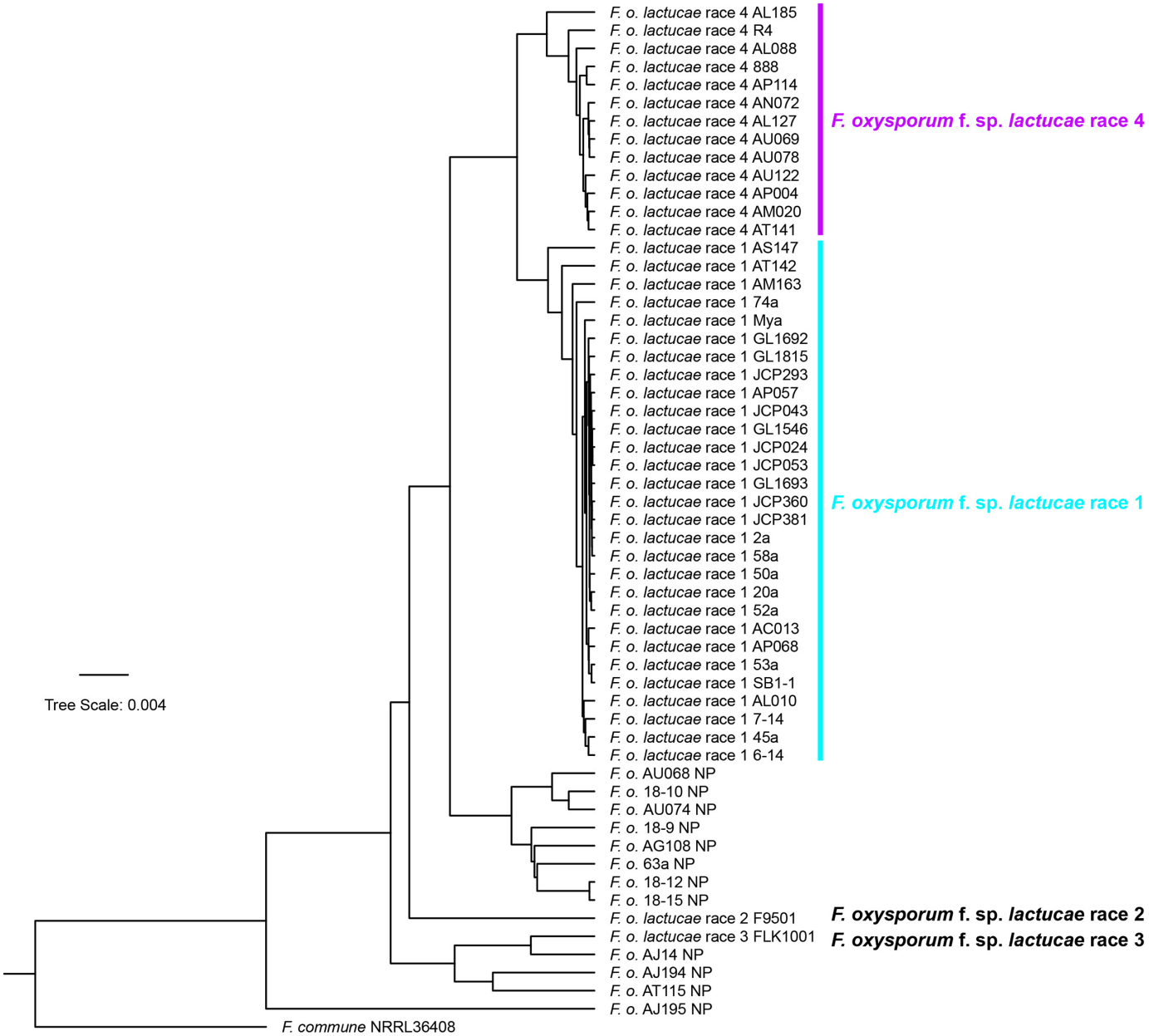

Fig. 3. Neighbor-joining phylogram depicting relationships of 44 *Fusarium oxysporum* f. sp. *lactucae* isolates and 12 *F. oxysporum* isolates recovered from diseased lettuce plants but unable to cause disease on lettuce (NP) based on pairwise *k*-mer comparisons using the Mash program (Ondov et al. 2016). One *F. commune* isolate, NRRL36408, was used as an outgroup taxon to root the phylogram. The Mash tree file in Newick format is provided in Supplementary File S1.

Using the HAWK program, a total of 1,942 unique contigs (≥150 bp) were identified by comparing *k*-mers in sequencing reads between JCP043 and R4, representing FOLac races 1 and 4, respectively. These unique contigs were subject to an exhaustive screening process to ensure that they were not present in nontarget *F. oxysporum* isolates as follows. In the first screening step, Illumina reads from 322 nontarget *F. oxysporum* isolates from Reference Panel 1 were mapped to these unique contigs to identify regions with zero read coverage. Then 90 unique sequences that had made it through the previous step were BLASTed against the genome assemblies of all the FOLac race 1 isolates, 322 isolates from Reference Panel 1, 446 GenBank isolates from Reference Panel 2, and all the sequences deposited in the NCBI nucleotide database. Finally, we obtained 15 unique diagnostic regions (Supplementary File S2) present only among FOLac race 1 isolates that did not match any other genomes they were screened against. The genomic coordinates of the unique sequences (Supplementary Table S4) revealed that some resided in close vicinity on the same contig. Based on the annotation of the FOLac race 1 genome (N. Li, *unpublished data*), none of the unique sequences were annotated as protein-coding genes.

Using the same sequence database as described above, we assessed the specificity of three previously developed PCR markers for race 1, including a 186-bp product amplified with the Hani3′–Hanilatt3revb primer pair (Pasquali et al. 2007), the FLA0101 marker (Shimazu et al. 2005), and two genotyping-by-sequencing markers (Fol1a [142 bp] and Fol1b [179 bp]; Mestdagh et al. 2023). When querying the Pasquali et al. (2007) marker against 797 *F. oxysporum* genome assemblies (351 in-house and 446 GenBank isolates), it was shown to be highly specific with only one off-target hit (98.9% sequence identity) against nonpath5 (GCA_020976685.1), a nonpathogenic isolate recovered from *Carthamus oxyacanthus* (wild safflower) (Supplementary Table S1). Like FOLac races 1 and 4, nonpath5 is a member of Lineage 3F (D. M. Geiser, *unpublished data*). It was technically challenging to analyze the FLA0101 marker via BLAST search because part of the sequence was highly repetitive, resulting in many fragments scattered in different contigs. This issue was resolved by mapping Illumina reads from individual isolates to the marker sequence, followed by visual inspection of the alignment result. We performed the analysis on four representative FOLac isolates, SB1-1 (race 1), F9501 (race 2), FLK1001 (race 3), and R4 (race 4). The results showed that SB1-1, F9501, and FLK1001 had continuous read coverage in the marker sequence despite variation in read depth (Supplementary Fig. S1), suggesting that the marker was not race 1 specific. The genotyping-by-sequencing markers developed by Mestdagh et al. (2023) were shown to lack specificity with Fol1a and Fol1b found in 19 and 2 other *formae speciales*, respectively, as well as various *F. oxysporum* isolates nonpathogenic on the host of recovery (Supplementary Table S1).

Development of a TaqMan qPCR assay for FOLac race 1

The 15 unique diagnostic regions were assessed for their eligibility to design both TaqMan qPCR and RPA assays, and five of them satisfied all the criteria (see the section "Marker development" in Materials and Methods). A small panel of six *F. oxysporum* isolates representing FOLac races 1 and 4, as well as nonpathogenic isolates of lettuce, were tested with conventional PCR to identify the best primer pair for specific amplification of FOLac race 1. After the initial PCR testing, we identified that one primer pair (Folac_TaqMan_F and Folac_TaqMan_R), amplifying an 89-bp fragment from the unique sequence Folac_R1_uniq-13 (Fig. 2), produced the clearest and most robust single product from FOLac race 1 isolates, with no false positives, at an optimal annealing temperature of 62°C. The TaqMan qPCR assay was also assessed by testing 29 FOLac race 1 isolates, which resulted in positive detection with an average C_t value of about 26 from 2 ng of genomic DNA.

The amplification of the FOLac race 1 marker exhibited a linear response to increasing amounts of DNA ($R^2 = 0.993$), with 2 pg as the detection limit (Fig. 4A). DNA in less than 2 pg was unable to be amplified consistently in the TaqMan assay, as C_t values varied widely between replicates. The efficiency of the assay was 90.22%. Subsequently, the specificity of the marker was evaluated against a panel of 142 nontarget *F. oxysporum* isolates from Reference Panel 1 (Table 3). All screened isolates were negative for the FOLac race 1 TaqMan assay at the concentration tested (2 ng DNA). The C_t values of all the isolates tested are shown in Supplementary Table S5.

The TaqMan qPCR assay was also evaluated with plant and soil DNA to determine whether the assay can be used for in planta diagnosis and soil detection of the pathogen, respectively. The target amplification was completely and partially inhibited with the presence of plant (data not shown) and soil DNA (Fig. 4C), respectively. Such inhibition was unlikely caused by IC primers and probes (Fig. 4A and C). By lowering the annealing temperature by 2°C, the TaqMan assay regained the ability to detect the pathogen from plant and soil substrates without compromising the amplification efficiency and assay sensitivity (Fig. 4B and D). However, with the presence of exogenous IC for soil detection, the C_t values obtained from 1 versus 2 pg of the target DNA were indistinguishable (Fig. 4D). A separate reaction without the exogeneous IC, as long as no inhibition of amplification was observed in the initial multiplexed assay, was able to resolve this issue (Fig. 4D).

RPA assay for FOLac race 1 exhibited levels of sensitivity and specificity similar to those of the TaqMan qPCR assay

RPA assays have been successfully deployed in disease diagnostics due to their high sensitivity, specificity, and rapid amplification at low and constant temperatures (37 to 42°C) with minimal sample preparation (Burkhardt et al. 2018, 2019; Miles et al. 2017; Pastrana et al. 2019). More importantly, it does not require highly trained personnel to operate and can be applied at the point of sample collection. In this study, an RPA assay was developed based on the same diagnostic region used for the TaqMan assay but with longer primer sequences and different probe modifications fitting the specifications of the RPA assay (Fig. 2). An initial screening with four different primer pairs resulted in one pair that gave the earliest onset of amplification for the pathogen while not amplifying other *F. oxysporum* isolates. The RPA assay was able to detect the target DNA in 2 pg with and without the presence of a crude plant extract, and detection remained linear at increasing amounts, with R^2 values of 0.9877 and 0.9954, respectively (Fig. 5). The presence of the crude plant extract slightly increased the time to the onset of amplification but did not affect the overall detection limit of the assay, with 2 pg of DNA amplifying before 13 min without a crude plant extract and before 15 min with it. The specificity of the RPA assay was evaluated using the same panel of *F. oxysporum* isolates as the TaqMan assay, which showed no false positives in nontarget isolates (Table 3).

Both diagnostic assays were 100% specific and sensitive for diseased lettuce samples

To test the efficacy of the TaqMan and RPA diagnostic assays on lettuce plants that were at the early development stage in field settings, 69 lettuce plants collected at the rosette stage from various lettuce production areas in Arizona and California were tested with both the TaqMan and the RPA assays. Of these samples, 63 showed symptoms associated with Fusarium wilt and had positive cultures isolated from the vascular tissue of the taproot, which later tested positive with the TaqMan assay, indicating that FOLac race 1 was present in those lettuce samples. All 63 plants tested positive with both diagnostic assays (Table 4). Four plants, although showing Fusarium wilt-like symptoms, tested negative for both assays. No morphologically *Fusarium*-like cultures were recovered from those plants (Table 4), suggesting that these were true negative samples. In a few cases, neither the pathogen nor the plant IC was detected in the TaqMan or the RPA assay, possibly due to bad DNA extrac-

tion or the presence of amplification inhibitors. In these cases, a second extraction from the same lettuce sample yielded a positive result.

During testing, we processed two asymptomatic lettuce samples that were obtained from lettuce fields where Fusarium wilt was present. Both samples tested positive for FOLac race 1 with the TaqMan assay but not with the RPA assay (Table 4). To ensure the samples were infected with FOLac race 1 and not false positive due to a cross-reaction with other microbes colonizing the plant, the samples were cultured on Komada's selective medium, and the resulting *Fusarium*-like cultures tested positive with the TaqMan assay (Table 4), indicating the presence of FOLac race 1 in those asymptomatic lettuce plants. The discrepancy in the test results between the two diagnostic assays could be attributed to the low level of FOLac present within the asymptomatic lettuce plants, which led to the tissue used for the RPA assay not containing enough of the pathogen to be detected. To test this, the two plant DNA samples used for the TaqMan assay were tested with the RPA assay and showed positive results with a prolonged time to the onset of amplification (15 min), suggesting that the pathogen load in the samples appeared quite low. Overall, both diagnostic assays with lettuce taproot tissue had diagnostic sensitivity and specificity of 100% (Table 4).

Soil quantification assay for FOLac race 1

The dilution series of the FOLac race 1-infested soil showed a wide distribution of CFU/g values, ranging from 2 to 7,280 CFU/g soil (Fig. 6A). Of the 21 soil samples examined, four populations were below 10 CFU/g soil, and 10 were above 1,000 CFU/g. Using the large-scale DNA extraction method developed by Matson et al. (2024), 100 µl of DNA elution was obtained from 15 g of soil, with DNA concentrations ranging from 1.44 ng/µl (corresponds to a 2 CFU/g soil sample) to 188 ng/µl (corresponds to a 7,280 CFU/g soil sample). All 21 samples, which could be quantified via plating, tested positive for the FOLac race 1 marker using 1 µl of soil DNA, with an R^2 value of 0.738 for regression analysis (Supplementary Fig. S2). However, six soil samples that had equal to or less than 40 CFU/g exhibited C_t values above 36, exceeding the upper limit for accurate quantification of the pathogen in a soil texture (Fig. 4D). Four microliters of the soil DNA from these samples was used to rerun the qPCR assay, which resulted in an average 1.72 C_t value decrease and the ability to quantify the pathogen below 10 CFU/g soil with an R^2 value of 0.88 (Fig. 6B). Sampling 4 µl of soil DNA

TABLE 3. Evaluation of the specificity of the TaqMan and the recombinase polymerase amplification (RPA) assays for *Fusarium oxysporum* f. sp. *lactucae* race 1

Species name and race[a]	Number of isolates (171 in total)	Results[b]	
		TaqMan	RPA
F. oxysporum f. sp. *lactucae* race 1	29	+	+
F. oxysporum f. sp. *lactucae* race 2	1	–	–
F. oxysporum f. sp. *lactucae* race 3	1	–	–
F. oxysporum f. sp. *lactucae* race 4	13	–	–
Nonpathogenic on lettuce	12	–	–
43 other *formae speciales* and different races	89	–	–
Nonpathogenic on other plant species	12	–	–
Isolates recovered from other plant species	4	–	–
Soil isolates	10	–	–

[a] *F. oxysporum* isolates used in this analysis are shown in Supplementary Table S5.
[b] Plus signs indicate positive results, and minus signs indicate negative results.

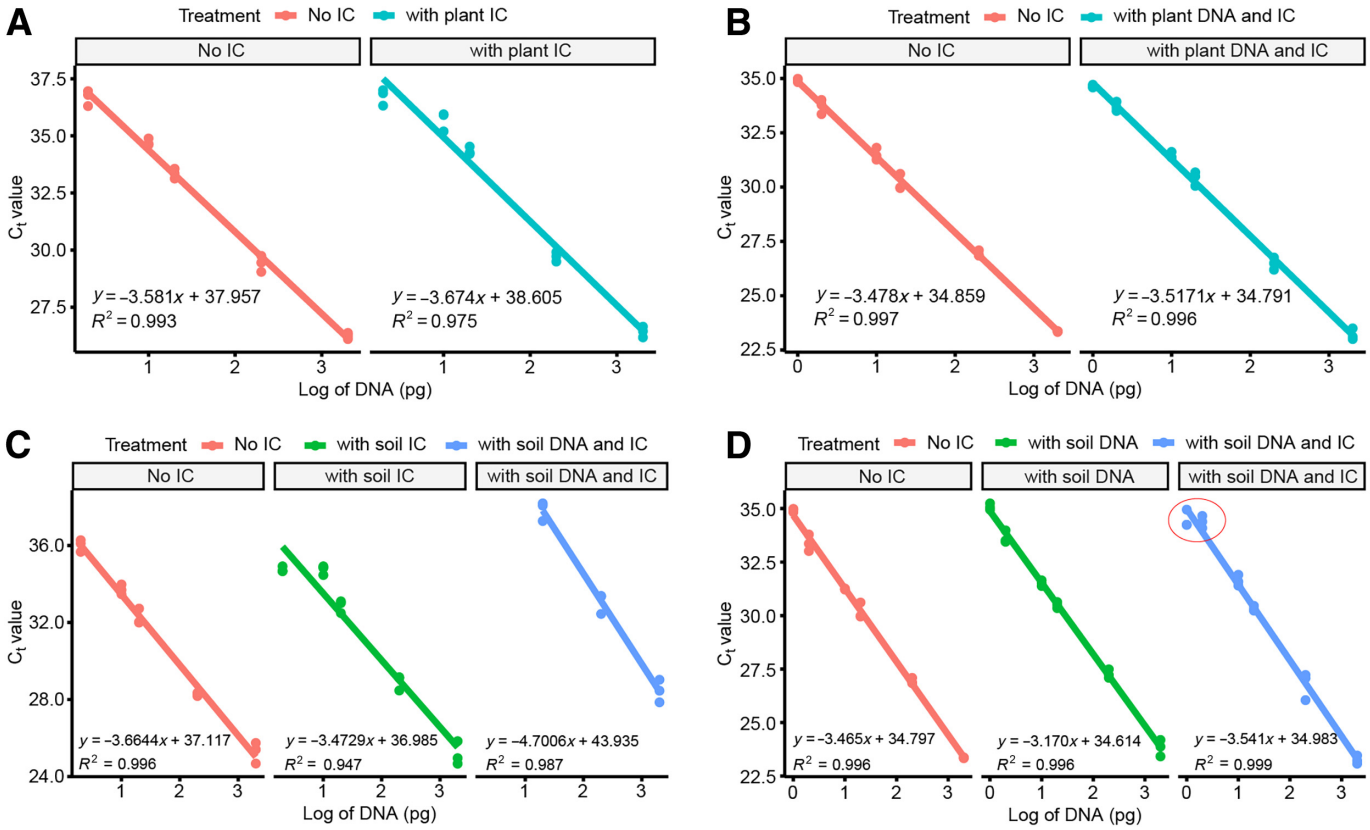

Fig. 4. Standard curves of *Fusarium oxysporum* f. sp. *lactucae* race 1 DNA dilution series with cycle threshold (C_t) value plotted against the log of the template DNA amount to evaluate amplification efficiency and sensitivity of the TaqMan assay under two different annealing temperatures (**A and C,** at 62°C; and **B and D,** at 60°C) in the presence and absence of endogenous plant (A and B) and exogenous (C and D) internal controls (ICs). C_t values indistinguishable between 1 and 2 pg of template DNA when soil IC is present are highlighted in a red circle. Regression analyses were performed for all experiments.

for those with low inoculum densities also improved the overall R^2 value to 0.8249 (Fig. 6A) from 0.738 (Supplementary Fig. S2).

Discussion

Pathogenic *F. oxysporum* are differentiated into *formae speciales* (singular *forma specialis*) based on their ability to cause disease in specific plant species (Armstrong and Armstrong 1981; Gordon and Martyn 1997). Many *formae speciales*, including FOLac, are polyphyletic, and the genes required for host-specific pathogenicity, including effector genes, often reside on gene-poor, transposon-rich accessory chromosomes that may be acquired through horizontal gene transfer (Henry et al. 2021; Ma et al. 2010; Yang et al. 2020). Because of the polyphyletic nature of FOLac, traditional markers that target the ribosomal DNA and highly conserved genes lack sufficient genetic variability to distinguish FOLac from closely related *formae speciales* and *F. oxysporum* nonpathogenic on the host of recovery (Claerbout et al. 2023; Gilardi et al. 2017; D. M. Geiser, *unpublished data*), let alone the two very closely related FOLac races 1 and 4. Accurate identification of FOLac race 1, which is prevalent in many lettuce production areas around the world and the only race detected in the United States, is critical for the lettuce industry as cultivar selection, planting, and disease management decisions heavily depend upon the nature of the pathogen present in the field.

The development of accurate molecular diagnostic tools benefits greatly from the availability of genomic data for both target and nontarget organisms (Steenwyk et al. 2023a). To meet this goal, we generated whole-genome sequence data from 29 FOLac race 1 isolates of broad geographical distribution, two isolates from Japan belonging to FOLac races 2 and 3, 13 FOLac race 4 isolates from several European countries, where the race is present, and 12 nonpathogenic isolates that were recovered from diseased lettuce plants but unable to cause disease. Compared with conventional phylogenomic analyses for phylogenetic inference of the sequenced isolates, which requires substantial computational resources (Steenwyk et al. 2023b), Mash provides a faster and computationally inexpensive approach for genomic comparison and clustering on a massive scale using a *k*-mer approach (Abram et al. 2021; Ondov et al. 2016). The Mash analysis revealed that FOLac was composed of four distinct lineages, with races 1 and 4 both being monophyletic lineages derived from a common ancestor (Fig. 3), a finding consistent with previous studies (Claerbout et al. 2023; Fujinaga et al. 2005; Gilardi et al. 2017; Mbofung et al. 2007). Besides its function for phylogenetic inference, the Mash analysis is also helpful in navigating the selection of ingroup and outgroup *F. oxysporum* isolates to be compared computationally and empirically, as the risk is too high if a false positive or false negative isolate is inadvertently introduced into the screening pipeline.

Sequence-based diagnostic assays have been developed for several *formae speciales* of *F. oxysporum*, with some exploiting sequence polymorphisms in the ribosomal DNA regions (ITS, IGS) and functional genes (De Sousa et al. 2015; Dita et al. 2010; Hirano and Arie 2006; Sasaki et al. 2015; Sharma et al. 2018) and others targeting unique genomic regions (Huang et al. 2016; Ortiz et al. 2017; Pastrana et al. 2019). Designing diagnostic markers that are absent in nontarget genomes is advantageous over those that rely on sequence polymorphisms for pathogen identification due to the reduced importance of carefully controlled annealing temperatures, which, due to calibration differences of thermal cyclers and other forms of lab-specific variation, can lead to erroneous results. With this in mind, we developed a novel approach that utilized the HAWK program (Rahman et al. 2018) for identifying unique genomic regions to target for diagnostic assays. The specificity of these sequences was validated following a hierarchical screening process that included an initial screen by mapping Illumina reads from a diverse panel of 322 nontarget *F. oxysporum* isolates, followed by a series of BLAST analyses against a database containing 797 *F. oxysporum* genome assemblies representing 64 *formae speciales*, as well as nonpathogens from plants and other diverse substrates. This led to the identification of 15 unique sequences that were present only among FOLac race 1 isolates and absent in the other *F. oxysporum* genomes that they were screened against. It is important to run the read mapping analysis for specificity, as it helps ensure reads that are not represented in the Illumina-based genome assembly are examined. Using read mapping, we demonstrated that the FOLac race 1 marker FLA0101 (Shimazu et al. 2005) was present in races 2 and 3 (Supplementary Fig. S1). The other previously developed markers also exhibited varying levels of sequence similarity to *F. oxysporum* isolates that are not FOLac race 1 (see Results; Supplementary Table S1). This highlights the importance of exploiting additional genomic resources that can be integrated into a rigorous screening process to ensure the specificity of the diagnostic marker. These options were unavailable when those markers were developed. The approach described above for identification of unique sequences should be applicable for development of diagnostic assays for additional *formae speciales* of *F. oxysporum* and other fungal taxa.

Although none of the unique sequences was annotated as a protein-coding gene (N. Li, *unpublished data*), the close vicinity of some of them located on the same contig (Supplementary Table S4) suggests that they may be located on lineage-specific accessory chromosomes or lineage-specific compartments of otherwise core

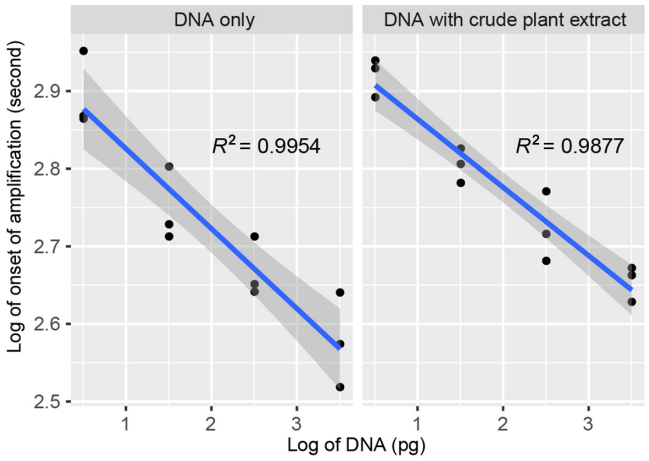

Fig. 5. Onset of amplification of the recombinase polymerase amplification assay using serial dilutions of purified DNA from three different *Fusarium oxysporum* f. sp. *lactucae* race 1 isolates ranging from 2 pg to 2 ng with and without a crude plant extract. The gray band around the regression line represents the 95% confidence interval.

TABLE 4. Lettuce samples tested with the TaqMan and the recombinase polymerase amplification (RPA) assays for the detection of *Fusarium oxysporum* f. sp. *lactucae* race 1, and the corresponding TaqMan results from the cultures isolated from each sample

Location	TaqMan[a]	RPA	Cultures isolated[b]	Number of samples
Symptomatic samples				
True positive				
Yuma, Arizona	+	+	+	28
California	+	+	+	35
True negative				
Yuma, Arizona	–	–	–	1
California	–	–	–	3
Asymptomatic samples				
Yuma, Arizona	+	–	+	1
California	+	–	+	1

[a] Plus signs indicate positive results, and minus signs indicate negative results.
[b] *Fusarium* cultures isolated on Komada's selective medium from vascular tissue of the taproot and tested with the TaqMan assay as evidence of pathogen presence.

chromosomes. Such accessory chromosomes and regions are common and possibly a universal trait in *F. oxysporum* pathogens (Ma et al. 2013; Yang et al. 2020). Of the 15 candidate loci identified, only five were qualified for the development of diagnostic assays as the remainder displayed a highly biased level of GC content, a characteristic of accessory genome regions (Ma et al. 2013). Our ongoing work to improve the genome assemblies of races 1 and 4 will help determine whether these unique loci reside on race-specific accessory chromosomes and potentially expand the pool of race-specific markers for diagnostic assay development.

Both TaqMan qPCR and RPA assays were developed and shown to be highly specific and sensitive when applied to purified fungal DNA (Table 3). We tested a wide range of *F. oxysporum* isolates representing 42 different *formae speciales* and 38 *F. oxysporum* isolates that were nonpathogenic on the host of recovery, and all of them were negative for both assays. For the TaqMan assay, high C_t values ranging from 37.35 to 45.69, above the detection limit of the TaqMan assay (C_t of 37), could be detected from some of the nontarget isolates (Supplementary Table S5); however, because the C_t value varied between two replicates (in most cases, one replicate yielded no signal) and did not change when tested with 10 and 20 ng of the same DNA sample (5× and 10× higher than the original amount), we considered those samples true negatives. Compared with the TaqMan assay, no background signal was observed when testing the same panel of fungal DNA using the RPA assay. However, the detection limit of both assays (2 pg) was 20-fold higher than those developed previously, which had sensitivity of 100 fg (Mestdagh et al. 2023), although similar levels of amplification efficiency were achieved (90.22% in this study versus ~92% described in Mestdagh et al. 2023; Sanna et al. 2022). Such differences in the detection limit could be attributed to the use of different types of real-time PCR master mix and presumably the integrity of tested DNA.

The detection limit of both diagnostic assays was also determined in the presence of environmental DNA, including plant (for both TaqMan and RPA assays) and soil (for TaqMan assay only) extracts, to mimic real testing conditions, as suggested in several studies (Bilodeau et al. 2012; Burkhardt et al. 2018; Kunjeti et al. 2016). One unique feature of the assays developed in this study that differs from the other detection assays focusing on FOLac race 1 is the ability to multiplex with IC for quality control purposes. The inclusion of IC into a single-tube reaction required some fine-tuning for the TaqMan assay (Fig. 4) but worked well for the RPA assay (Fig. 5). Under the initial testing condition, amplification of the target sequence was significantly inhibited when environmental DNA was added to the TaqMan assay (Fig. 4A and C), suggesting that something else in the environmental DNA, not the IC primers and probes, interfered with pathogen detection. Haudenshield and Hartman (2011) showed that the detection of *F. virguliforme*, the soybean sudden death syndrome pathogen, was greatly compromised by adding an exogenous IC to the master mix, although the potential cause was not investigated. This was discussed in greater detail in Bilodeau et al. (2012) and Kunjeti et al. (2016); it was believed to be due to competition for amplification between the IC and target DNA and could be ameliorated by using a low amount of exogenous IC. Because we started with a level of exogenous IC in the master mix that was not found to interfere with target amplification at the concentration present, another factor was likely involved in the inhibition we encountered. The negative impact of the DNA extracted from lettuce plants or soil was reversed by reducing the annealing temperature (Fig. 4B and D), which might have relaxed the amplification stringency, as supported by the observed 5% (with plant DNA and IC) and 28.4% (with soil DNA and IC) increase in the amplification efficiency of the pathogen, as well as a significant drop of the C_t value under the new annealing temperature (Fig. 4). This modification also improved the detection limit from 2 to 1 pg (Fig. 4). Further research is needed to uncover the components present in the environmental DNA that inhibit the FOLac race 1 marker from amplifying at 62°C.

The detection of FOLac race 1 in lettuce samples obtained from commercial lettuce growers in California and Arizona indicated that both assays were useful tools for in planta Fusarium wilt diagnostics, which showed 100% sensitivity and 100% specificity on the 69 lettuce samples tested (Table 4). With the plant IC multiplexed with the pathogen assay, several false negative samples were identified due to the lack of amplification from the plant IC. These samples

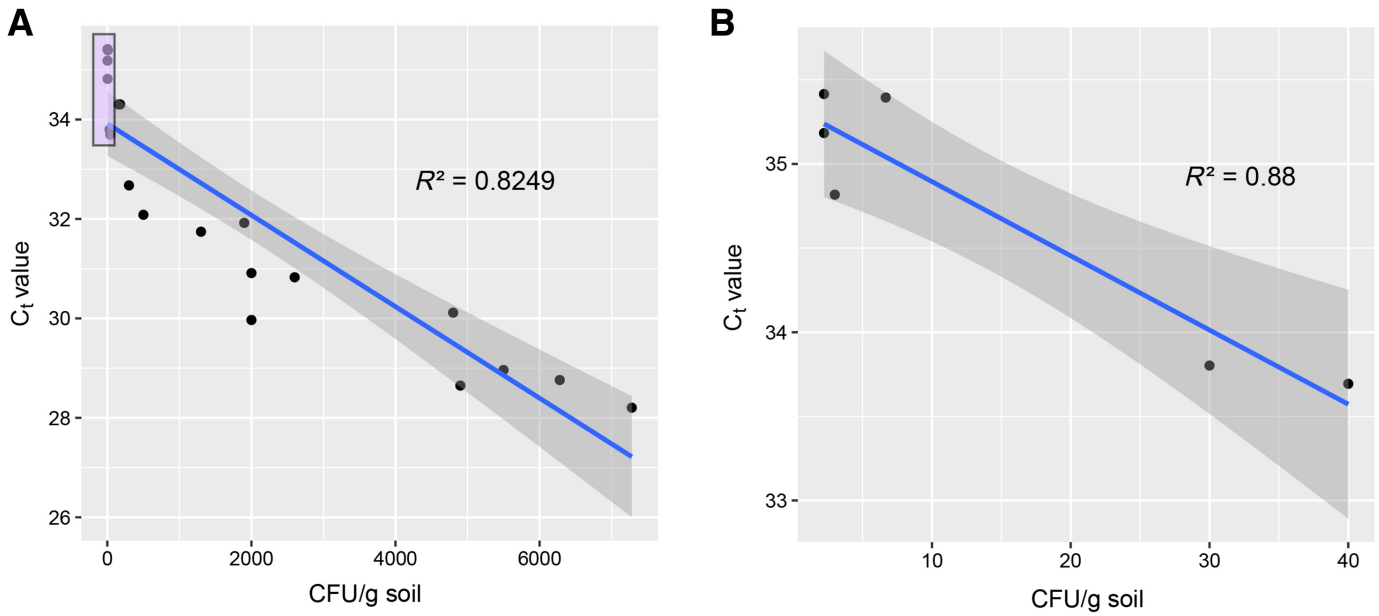

Fig. 6. Relationship of TaqMan quantitative PCR (qPCR) cycle threshold (C_t) values and measured CFU/g values for *Fusarium oxysporum* f. sp. *lactucae* race 1. **A,** Regression analysis was performed on all 21 soil samples used in the 1:1 dilution series. The qPCR assay was run with one (greater than 40 CFU/g) or four (equal or less than 40 CFU/g) microliters of soil DNA. The datapoints in the shaded purple area represent samples at or below 40 CFU/g. A separate regression analysis of these soil samples is shown in **B**.

were found to be true positives with re-extracted plant DNA, highlighting the importance of incorporating IC for diagnostic assays. In confirmation, all samples were plated on Komada's medium, and the recovered fungal cultures were positive for the TaqMan assay (Table 4). In addition, both assays have the potential to monitor for the presence of FOLac race 1 in asymptomatic lettuce samples (Table 4). Although it initially failed to detect the pathogen in asymptomatic plants, the RPA assay later showed positive results using the extracted plant DNA that tested positive with the TaqMan assay. This suggests that the false negative result obtained from the RPA assay might be due to the low amount of pathogen DNA in that crude plant extract. A broader panel of asymptomatic lettuce plants, including resistant lettuce cultivars, is needed to fully assess the efficacy of both assays on field samples. Because all lettuce samples surveyed in this study were at the early development stage, the ability for early detection of the pathogen in plants prior to expressing typical symptoms of Fusarium wilt would allow growers to quickly respond to the disease with effective management strategies and minimize the risk of disease spread (Buja et al. 2021).

Developing a soil quantification assay for FOLac race 1 with high sensitivity and accuracy remains a challenge. Traditional soil plating on Komada's medium represents a time-consuming and nonspecific approach due to the lack of morphological characteristics to distinguish *formae speciales*, including FOLac, from other *F. oxysporum* isolates that may be present in the soil as saprophytes. Here, we provided a solution to this with the FOLac race 1-specific TaqMan qPCR assay. At the beginning of the soil quantification assay, attempts were made to construct a drug-resistant mutant of FOLac race 1 so that contamination issues can be easily avoided in the plating assay. However, it was unsuccessful after multiple trials. A wild-type strain was then used for infesting an autoclaved soil with attention made to not introducing contamination into the system. Taking advantage of a newly developed DNA extraction method (Matson et al. 2024) that can substantially increase detection sensitivity of *F. oxysporum* f. sp. *fragariae* in soils by sampling large quantities of soils for higher DNA yield, we utilized the method for extracting DNA from artificially infested soils carrying various amounts of chlamydospores of FOLac race 1. Our soil dilution series examining the relationship between inoculum densities and C_t values showed that the qPCR assay could reliably detect the pathogen in soils down to approximately 2 CFU/g with an average C_t value of 35.30, although 4 µl of DNA added to the amplification master mix is needed to achieve this level of sensitivity (Fig. 6). Our result differed slightly from the *F. oxysporum* f. sp. *fragariae* quantification assay, in which 2 µl of a soil DNA extraction was enough to detect the pathogen at a level below 10 CFU/g soil. This may be attributed to the difference in soil texture, which was previously shown to affect the extraction efficiency (Matson et al. 2024). Given that only one soil type was evaluated in this study, a comprehensive assessment of different soil types that are commonly used in lettuce cultivation (heavy versus loamy soil texture) would be needed to fully understand the sensitivity of the qPCR-based soil quantification assay across different conditions.

The genomics-informed approach taken here serves as an example of how serious issues that impede traditional and molecular diagnostics development (inconsistent pathological and morphological phenotypes; high genomic similarity between different pathotypes) can be overcome. The same framework utilized here can be used as a starting point for developing similar markers for other *F. oxysporum formae speciales* that currently exist or may arise in the future. New resources can be developed for other plant pathogen groups (Geiser et al. 2023) or even to human pathogens where parallel challenges exist (Kowalski and Cramer 2020). Central to this effort is the creation and curation of genomic data from many hosts and substrates, as well as inclusion of both pathogens and nonpathogens that occur on the same host, utilizing both publicly available data and new data targeted to a specific pathogen.

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