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Use of Comparative Genomics-Based Markers for Discrimination of Host Specificity in *Fusarium oxysporum*

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ABSTRACT The polyphyletic nature of many *formae speciales* of *Fusarium oxysporum* prevents molecular identification of newly encountered strains based on conserved, vertically inherited genes. Alternative molecular detection methods that could replace labor- and time-intensive disease assays are therefore highly desired. Effectors are functional elements in the pathogen-host interaction and have been found to show very limited sequence diversity between strains of the same *forma specialis*, which makes them potential markers for host-specific pathogenicity. We therefore compared candidate effector genes extracted from 60 existing and 22 newly generated genome assemblies, specifically targeting strains affecting cucurbit plant species. Based on these candidate effector genes, a total of 18 PCR primer pairs were designed to discriminate between each of the seven Cucurbitaceae-affecting *formae speciales*. When tested on a collection of strains encompassing different clonal lineages of these *formae speciales*, nonpathogenic strains, and strains of other *formae speciales*, they allowed clear recognition of the host range of each evaluated strain. Within *Fusarium oxysporum* f. sp. *melonis* more genetic variability exists than anticipated, resulting in three *F. oxysporum* f. sp. *melonis* marker patterns that partially overlapped with the cucurbit-infecting *Fusarium oxysporum* f. sp. *cucumerinum*, *Fusarium oxysporum* f. sp. *niveum*, *Fusarium oxysporum* f. sp. *momordicae*, and/or *Fusarium oxysporum* f. sp. *lagenariae*. For *F. oxysporum* f. sp. *niveum*, a multiplex TaqMan assay was evaluated and was shown to allow quantitative and specific detection of template DNA quantities as low as 2.5 pg. These results provide ready-to-use marker sequences for the mentioned *F. oxysporum* pathogens. Additionally, the method can be applied to find markers distinguishing other host-specific forms of *F. oxysporum*.

IMPORTANCE Pathogenic strains of *Fusarium oxysporum* are differentiated into *formae speciales* based on their host range, which is normally restricted to only one or a few plant species. However, horizontal gene transfer between strains in the species complex has resulted in a polyphyletic origin of host specificity in many of these *formae speciales*. This hinders accurate and rapid pathogen detection through molecular methods. In our research, we compared the genomes of 88 strains of *F. oxysporum* with each other, specifically targeting virulence-related genes that are typically highly similar within each *forma specialis*. Using this approach, we identified marker sequences that allow the discrimination of *F. oxysporum* strains affecting various cucurbit plant species through different PCR-based methods.

KEYWORDS cucurbits, genome analysis, host range, molecular markers, pathogen detection, pathogenic fungi

Accurate and rapid pathogen detection is necessary to take appropriate action against plant diseases. *Fusarium oxysporum* is a soilborne fungus that includes both nonpathogenic and plant-pathogenic strains. Pathogenic strains of *F. oxysporum*

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TABLE 1 *Formae speciales* of *F. oxysporum* affecting members of the Cucurbitaceae family

Forma specialis	Abbreviation in strain designations	Host	Reference(s)
F. oxysporum f. sp. *cucumerinum*	Focuc	Cucumber (*Cucumis sativus*)	66
F. oxysporum f. sp. *melonis*	Fomln	Muskmelon (*Cucumis melo*)	67
F. oxysporum f. sp. *niveum*	Foniv	Watermelon (*Citrullus lanatus*)	68
F. oxysporum f. sp. *radicis-cucumerinum*	Forcu	Multiple cucurbits (including cucumber, melon, watermelon, and gourd)	69
F. oxysporum f. sp. *momordicae*	Fomom	Bitter gourd (*Momordica charantia*)	70
F. oxysporum f. sp. *lagenariae*	Folag	Calabash gourd (*Lagenaria* spp.)	32, 71
F. oxysporum f. sp. *luffae*	Foluf	Sponge gourd (*Luffa cylindrica*)	7

cause vascular wilt and cortical rot disease in a wide variety of agricultural crop species. They are classified into host-specific forms (*formae speciales*) and are often further subdivided into races based on their capacity to infect different cultivars of a plant species (1–3).

Fusarium wilt and root rot in cucurbits are among the most prominent and destructive diseases affecting this plant family (4–6). In total, seven cucurbit-infecting *formae speciales* have been described: *Fusarium oxysporum* f. sp. *cucumerinum*, *Fusarium oxysporum* f. sp. *melonis*, *Fusarium oxysporum* f. sp. *niveum*, *Fusarium oxysporum* f. sp. *radicis-cucumerinum*, *Fusarium oxysporum* f. sp. *lagenariae*, *Fusarium oxysporum* f. sp. *momordicae*, and *Fusarium oxysporum* f. sp. *luffae* (Table 1). The last three are mostly restricted to Southeast Asia (7), while the *formae speciales* affecting cucumber, melon, and watermelon are globally distributed and more important from an economic standpoint (4, 7, 8).

Currently, there are no effective curative treatments for *Fusarium* disease (9). Use of resistant varieties or rootstocks is the only practical measure for controlling the disease in the field (10–12). In greenhouses, soil sterilization by fumigation with methyl bromide can be performed (10, 13). Most efforts are directed toward prevention of the disease. Routine methods that provide reliable subspecific identification, sensitive detection, and accurate quantification of *F. oxysporum* are of high importance (14) and could prevent unnecessary efforts to suppress harmless fungal populations (15). Development of these types of markers has thus far been complicated by the polyphyletic nature of most *formae speciales* of *F. oxysporum* (14).

As many *F. oxysporum* strains have been found to be nonpathogenic, endophytic, or even applicable as biocontrol strains (16–18), discrimination between pathogenic and abundantly present nonvirulent strains is very important (19). Discrimination of *F. oxysporum formae speciales* and races is routinely done through labor- and time-intensive disease assays (20–22). Molecular detection methods are therefore highly desired.

Formae speciales are often of polyphyletic origin (23), and pathogenic strains may share a higher level of sequence similarity of conserved genes with strains that are nonpathogenic or pathogenic toward another host (24, 25). Diagnostics based on genes like that encoding translation elongation factor 1-alpha (*EF1*α) or the ribosomal intergenic spacer (IGS) are therefore only useful to discriminate between fungal species (26, 27). In several cases they have been suggested for subspecies discrimination, but these often prove to be unreliable for this purpose (8, 27, 28).

Several molecular markers for the cucurbit-infecting *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *radicis-cucumerinum*, *F. oxysporum* f. sp. *niveum*, and *F. oxysporum* f. sp. *luffae* have been developed. These are all based on random amplified polymorphic DNA (RAPD) fragments, resulting in sequence-characterized amplified region (SCAR) markers. SCAR markers are suboptimal for *forma specialis* discrimination because they are based on genomic regions that are not necessarily required for virulence. Furthermore, as they can be localized anywhere on the genome, there are often little to no sequence data available in public databases for comparison with other sequences. The robustness of the markers can be verified only by screening against a large collection of strains (14).

Interestingly, closer inspection of previously developed *forma specialis*-distinguishing SCAR markers showed that the selected sequences were often (part of) a transposable element, such as *Fot1* (*Fusarium oxysporum* f. sp. *albedinis*, *Fusarium oxysporum* f. sp. *chrysanthemi*, and *Fusarium oxysporum* f. sp. *dianthi*) and *Folyt1* (*F. oxysporum* f. sp. *radicis-cucumerinum*) and *Impala* (*Fusarium oxysporum* f. sp. *ciceris* and *F. oxysporum* f. sp. *dianthi*) (29), or pathogenicity-associated genes like *FTF1* (*Fusarium oxysporum* f. sp. *phaseoli*) (30). A race 1-specific *Fusarium oxysporum* f. sp. *lactucae* marker was developed by amplifying and cloning regions between long terminal repeats of retrotransposons in the genome (31). For *F. oxysporum* f. sp. *lagenariae*, *F. oxysporum* f. sp. *momordicae*, and *F. oxysporum* f. sp. *melonis*, only DNA fingerprinting results have been described thus far (32).

It was recently shown that host specificity is associated with the suite of effector genes present in the genomes of *F. oxysporum* strains (33). Both presence-absence polymorphisms and the sequence type of individual effector genes turned out to be predictive for a strain's host range. These genes therefore form the most solid base for discrimination of *formae speciales* within the *F. oxysporum* species complex (FOSC) (14, 20, 25). Indeed, use of virulence genes to identify fungal plant pathogens has proven successful in the past for other *Fusarium* species (34, 35). Within the FOSC, this approach has been applied to distinguish *Fusarium oxysporum* f. sp. *cubense* tropical race 4 by targeting a candidate effector gene (36). Additionally, *Fusarium oxysporum* f. sp. *lycoperici* and *F. oxysporum* f. sp. *cubense* can be discriminated from other *formae speciales* through the use of PCR primers designed to detect specific *secreted in xylem* (*SIX*) effector gene sequences (15, 25, 37). At the time of these studies, however, no (or limited) comparative genomics analyses could be performed due to the lack of available genome sequences. All *SIX* genes have homologs in other host-pathogenic forms of *F. oxysporum* (e.g., *SIX1*, *SIX5*, and *SIX6* [33, 38–40]). For these, marker specificity could not be evaluated beforehand and cross-reaction with nontarget *formae speciales* was found (25).

Since it is not yet viable to sequence every individual strain encountered, we decided to design effector candidate-based markers. In this way, we aimed to be able to distinguish cucurbit-affecting *formae speciales* from (i) each other, (ii) other *formae speciales*, and (iii) nonpathogenic strains. Therefore, we used whole-genome sequences of a number of representative cucurbit-infecting *F. oxysporum* strains as a starting point and identified putative effector genes suitable as markers. An advantage of using molecular markers over whole-genome sequencing is that they can also be applied to infected soil or plant tissue samples; the fungus does not need to be isolated and cultured (28). Techniques such as TaqMan real-time PCR even allow for a quantitative evaluation of pathogen abundance, e.g., on DNA isolated from soil (41).

The genetic bases for host specificity of FOSC strains toward plants belonging to the Cucurbitaceae family are similar (33), making these *formae speciales* relatively difficult to separate. This means that this is a good test case for host specificity discrimination, and the results presented here can be exemplary for application to other plant species where disease caused by *F. oxysporum* is a pressing problem.

RESULTS

Several cucurbit-infecting *formae speciales* have a polyphyletic origin. In order to be able to select *forma specialis*-wide marker sequences, it is necessary to collect the genetic variety for that *forma specialis* as completely as possible. We made use of 66 previously published genome sequences and added *de novo* genome assemblies generated from Illumina paired-end read data of 22 new strains (see Data S2 in the supplemental material).

In order to assess the genetic diversity of the *formae speciales* under investigation, we generated a phylogenetic tree based on over 400 core genomic gene sequences from each of their genomes (Fig. 1). This showed that *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *niveum*, and *F. oxysporum* f. sp. *lagenariae* occupied multiple clades in the tree (5, 3, 3, and 3, respectively), indicating that they

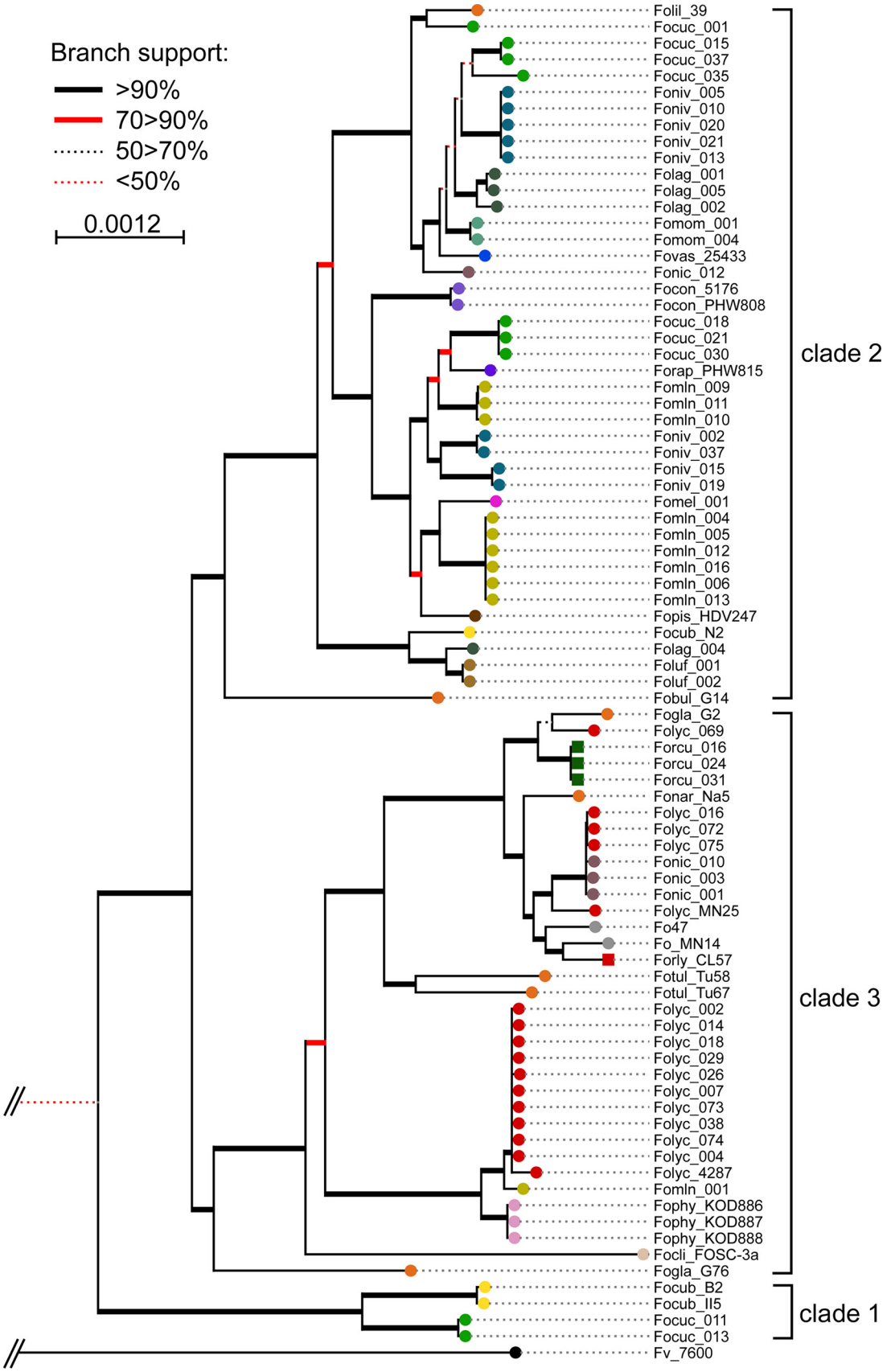

FIG 1 *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *niveum*, and *F. oxysporum* f. sp. *lagenariae* are of polyphyletic origin. A total of 441 conserved core genes from all genomes were extracted, aligned, and concatenated into

(Continued on next page)

belong to different clonal lines. In our set of strains, we have 6 of 7 described *F. oxysporum* f. sp. *cucumerinum* vegetative compatibility groups (VCGs) (6, 42), 3 of 8 *F. oxysporum* f. sp. *melonis* VCGs (43), all 3 *F. oxysporum* f. sp. *niveum* VCGs (43), and both *F. oxysporum* f. sp. *radicis-cucumerinum* VCGs (43). For *F. oxysporum* f. sp. *lagenariae* (3 VCGs described [44]), *F. oxysporum* f. sp. *momordicae* (4 VCGs described [44]), and *F. oxysporum* f. sp. *luffae* (unknown number of VCGs), no VCG information was available for our strains, although they group into three, one, and one clade(s), respectively (Fig. 1).

Candidate effector gene phylogenies display clear grouping of host specificity. Unlike conserved core genes, virulence-related genes tend to be identical across members belonging to the same polyphyletic *forma specialis* of *F. oxysporum* (15, 33). For this reason, they have predictive value for a strain's host range. *Forma specialis* markers are essentially the smallest possible set of effector genes that is shared by all strains of a *forma specialis* and absent or different in sequence (at least as a set) in all other strains (33).

We extracted the sequences of the candidate effector genes from the work of van Dam et al. (33) from all assemblies and generated a multiple-sequence alignment (MSA; see Data S1 in the supplemental material) and phylogenetic tree for each of them (three examples in Fig. 2 and continued in Data S2). A custom python script identified those genes in which all members of a *forma specialis* grouped together in a separate clade. From the genes displaying such grouping, the genes that facilitated the best discrimination were selected based on manual inspection of the MSA to come to a final selection of marker sequences per *forma specialis* (Table 2).

Some of the selected genes show multiple *forma specialis*-specific clades; therefore, multiple markers targeting different *forma specialis* could be designed on these genes. An example is candidate effector 99, a hypothetical protein-encoding gene that is used as a marker for *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *lagenariae*, *F. oxysporum* f. sp. *cucumerinum*, and *F. oxysporum* f. sp. *luffae* (Fig. 2B). *F. oxysporum* f. sp. *melonis* strain Fomln010 possesses a copy identical to both candidate effector 99 homologs present in the *F. oxysporum* f. sp. *niveum* strains as well as a copy identical to the *F. oxysporum* f. sp. *lagenariae* gene sequence. To still be able to distinguish these *formae speciales* from one another, it is therefore of importance to use multiple markers for each *forma specialis*.

Discrimination of cucurbit-infecting *formae speciales* by PCR. PCR primers were designed specifically on polymorphic regions of the selected DNA sequences (Table 2; see also Data S1), aiming to generate a PCR product sized above 120 and below 700 nucleotides (nt) for quick and reliable application. The *Fusarium* extracellular matrix 1 gene (*FEM1*) (45) was taken along as a positive control. To verify the applicability of the markers, PCRs were executed for each of the primer pairs on a subset of the strains that were used for marker design, i.e., of which the host range has been confirmed and the genome had been sequenced. This included strains belonging to the cucurbit-infecting *formae speciales*, several other *formae speciales* (*Fusarium oxysporum* f. sp. *vasinfectum*, *Arabidopsis* infecting, *F. oxysporum* f. sp. *lycopersici*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Fusarium oxysporum* f. sp. *nicotianae*, *Fusarium oxysporum* f. sp. *melongenae*, *Physalis* infecting, *F. oxysporum* f. sp. *cubense*, *Fusarium oxysporum* f. sp. *pisi*, *Fusarium oxysporum* f. sp. *tulipae*, and *Fusarium oxysporum* f. sp. *gladioli*) and two nonpathogenic *F. oxysporum* strains: Fo47 (16) and MN14 (33). The strains were selected based on their differential phylogenetic distribution in Fig. 1 as well as the presence and absence of selected marker sequences in their genome assembly.

FIG 1 Legend (Continued)
a multiple-sequence alignment. Phylogeny was inferred with 100 bootstrap iterations. All strains fall within the three main clades of the FOSC. Focuc, *F. oxysporum* f. sp. *cucumerinum*; Fomln, *F. oxysporum* f. sp. *melonis*; Foniv, *F. oxysporum* f. sp. *niveum*; Forcu, *F. oxysporum* f. sp. *radicis-cucumerinum*; Folag, *F. oxysporum* f. sp. *lagenariae*; Foluf, *F. oxysporum* f. sp. *luffae*; Fomom, *F. oxysporum* f. sp. *momordicae*. For abbreviations of other *formae speciales*, see Data S1.

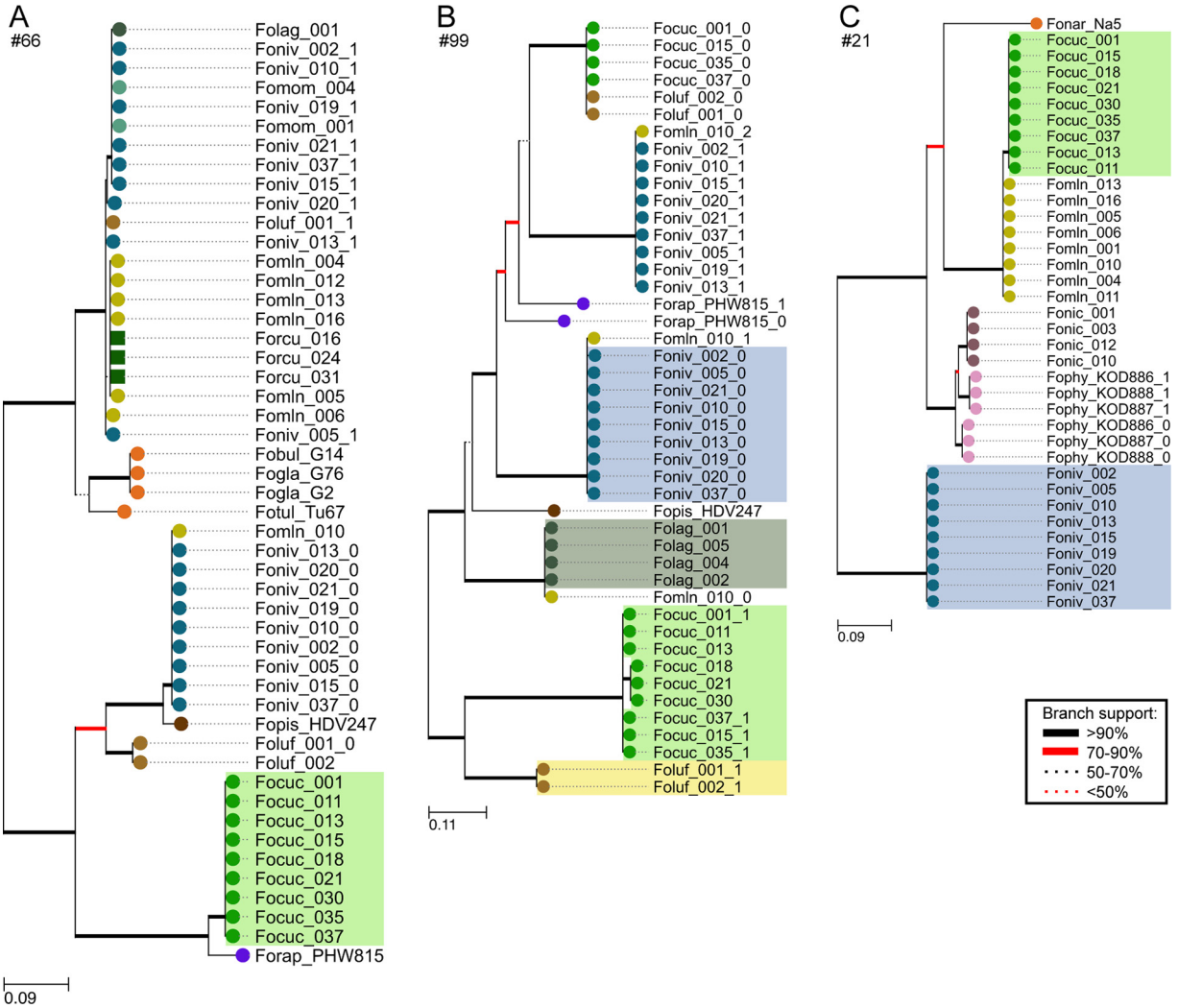

FIG 2 Phylogenetic trees of three genes selected as markers for *F. oxysporum* f. sp. *cucumerinum*: 66 (A), 99 (B), and 21 (C). Separation of a clade that includes all strains belonging to a *forma specialis* indicates sequence similarity within and sequence dissimilarity between *formae speciales*. Colored areas in the tree reflect the target *forma specialis* of the marker. Hypothetical protein-encoding genes 99 and 21 are used as markers for multiple *formae speciales*.

All except one of the *forma specialis*-specific PCR markers behaved like expected (Table 3), showing PCR products only in the expected combinations of genomic DNA and marker primers. One false-positive PCR product was found, in the combination of *F. oxysporum* f. sp. *pisi* HDV247 and marker 130 (*F. oxysporum* f. sp. *momordicae*). In the genome assembly of HDV247, this gene was found to be present with 97% sequence similarity, although the downstream region of this gene provided sufficient sequence diversity for primer design (Data S1).

Marker 94, targeting all cucurbit-affecting *formae speciales*, gave a band of the correct size for all cucurbit-affecting isolates tested, except Fomln010. Furthermore, strain Fomln010 displayed an atypical *F. oxysporum* f. sp. *melonis* marker pattern, as it yielded PCR products that were not seen in the other *F. oxysporum* f. sp. *melonis* isolates for *F. oxysporum* f. sp. *niveum* markers 99 and 100 and *F. oxysporum* f. sp. *lagenariae* marker 99. This pattern, designated pattern B in Table 3, was not unexpected, since presence of identical sequences for these three markers as well as absence of the gene encoding hypothetical protein 94 had been observed in the genome assembly of Fomln010 (sequence similarity of marker 99 shown in Fig. 2B). The presence of identical effector candidate sequences across *formae speciales* affecting similar plant species was

TABLE 2 Selected marker genes and their respective target *formae speciales*

Gene ID[a]	Target gene	Target *forma specialis*[b]	Gene tree illustration
Positive control	*FEM1*	Positive control	
94	HPEG[c]	All cucurbit-infecting *formae speciales*	Data S2A
13	*SIX13*	*radicis-cucumerinum*	Data S2B
70	HPEG	*radicis-cucumerinum*	Data S2C
66	HPEG	*cucumerinum*	Fig. 2A
99	HPEG	*cucumerinum*	Fig. 2B
21	Fom effector 7	*cucumerinum*	Fig. 2C
1	*SIX1*	*melonis*	Data S2D
20	Fom effector 6	*melonis*	Data S2E
18	Fom effector 3	*melonis* + *niveum*	Data S2G
99	HPEG	*niveum*	Fig. 2B
100	HPEG	*niveum*	Data S2H
21	Fom effector 7 (pseudogenized)	*niveum*	Fig. 2C
98	HPEG	*momordicae*	Data S2I
130	HPEG	*momordicae*	Data S2J
1	*SIX1*	*lagenariae* + *momordicae*	Data S2D
71	HPEG	*lagenariae*	Data S2F
99	HPEG	*lagenariae*	Fig. 2B
99	HPEG	*luffae*	Fig. 2B

[a]ID, identifier.
[b]All *formae speciales* listed are *F. oxysporum formae speciales*; to save space, a shortened form of the organism name is used.
[c]HPEG, hypothetical protein-encoding gene.

not surprising since they share part of their genetic toolset allowing for pathogenic colonization of these plants (33). It does, however, make marker selection more challenging. While screening for specific differentiation of, for instance, *F. oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *niveum*, it is therefore important to check multiple markers.

Evaluating *forma specialis* classification using markers. After testing of the markers on sequenced strains to verify that they worked as anticipated, an extended set of strains originating from around the world (strain information in Data S1) was screened. Most strains were isolated from *Fusarium*-affected cucurbit plants and were described as one of the pathogenic forms listed in Table 1. The aim was to either confirm or reject their reported host specificity with our markers. A number of strains isolated from noncultivated soil samples was also taken along. The expectation was that these nonspecialized strains do not possess many effector genes and therefore would test negative for all of the 18 markers.

As can be seen from Table 4, marker analysis confirmed the reported *forma specialis* of most strains that were tested. However, some strains behaved differently than expected. For example, PCR products were identified for Fomln017, Fomln021, Fomln024, and Fomln026 for *cucumerinum* markers 66 and 21, as well as *F. oxysporum* f. sp. *momordicae*/*F. oxysporum* f. sp. *lagenariae* marker 1. Intriguingly, none of the *F. oxysporum* f. sp. *melonis* markers tested positive in these strains (marker pattern C in Table 4). Additionally, a third *F. oxysporum* f. sp. *melonis* pattern was observed with strain Fomln023 that was nearly identical to the pattern of Fomln010 (pattern B in Table 3). Finally, *F. oxysporum* f. sp. *melonis* marker 1 cross-reacted with Foniv041 genomic DNA, showing that this marker is not 100% specific for *F. oxysporum* f. sp. *melonis*.

Cucurbit marker 94 did not test positive for four individual *F. oxysporum* f. sp. *melonis* strains and one *F. oxysporum* f. sp. *momordicae* strain. However, while it does not detect all cucurbit-infecting strains, it did not result in false positives (Tables 3 and 4), meaning that it can still be used in addition to the other *forma specialis*-specific markers.

Several strains showed a marker pattern typically observed for another *forma specialis*, indicating that their reported host specificities might not be accurate. Strains Focuc014 and Focuc040, reported as *F. oxysporum* f. sp. *cucumerinum*, clearly showed

TABLE 3 PCR markers allowing discrimination of cucurbit-affecting *formae speciales* of *F. oxysporum*[a]

Strain	*forma specialis*	FEM (+)	94 cucurbits	13 Forcu	70 Forcu	66 Focuc	99 Focuc	21 Focuc	1 Fomln	20 Fomln	18 Fomln + Foniv	99 Foniv	100 Foniv	21 Foniv	98 Fomom	130 Fomom	1 Fomom + Folag	71 Folag	99 Folag	99 Foluf	Marker profile
Forcu016	*radicis-cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*
Forcu031	*radicis-cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*
Focuc001	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*
Focuc013	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*
Focuc015	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*
Focuc018	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*
Focuc035	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*
Fomln001	*melonis*	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*
Fomln006	*melonis*	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*
Fomln010	*melonis*	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	-	*melonis "B"*
Foniv002	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*
Foniv010	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*
Foniv015	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*
Foniv020	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*
Fomom001	*momordicae*	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	*momordicae*
Fomom004	*momordicae*	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	*momordicae*
Folag001	*lagenariae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	*lagenariae*
Folag004	*lagenariae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	*lagenariae*
Foluf001	*luffae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*luffae*
Foluf002	*luffae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	*luffae*
Fovas 25433	*vasinfectum*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Focon5176	*conglutinans*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Folyc 4287	*lycopersici*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Folyc MN25	*lycopersici*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Folyc069	*lycopersici*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Folyc072	*lycopersici*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Forly CL57	*radicis-lycopersici*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fonic001	*nicotianae*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fomel001	*melongenae*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fophy KOD886	*physali*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Focub II5	*cubense*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fopis HDV247	*pisi*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-
Fotul Tu67	*tulipae*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fogla G76	*gladioli*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fo47	non-pathogenic	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FoMN14	non-pathogenic	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Symbols: +, positive test result; −, negative test result; ±, weak positive test result (very faint PCR product of the expected size present).

a positive result for both *F. oxysporum* f. sp. *radicis-cucumerinum* markers and an absence of all three *F. oxysporum* f. sp. *cucumerinum* markers, suggesting that they are in fact *F. oxysporum* f. sp. *radicis-cucumerinum* strains. Another interesting candidate was strain 14150, reportedly an isolate belonging to "*F. oxysporum* f. sp. *cucurbitacearum*," a *forma specialis* proposed to encompass all *formae speciales* affecting cucurbits (46). This strain also showed the marker pattern typically observed for *F. oxysporum* f. sp. *radicis-cucumerinum*. Four strains (one reported as *F. oxysporum* f. sp. *cucumerinum* and three as *F. oxysporum* f. sp. *niveum*) displayed an absence of all 18 markers tested, while another strain (reported as *F. oxysporum* f. sp. *cucumerinum*) tested positive only for *F. oxysporum* f. sp. *cucumerinum* marker 21, suggesting that they are not capable of infecting any of the cucurbit plants. As expected, each of the environmental strains tested negative for all of the markers.

Disease assays confirm marker predictions. The strains of which the reported *forma specialis* did not match the marker pattern were tested in a bioassay on susceptible cucurbit varieties to evaluate their actual host range (Table 4, rightmost column; Data S3). Strains Focuc014, Focuc040, and 14150 caused severe crown rot symptoms in both cucumber and melon, meaning that they are in fact *F. oxysporum*

TABLE 4 PCR testing of the markers on a set of 48 worldwide isolates for verification of their reported *formae speciales*

Strain	Reported f.sp.	FEM (+)	94 cucurbits	13 Forcu	70 Forcu	66 Focuc	99 Focuc	21 Focuc	1 Fomln	20 Fomln	18 Fomln + Foniv	99 Foniv	100 Foniv	21 Foniv	98 Fomom	130 Fomom	1 Fomom + Folag	71 Folag	99 Folag	99 Foluf	Marker profile [a]	Bio-assay result [b]
Focuc014	*cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	*radicis-cucumerinum*
Focuc040	*cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	*radicis-cucumerinum*
Forcu005	*radicis-cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	not tested
Forcu017	*radicis-cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	not tested
Forcu020	*radicis-cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	not tested
Forcu028	*radicis-cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	not tested
Forcu029	*radicis-cucumerinum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	not tested
14150	*cucurbitacearum*	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*radicis-cucumerinum*	*radicis-cucumerinum*
Focuc009	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Focuc010	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Focuc016	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Focuc017	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Focuc026	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Focuc027	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Focuc036	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Focuc038	*cucumerinum*	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	*cucumerinum*	not tested
Fomln018	*melonis*	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*	not tested
Fomln019	*melonis*	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*	not tested
Fomln020	*melonis*	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*	not tested
Fomln027	*melonis*	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*	not tested
Fomln025	*melonis*	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*	*melonis*
Fomln002	*melonis*	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*	non-pathogenic
Fomln003	*melonis*	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	*melonis "A"*	not tested
Fomln023	*melonis*	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	*melonis "B"*	*melonis/niveum*
Fomln024	*melonis*	+	+	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-	*melonis "C"*	*melonis*
Fomln017	*melonis*	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	*melonis "C"*	*melonis*
Fomln021	*melonis*	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	*melonis "C"*	*melonis*
Fomln026	*melonis*	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	*melonis "C"*	*melonis*
Foniv011	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*	not tested
Foniv017	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*	not tested
Foniv018	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*	not tested
Foniv033	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*	not tested
Foniv039	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*	not tested
Foniv040	*niveum*	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	*niveum*	not tested
Foniv041	*niveum*	+	+	-	-	-	-	-	±	-	+	+	+	+	-	-	-	-	-	-	*niveum*	not tested
Fomom002	*momordicae*	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	*momordicae*	not tested
Fomom003	*momordicae*	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	*momordicae*	not tested
Folag003	*lagenariae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	*lagenariae*	not tested
Folag006	*lagenariae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	*lagenariae*	not tested
Folag007	*lagenariae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	*lagenariae*	not tested
Folag008	*lagenariae*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	*lagenariae*	not tested
Focuc022	*cucumerinum*	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	non-pathogenic
Focuc028	*cucumerinum*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	non-pathogenic
Focuc039	*cucumerinum*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	non-pathogenic
Foniv034	*niveum*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	non-pathogenic
Foniv035	*niveum*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	non-pathogenic
Foniv038	*niveum*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	non-pathogenic
RBG1687	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG1693	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5713	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5786	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5789	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5791	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5798	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5820	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5824	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
RBG5827	environmental	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	non-pathogenic	not tested
H_2O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		

[a]"Non-pathogenic" means not pathogenic toward any of the seven hosts listed in Table 1.
[b]"Non-pathogenic" means no symptom development in susceptible plant hosts of the originally reported *forma specialis*.

f. sp. *radicis-cucumerinum* strains, as predicted by our PCR analysis. The strains that were predicted to be nonpathogenic based on their marker patterns indeed did not cause symptom development when tested on susceptible cucumber (Focuc028 and Focuc039) or watermelon (Foniv034, Foniv035, and Foniv038) plants. Strains Fomln017, Fomln021, Fomln024, and Fomln026 (profile C) as well as Fomln023 (profile B) were all

able to cause disease in susceptible musk melon plants, even though their marker pattern was different from the most common profile in our set of isolates (profile A [Tables 3 and 4]). Fomln023, which tested positive for two of the three *F. oxysporum* f. sp. *niveum* markers, was also tested on susceptible watermelon plants. This strain was found to also be capable of causing disease in these plants, whereas Fomln010, with an almost identical marker pattern, was not (33). Fomln002 did not cause symptoms in susceptible melon plants, showing that possessing effector gene sequences alone is not always sufficient for pathogenicity and false positives may show up.

The fact that the bioassay data confirmed the suspected *forma specialis* predicted by the reported markers indicates that they provide a robust tool for identifying whether an isolate indeed belongs to the suspected *forma specialis* or not. PCR cross-reaction between *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *melonis*, and *F. oxysporum* f. sp. *niveum* markers and the cross-pathogenicity of strain Fomln023 suggest a shared evolutionary origin of the *formae speciales* affecting cucumber, melon, and watermelon.

Specific detection of *F. oxysporum* f. sp. *niveum* using a TaqMan assay. TaqMan real-time PCR has added benefits over traditional PCR: samples can easily be multiplexed, the fluorescent probe provides additional sequence specificity, and the technique allows for quantification of a target DNA sequence, for example, on DNA isolated from soil or diseased plant tissue. A TaqMan experiment was conducted using two of the marker genes in this study, *F. oxysporum* f. sp. *niveum* markers 21 and 100. These markers showed good specificity and displayed no cross-reaction with nontarget strains in the PCRs (Tables 3 and 4). TaqMan-specific primers and probes were designed in such a way that 116- and 138-bp *F. oxysporum* f. sp. *niveum*-specific amplicons were formed, respectively. As a fluorescent dye, hexachlorofluorescein (HEX; $\lambda_{emission} = 556$ nm), was used for marker 21 and 6-carboxyfluorescein (FAM; $\lambda_{emission} = 518$ nm) was used for marker 100. As an internal control for sample/DNA quality that would allow for normalization of the tested markers during multiplexing experiments, a set of primers and a probe with a different fluorescent dye (6-carboxytetramethylrhodamine [TAMRA]; $\lambda_{emission} = 580$ nm) was designed on a region of *EF1α* conserved in all *F. oxysporum* strains. To test the efficiency of the primers and probe sets, a dilution series of *F. oxysporum* genomic DNA was made and used as the template in a TaqMan assay.

A linear relationship was found between Foniv002 genomic DNA concentration and real-time quantification cycles (Data S4; $R^2_{marker21} = 0.999$; $R^2_{marker100} = 0.998$; $R^2_{EF1\alpha} = 0.999$). The pathogen could be detected at template concentrations as low as ~2.5 pg (Data S4).

The TaqMan assay was performed on isolates for which marker genes 21 and 100 were identified in the genome assembly (Fig. 2C; see also Data S2H). Each sequence type was included, with the addition of strains of *Fusarium proliferatum* and a *Fusarium* sp. that were identified to have candidate effector 100 in a recent study (47). *F. oxysporum* f. sp. *lycopersici* 4287, *F. oxysporum* f. sp. *cubense* II5, and biocontrol strain Fo47 were included as negative controls, since these do not have either of the marker genes. No cross-reactions were found, except in the case of Fomln010, which possesses a gene sequence identical to marker 100 in *F. oxysporum* f. sp. *niveum* isolates (Fig. 3). These results show the applicability of the TaqMan assay for specific detection of *F. oxysporum* f. sp. *niveum* DNA in very small quantities.

DISCUSSION

In the current study, we tried to make use of comparative genomics to design robust markers based on candidate effector genes. Effectors are functional elements in the pathogen-host interaction and have been found to show very limited sequence diversity between members of the same *forma specialis* (48, 49). This means that they form ideal targets for marker design (20). Effector gene sequences are often different between *formae speciales*, although several cases of identical gene sequences have been found in a previous study in our lab (33). For example, the *SIX6* and *SIX11* homologs present in some isolates belonging to *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *melonis*, and *F. oxysporum* f. sp. *radicis-cucumerinum* have 100% nucleotide

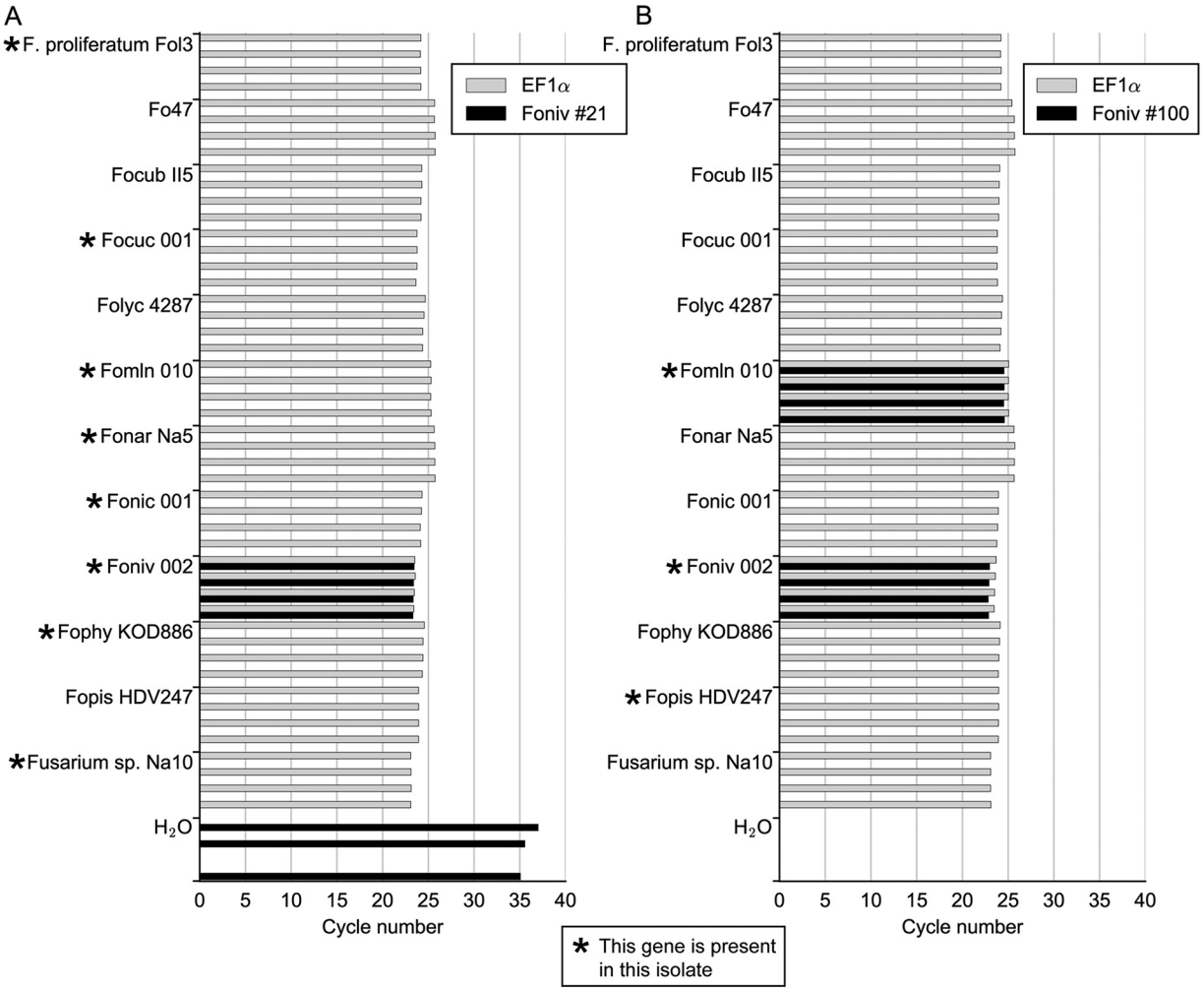

FIG 3 TaqMan primer-probe combinations show amplification of *F. oxysporum* f. sp. *niveum* DNA (Foniv002) when markers 21 (A) and 100 (B) are tested in duplex with *EF1α*. No amplification of these markers was detected in any of the non-*F. oxysporum* f. sp. *niveum* strains, with the exception of Fomln010, which has an identical gene sequence for hypothetical protein-encoding gene 100. High threshold cycle (C_T) values (≥35 cycles) under the detection threshold in the water control of marker 21 are probably caused by primer-dimer formation. Four technical replicates were used per sample, each represented by a bar.

identity. These sequences can therefore not be used for differentiation of these *formae speciales*. They do, however, give insight into the evolutionary history of pathogenicity of *F. oxysporum* toward cucurbits; the presence of sequences that are completely identical between relatively distantly related strains implies recent horizontal transfer of genetic material.

The benefit of using comparative genomics for marker design is that the specificity of the designed markers can directly be evaluated in other genome assemblies (as opposed to RAPD-derived marker sequences). Within the FOSC, one study has reported the use of comparative genomics for *forma specialis* marker development. This resulted in markers based on unique (random) sequences distinguishing *F. oxysporum* f. sp. *conglutinans* from 19 other *formae speciales* of *F. oxysporum* (50).

Our goal was to differentiate between *formae speciales* affecting the Cucurbitaceae family. The respective hosts are highly similar to each other, and incidental cross-pathogenicity between these *formae speciales* has been described (51–53). We designed a set of 18 primer pairs aiming to discriminate seven cucurbit-infecting *formae speciales* from each other as well as from other host-specific forms and nonpathogenic strains of *F. oxysporum*. We found that for *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *radicis-cucumerinum*, *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *lagenariae*,

F. oxysporum f. sp. *momordicae*, and *F. oxysporum* f. sp. *luffae*, the marker sets allowed clear recognition of the host range of each evaluated strain. Marker 94, designed on a gene encoding a hypothetical protein present in all cucurbit-infecting *formae speciales*, was positive for all target strains, with the exception of several *F. oxysporum* f. sp. *melonis* strains and one *F. oxysporum* f. sp. *momordicae* strain. This gene was not identified in the genome sequence of Fomln010.

Within *F. oxysporum* f. sp. *melonis* strains, more genetic variability exists than what had been taken into account as a starting point used for marker design (the 10 *F. oxysporum* f. sp. *melonis* strains with a sequenced genome). Indeed, *F. oxysporum* f. sp. *melonis* has been described as a highly heterogeneous *forma specialis*, encompassing at least eight VCGs (33, 43, 54). Several *F. oxysporum* f. sp. *melonis* strains showed overlap in their effector gene contents with cucurbit-infecting *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *momordicae*, and/or *F. oxysporum* f. sp. *lagenariae* (Table 4). So far, no SCAR or other marker sequences have been reported for *F. oxysporum* f. sp. *melonis*, possibly due to its heterogeneous nature. Two marker patterns were observed that were different from the marker patterns found in the majority of our *F. oxysporum* f. sp. *melonis* strains. Two strains (Fomln010 and Fomln023) tested positive for *F. oxysporum* f. sp. *melonis* as well as *F. oxysporum* f. sp. *niveum* markers (pattern B). Interestingly, Fomln023 was capable of causing severe wilting symptoms both in melon and in watermelon, while Fomln010 was not (33). This raises the question of whether the separation of these *formae speciales* is justified, similar to the question of whether strains pathogenic toward both cucumber and melon should be regarded as *F. oxysporum* f. sp. *cucumerinum* or *F. oxysporum* f. sp. *melonis*. Cafri et al. (53) decided in their study that since the *F. oxysporum* f. sp. *cucumerinum* strains they tested were more aggressive toward cucumber than melon and no cross-pathogenicity was found the other way around, these *formae speciales* should indeed remain distinct. In the case of strain Fomln023 in the current study, disease severities were comparable between watermelon and melon plants, indicating that this strain is a "bridging" *forma specialis*, and its marker gene pattern reflects this. Isolates bridging multiple host species are not commonly described in the literature, although most isolates admittedly are not tested against a large variety of plant species to confirm host specificity. It would be interesting to compare the genomes of strains with a wider host range with those that are highly specific to one plant species, which may have implications for the current nomenclature system within the FOSC.

We recently demonstrated that clustering isolates based on patterns of presence or absence of candidate effector genes divided *F. oxysporum* f. sp. *cucumerinum* into two groups, separated from each other by *F. oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *niveum* strains (33). The cucurbit-infecting isolates formed a supercluster from other *formae speciales*, indicating that they share a significant number of effector genes between them. Not much is known regarding the evolution of host specificity of *F. oxysporum* toward cucurbits, but Fomln023 might contain accessory genetic material originating from both an *F. oxysporum* f. sp. *niveum* and an *F. oxysporum* f. sp. *melonis* strain. Likewise, strains Fomln017, Fomln021, Fomln024, and Fomln026 tested positive for two *F. oxysporum* f. sp. *cucumerinum* markers and the *F. oxysporum* f. sp. *melonis* markers used all tested negative (pattern C). This indicates that these *F. oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *cucumerinum* strains also share accessory genetic material. However, Fomln017 is, like most *F. oxysporum* f. sp. *melonis* isolates, highly specific to melon plants. It would be interesting to further investigate these strains, for example, through long-read sequencing of their genomes and analysis of their pathogenicity chromosome(s) compared to those of other *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *melonis*, and *F. oxysporum* f. sp. *niveum* strains. This could shed light on how pathogenicity toward cucurbits has evolved in the FOSC.

Horizontal gene and chromosome transfer has been described as an important contributor to genetic diversity and the generation of new (pathogenic) clonal lines in fungi (55, 56). The different effector sequences and presence/absence patterns between and even within some cucurbit-infecting *formae speciales* suggest that it is

possible that multiple horizontal transfers of accessory genome material have taken place in the evolutionary trajectory, resulting in pathogenicity of *F. oxysporum* toward cucurbits. This is in contrast to the case with *F. oxysporum* f. sp. *lycopersici*: the four clonal lines that were tested in the work of van Dam et al. all have nearly identical sets of effectors and effector gene sequences (25, 33).

Minor cross-reaction (a much lighter band) was found with one of the markers (*F. oxysporum* f. sp. *momordicae* marker 130) with an unrelated *forma specialis*. *F. oxysporum* f. sp. *pisi* HDV247 (as well as *F. oxysporum* f. sp. *raphani* PHW815) indeed possesses this gene, although its downstream flank on which the reverse primer (FP7336) was designed was deemed to be sufficiently different from the copy in *F. oxysporum* f. sp. *momordicae*; only the four 5′ nucleotides matched between these two sequences. The forward primer contained only a single nucleotide polymorphism (SNP), meaning that it probably binds in the nontarget sequence of *F. oxysporum* f. sp. *pisi*, too. A similar observation was made for *F. oxysporum* f. sp. *melonis* marker 1 and Foniv041 genomic DNA. For this isolate, however, no genome sequence is available. Through quantitative PCR techniques such as TaqMan, (cross-)reactions with a significantly smaller amount of product can probably be distinguished from genuine positives.

As a proof of concept, a TaqMan test was developed for two of the markers. The TaqMan real-time PCR technique has several advantages over traditional PCR. Since it makes use of a sequence-specific fluorescently tagged probe in addition to the primer sequences, marker specificity is potentially higher. Additionally, the technique allows for quantification of the targeted DNA sequence (and thus of the pathogen in soil or infected plant tissue). Quantification of pathogenic *F. oxysporum* propagules in soil, seeds, or plant tissues may aid in deciding if and when to take action. Also, it is possible to test multiple markers by multiplexing, using several different fluorescent dyes at once (57–59). The markers that were tested in duplex for *F. oxysporum* f. sp. *niveum* behaved like expected: no amplification was identified in other strains (except Fomln010 with marker 100), even those that do possess the target gene. The technique allows for identification of sequences slightly different from the target sequence; the cycle number of a single copy marker with SNPs would be distinguishably higher than that of a positive-control single-copy gene like *EF1*α.

These findings illustrate the hurdles that can be experienced in the process of designing *forma specialis*-specific markers based on candidate effector genes, specifically if the *formae speciales* infect members of the same plant family and possibly arose through a shared and recent evolutionary history. Nonetheless, the combination of marker sequences described here can be used with relatively high fidelity to discriminate the seven cucurbit-affecting *formae speciales*, particularly when multiple markers are tested simultaneously in the analysis. It is possible—perhaps even likely—that more diversity exists among the seven *formae speciales* targeted in this study, since for several of the *formae speciales* not all VCGs were sampled for genome sequencing due to unavailability of these strains. This means that the markers might require revision in the future. The availability of more whole-genome sequences like the ones generated in this study will allow easier marker design and comparison in the future.

MATERIALS AND METHODS

Fungal strains and accession numbers. An overview of the strains that were used in this study and their respective genome assembly or nucleotide sequence accession numbers can be found in Table 5.

Whole-genome sequencing and *de novo* assembly. *F. oxysporum* genomic DNA was isolated through phenol-chloroform extraction from freeze-dried mycelium that was harvested from 5-day-old NO_3 medium (0.17% yeast nitrogen base, 3% sucrose, 100 mM KNO_3) cultures as described in detail in reference 33. Library preparation of insert size 550 bp and Illumina HiSeq 2500 paired-end sequencing were performed at Keygene N.V. (Wageningen, Netherlands).

Sequencing reads were trimmed for quality and to remove adapter sequences with FastqMcf v1.04.676 (https://github.com/ExpressionAnalysis/ea-utils/blob/wiki/FastqMcf.md; quality threshold = 20). *De novo* assemblies were generated using CLC-workbench 8.0. Default settings were used, except "minimum contig length = 500."

For generating a core phylogeny, homologs of 15,956 Fol4287 core genes (including introns) were searched in all genomes using BLASTN with default parameters. We selected all sequences that overlapped >70% with the query sequence and with more than 80% identity to the query. We then

TABLE 5 Overview of fungal strains used in this study and their NCBI genome accession numbers

Strain	Original designation	*Forma specialis* or description	VCG	Race	Origin of strain	Source or reference[a]	GenBank assembly accession no.
Folyc 4287	NRRL34936	*lycopersici*	0030	2	Spain	Broad Institute	GCA_000149955.2
Focon 5176	Fo5176	On *Brassica*			Australia	Broad Institute	GCA_000222805.1
Folyc MN25	NRRL54003	*lycopersici*	0033	3	USA	Broad Institute	GCA_000259975.2
Fopis HDV247	NRRL37622	*pisi*				Broad Institute	GCA_000260075.2
Forly CL57	NRRL26381	*radicis-lycopersici*	0094		USA (Florida)	Broad Institute	GCA_000260155.3
Fovas 25433	NRRL25433	*vasinfectum*			China	Broad Institute	GCA_000260175.2
Focub II-5	NRRL54006	*cubense*	01213	TR4	Indonesia	Broad Institute	GCA_000260195.2
Focon PHW808	NRRL54008	*conglutinans*	0101	2		Broad Institute	GCA_000260215.2
Forap PHW815	NRRL54005	*raphani*	0102			Broad Institute	GCA_000260235.2
Fomln001	NRRL26406	*melonis*	0136	1	Mexico	Broad Institute	GCA_000260495.2
Fo47	NRRL54002	Nonpathogen, biocontrol			France	Broad Institute	GCA_000271705.2
Focli FOSC 3-a	NRRL32931	Clinical isolate, from human blood			USA (Massachusetts)	Broad Institute	GCA_000271745.2
Focub N2	N2	*cubense*		1	China	72	GCA_000350345.1
Focub B2	B2	*cubense*		4	China	72	GCA_000350365.1
Focuc013	9904-1	*cucumerinum*	0186		China	22	MABJ01000000
Focuc015	9906-3	*cucumerinum*	0184		China	22	MABK01000000
Focuc021	ATCC 16416	*cucumerinum*	0180		USA (Florida)	22	MABL01000000
Focuc018	Afu-50(B)	*cucumerinum*	0180		Crete, Greece	22	MABM01000000
Focuc030	FOCU-22P	*cucumerinum*	0180		Israel	22	MABN01000000
Focuc035	NETH 11179	*cucumerinum*	0181		Netherlands	22	MABO01000000
Focuc037	Tf-213	*cucumerinum*	0185		Japan	22	MABP01000000
Forcu016	33	*radicis-cucumerinum*	0260		Canada	22	MABQ02000000
Forcu024	Afu-11(A)	*radicis-cucumerinum*	0260		Crete, Greece	22	MABR01000000
Forcu031	AK-2	*radicis-cucumerinum*	0261		Crete, Greece	22	MABS01000000
Focuc011	9903-1	*cucumerinum*	0186		China	22	MABT01000000
Fomln005	Fom 0123	*melonis*	0134	1	Spain	73	MAKY01000000
Focuc001	Foc-1	*cucumerinum*	0183		Japan	B.L.	MAKZ01000000
Fomln004	Fom 0122	*melonis*	0134	0	Spain	73	MALA01000000
Fomln006	Fom 0124	*melonis*	0134	2	Spain	73	MALB01000000
Fomln009		*melonis*	0135	2	Israel	73	MALC01000000
Fomln010		*melonis*		1	Israel	73	MALD01000000
Fomln012	ML2	*melonis*	0134	0		73	MALE01000000
Fomln016	Fom26	*melonis*	0134	1		73	MALF01000000
Fomln013		*melonis*	0134	2	Spain	73	MALG01000000
Folyc004	IPO1530/B1	*lycopersici*	0030	1	Netherlands	74	MALH01000000
Folyc007	D2	*lycopersici*	0030	2	France	74	MALI01000000
Folyc014	LSU-3	*lycopersici*	0030	1	USA (Louisiana)	74	MALJ01000000
Folyc026	BRIP 14844 (M1943)	*lycopersici*	0030	3	Australia	74	MALK01000000
Folyc018	LSU-7	*lycopersici*	0030	2	USA (Louisiana)	74	MALL01000000
Folyc016	BFOL-51	*lycopersici*	0031	1	USA (Louisiana)	74	MALM01000000
Folyc029	5397	*lycopersici*	0030	3	USA (Florida)	74	MALN01000000
Folyc038	CA92/95	*lycopersici*	0030	3	USA (California)	25	MALO01000000
Folyc069	DF0-23	*lycopersici*	0035	2	USA (California)	75	MALP01000000
Folyc072	DF0-38	*lycopersici*	0031	2	USA (California)	75	MALQ01000000
Folyc073	DF0-40	*lycopersici*	0030	2	USA (California)	75	MALR01000000
Folyc074	DF0-41	*lycopersici*	0030	3	USA (California)	75	MALS01000000
Folyc075	DF0-62	*lycopersici*	0031	2	USA (California)	75	MALT01000000
FoMN14	MN-14	Nonpathogen, from tomato plant			USA (California)	76	MALU01000000
Foniv002	CBS 418.90	*niveum*			Israel	22	MALX01000000
Foniv005	TX-471-1	*niveum*	0080	0	USA (Texas)	51	MALY01000000
Foniv010	F-016-1	*niveum*	0082	1	USA (Maryland)	51	MALZ01000000
Foniv013	F-014-2	*niveum*	0082	2	USA (Maryland)	51	MAMA01000000
Foniv015	F-063-1	*niveum*	0082	2	USA (Maryland)	51	MAMB01000000
Foniv019	TX-X1D	*niveum*	0082	2	USA (Texas)	51	MAMC01000000
Foniv020	F-099-1	*niveum*	0083	2	USA (Delaware)	51	MAMD01000000
Foniv021	MD-ZE622	*niveum*		3	USA (Maryland)	51	MAME01000000
Foniv037	NRRL38539	*niveum*			Israel	77	MAMF01000000
Folyc002	WCS862/E241	*lycopersici*	0030	2	Netherlands	74	MAMG01000000
Fomln011		*melonis*		0	Israel	73	MAMH01000000
Fogla G14	G14	*gladioli*	0341		Netherlands	78	NJCM01000000
Fogla G2	G2	*gladioli*	0340		France	78	NJCL01000000

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TABLE 5 (Continued)

Strain	Original designation	*Forma specialis* or description	VCG	Race	Origin of strain	Source or reference[a]	GenBank assembly accession no.
Fogla G76	G76	*gladioli*	0343		Italy	78	NJCK01000000
Folag001	01-03008	*lagenariae*			Japan	32	NJCJ01000000
Folag002	03-05118	*lagenariae*			Japan	32	NJCI01000000
Folag004	Lag:3-1 (JCM9293)	*lagenariae*			Japan	32	NJCH01000000
Folag005	Lag:1-1	*lagenariae*			Japan	32	NJCG01000000
Folil Fol39	Fol39	*lilii*			Netherlands	79; J.V.D.	NJCF01000000
Foluf001	Fol-114	*luffae*			Taiwan	25	NJCE01000000
Foluf002	Fol-167	*luffae*			Taiwan	25	NJCD01000000
Fomel001	J-71	*melongenae*				IPO	NJCC01000000
Fomom001	NRRL26413	*momordicae*			Taiwan	ARS	NJCB01000000
Fomom004	90NF2-1 (JCM9292)	*momordicae*			Japan	32	NJCA01000000
Fonar Na5	Na5	*narcissi*	2		Netherlands	79; J.V.D.	NJCV01000000
Fonic001	FON-1	*nicotianae*			USA (Connecticut)	80	NJBZ01000000
Fonic003	10913	*nicotianae*	0373		USA (Maryland)	80	NJBY01000000
Fonic010	Ft-Rob	*nicotianae*	0378		USA (North Carolina)	80	NJBX01000000
Fonic012	Ft-1512	*nicotianae*			USA (North Carolina)	80	NJCU01000000
Fophy KOD886	KOD886	*physali*			USA (California)	K.O.	NJBW01000000
Fophy KOD887	KOD887	*physali*			USA (California)	K.O.	NJBV01000000
Fophy KOD888	KOD888	*physali*			USA (California)	K.O.	NJBU01000000
Fo Tu58	Tu58	Nonpathogenic, from symptomatic tulip)			Netherlands	79; J.V.D.	NJBT01000000
Fotul Tu67	Tu67	*tulipae*			Netherlands	79; J.V.D.	NJBS01000000
14150	14150	*cucurbitacearum* (redesignated *radicis-cucumerinum)*			Netherlands	NAKT	Not available
Focuc009	0020	*cucumerinum*	0187		China	22	Not available
Focuc010	9901-2	*cucumerinum*	0186		China	22	Not available
Focuc014	9906-2	*cucumerinum* (redesignated *radicis-cucumerinum*)	0184		China	22	Not available
Focuc016	9909-2	*cucumerinum*	0185		China	22	Not available
Focuc017	9909-3	*cucumerinum*	0186		China	22	Not available
Focuc022	ATCC 36330	*cucumerinum* (redesignated a nonpathogen)	0180		Israel	22	Not available
Focuc026	ATCC 201950	*cucumerinum*	0180		USA (Florida)	22	Not available
Focuc027	Cu:4-1 Koma 4	*cucumerinum*	0181		Japan	22	Not available
Focuc028	Cu:5-0 Koma 5	*cucumerinum* (redesignated a nonpathogen)	0183		Japan	22	Not available
Focuc036	NRRL26437	*cucumerinum*			USA (South Carolina)	ARS	Not available
Focuc038	NRRL26744	*cucumerinum* (redesignated a nonpathogen)			Japan	ARS	Not available
Focuc039	NRRL38591	*cucumerinum*			New Zealand	ARS	Not available
Focuc040	07-08969	*cucumerinum* (redesignated *radicis-cucumerinum*)			Netherlands	NAKT	Not available
Folag003	07-27503	*lagenariae*			Japan	32	Not available
Folag006	Lag:7-1	*lagenariae*			Kumamoto, Japan	32	Not available
Folag007	No. 87	*lagenariae*			Tochigi, Japan	32	Not available
Folag008	No. 134	*lagenariae*			Tochigi, Japan	32	Not available
Fomln002	CBS 420.90	*melonis*			Israel	22	Not available
Fomln003	CBS 423.90	*melonis*			Israel	22	Not available
Fomln017	NRRL22518	*melonis*			USA	81	Not available
Fomln018	NRRL22519	*melonis*			France	81	Not available
Fomln019	NRRL22520	*melonis*			USA	81	Not available
Fomln020	NRRL22521	*melonis*			Belgium	81	Not available
Fomln021	NRRL26172	*melonis*			China	ARS	Not available
Fomln023	NRRL26174	*melonis*			China	ARS	Not available
Fomln024	NRRL26745	*melonis*			Japan	ARS	Not available
Fomln025	NRRL26746	*melonis*			Japan	ARS	Not available
Fomln026	NRRL38516	*melonis*			New Zealand	ARS	Not available
Fomln027	NRRL38524	*melonis*			New Zealand	ARS	Not available
Fomom002	NRRL26748	*momordicae*			Japan	82	Not available
Fomom003	90NF1-2	*momordicae*			Kagoshima, Japan	32	Not available
Foniv011	F-086-1	*niveum*	0082	1	USA (Maryland)	83	Not available
Foniv017	F-097-2	*niveum*	0082		USA (Delaware)	51	Not available

(Continued on next page)

TABLE 5 (Continued)

Strain	Original designation	*Forma specialis* or description	VCG	Race	Origin of strain	Source or reference[a]	GenBank assembly accession no.
Foniv018	F-100-2	*niveum*	0082	2	USA (Delaware)	51	Not available
Foniv033	NRRL26747	*niveum*			Japan	ARS	Not available
Foniv034	NRRL36275	*niveum* (redesignated a nonpathogen)				ARS	Not available
Foniv035	NRRL38278	*niveum* (redesignated a nonpathogen)			USA	ARS	Not available
Foniv036	NRRL38503	*niveum*			New Zealand	ARS	Not available
Foniv038	NRRL38552	*niveum* (redesignated a nonpathogen)			Israel	ARS	Not available
Foniv039	LB	*niveum*		0		NAKT	Not available
Foniv040	IPO 30288	*niveum*		1		IPO	Not available
Foniv041	CBS 419.90	*niveum*			Israel	22	Not available
Forcu005	14	*radicis-cucumerinum*			Canada	22	Not available
Forcu017	34	*radicis-cucumerinum*			Canada	22	Not available
Forcu020	38	*radicis-cucumerinum*			France	22	Not available
Forcu028	Afu-58	*radicis-cucumerinum*	0260		Crete, Greece	22	Not available
Forcu029	Afu-68(A)	*radicis-cucumerinum*	0260		Crete, Greece	22	Not available
RBG1687	RBG1687	Nonpathogen, from *Wollemia nobilis* seedling leaves			Australia	M.L.	Not available
RBG1693	RBG1693	From flannel flower roots			Australia	M.L.	Not available
RBG5713	RBG5713	Nonpathogen, from soil			Australia	49	Not available
RBG5786	RBG5786	Nonpathogen, from soil			Australia	49	Not available
RBG5789	RBG5789	Nonpathogen, from soil			Australia	49	Not available
RBG5791	RBG5791	Nonpathogen, from soil			Australia	49	Not available
RBG5798	RBG5798	Nonpathogen, from soil			Australia	49	Not available
RBG5820	RBG5820	Nonpathogen, from soil			Australia	49	Not available
RBG5824	RBG5824	Nonpathogen, from soil			Australia	49	Not available
RBG5827	RBG5827	Nonpathogen, from soil			Australia	49	Not available
F. proliferatum Fol3	Fol3	From a *Lilium* sp.			Netherlands	47	NJCT01000000
Fusarium sp. strain Na10	Na10	From a *Narcissus* sp.			Netherlands	47	NJCS01000000

[a]Abbreviations and initials: NAKT, NAKtuinbouw, Netherlands Inspection Service for Horticulture, Roelofarendsveen, Netherlands; B.L., Bart Lievens, Scientia Terrae Research Institute, Belgium; J.V.D., Joop van Doorn, PPO Research Centre, Lisse, Netherlands; M.L., Matthew Laurence, Plant Disease Diagnostic Unit of the Royal Botanic Gardens and Domain Trust, Sydney, Australia; ARS, Agricultural Research Service, USDA, USA; IPO, Plant Research International (formerly Instituut voor Planteziektenkundig Onderzoek), Wageningen, Netherlands; K.O., Kerry O'Donnell, USDA ARS, Peoria, IL.

selected query genes for which we found only a single hit in each genome, leaving us with 440 genes. We used ClustalO (60) to construct a multiple-sequence alignment for each gene and a custom python script to concatenate these alignments. This alignment was subsequently trimmed using trimAl-strictplus. We used PhyML v20120412 (61) with 100 bootstraps to infer phylogeny and ETE v3.0.0b35 (62) to visualize the tree.

Marker discovery and primer design. A custom python script was written to extract the sequence (plus 150 bp up- and downstream) of candidate effector genes from each of the genome assemblies using BLASTN with default parameters. MUSCLE v3.8.31 (63) was used to generate alignments of each gene, and phylogeny was inferred using PhyML v20120412 with 100 bootstraps. Another python script was used to traverse the tree in ETE v3 to identify instances where all isolates belonging to a *forma specialis* were clustering together in a separate clade, indicating sequence similarity that could potentially be used for primer design. Highlighting, drawing, and rendering of the gene trees were done using ETE v3. Visual inspection of each of the gene trees allowed for the selection of a final set of marker genes per *forma specialis*. Scripts are available upon request.

Primers were designed manually based on the sequence alignment per gene (see Data S1 in the supplemental material). In cases where only a few SNPs were identified to separate host specificity of isolates, we aimed to target the mismatching nucleotides toward the 3′ end of the primer, as described in reference 64.

DNA isolation. Genomic DNA isolation for testing of markers was performed using 10- to 20-day-old mycelium scraped off a peptone-dextrose agar (PDA) plate as starting material. The tissue was disrupted by shaking it in a TissueLyser (Qiagen) for 2 min at 30 Hz in the presence of 400 μl of Tris-EDTA (TE), 300 μl of phenol-chloroform (1:1), and glass beads. The aqueous phase was transferred to a fresh tube, and an equal volume of chloroform was added. The DNA in the aqueous phase was transferred to a fresh tube and diluted 10× with sterile Milli-Q water prior to use in PCR. DNA quantity was estimated for the TaqMan standard curve using a Qubit fluorometer (Thermo Fisher Scientific).

PCR. PCR was executed using Sphaero-Q Super *Taq* (Gorinchem, Netherlands) in 20-μl reaction volumes which included the following components (final concentration): 1× Sphaero-Q Super *Taq*

TABLE 6 Primers and annealing temperatures used in this study

Gene ID	Target gene	Target *forma specialis*	Primer name	Primer sequence (5′–3′)	T_{ann} (°C)	Product size (nt)
+	*FEM1*	Positive control	fp157	ATGAAGTACACTCTCGCTACC	54	274
			fp158	GGTGAAAGTGAAAGAGTCACC		
94	HPEG	All cucurbit infecting	fp7304	GCCTCATTGAAGTTTCAACA	54	346
			fp7321	TGGTAAAGGACACGACCATT		
13	*SIX13*	*radicis-cucumerinum*	fp7305	TTGCCCAAAATGGCATGTTT	56	328
			fp7322	CATTGACACTGTAAGTGGG		
70	HPEG	*radicis-cucumerinum*	fp7306	TACAACCTCTCTCTTTCCTT	54	454
			fp7323	GCTGAATTCTAGCAGAGAAT		
66	HPEG	*cucumerinum*	fp7307	CCGTTATGGCCAGAGATC	54	425
			fp7324	CCAACAAACAGAGCAAAACTAA		
99	HPEG	*cucumerinum*	fp7308	CTACCAATCTCTCCTGAGTG	54	445
			fp7325	GTCGATTGCAGTGCTAGTCT		
21	Fom effector 7	*cucumerinum*	fp7309	CAGTCTAACCCTGTCTCATT	54	381
			fp7326	CGCCAATAGATAGTGATGGA		
1	*SIX1*	*melonis*	fp7310	CCTCTCAGTCCTTGGGTCT	54	397
			fp7327	ACTCGCTTCAGCTTACCGA		
20	Fom effector 6	*melonis*	fp7406	TGAAAGTCTTGGCGGGTGT	56	305
			fp7328	TCCTCTCCATCCTCATCAGT		
18	Fom effector 3	*melonis* + *niveum*	fp7312	TTAGTGCAGCTTTTCTCCTC	54	299
			fp7329	AGTGGTTAGTCAAGTGGTAA		
99	HPEG	*niveum*	fp7313	TGCCGGGCTAGTTAATATAGT	54	406
			fp7330	ACCATTTTTCTGTTGGGGTTG		
100	HPEG	*niveum*	fp7314	ATTTTGCTAGCTTCAGCAGTT	54	482
			fp7331	ATCCTGAACGGTGACTAGAG		
21	Fom effector 7	*niveum*	fp7315	CGCTCGCTATAATTCAAACG	54	139
			fp7332	GGAGGAGCACTACAACTAAT		
71	HPEG	*lagenariae*	fp7407	TAGTCCAATCTGCCTCAGCAA	54	270
			fp7410	GGAAGTGAGCATTCTTCCGTA		
99	HPEG	*lagenariae*	fp7408	TCGTATCTCTCAGTAGTATGG	54	367
			fp7411	AATGGATACCTTATAAGGGCT		
1	*SIX1*	*lagenariae* + *momordicae*	fp7409	TTGGGATTGCGGCTTATGCT	56	463
			fp7412	AAAGTGGTACACTCCGTGC		
98	HPEG	*momordicae*	fp7318	AGGTGCAGCGTTTTTAGGT	60	469
			fp7335	GAGGGCTGGTTGAGAACTA		
130	HPEG	*momordicae*	fp7319	TCTACGCTTCGAGGATGGTA	56	368
			fp7336	TCGTTTAGACGACTACAACC		
99	HPEG	*luffae*	fp7320	TACTCTCCTAGAGTCAGTCT	54	606
			fp7337	CACGCCATCATCCTTTATTC		

buffer, 0.25 U of Sphaero-Q Super *Taq*, 5 pmol of each primer, deoxynucleoside triphosphates (dNTPs; 0.2 mM each), and 1 μl of template DNA. The following PCR program was used: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at annealing temperature (T_{ann}), and 40 s at 72°C; 5 min at 72°C; and a pause at 16°C. The PCR primer sequences and corresponding annealing temperatures are listed in Table 6. *Fusarium FEM1* primers were used as a positive control and sterile Milli-Q was used as a negative control for each of the primer combinations instead of template DNA.

TaqMan real-time PCR. TaqMan reverse transcription-PCRs (RT-PCRs) were performed on a QuantStudio 3 system (Thermo Fisher Scientific). Primers and probes were designed using Primer3web v4.0.0 (http://primer3.ut.ee/), and their sequences can be found in Table 7. A total volume of 10 μl of the reaction mixture included the following components (final concentrations): 1× Sphaero-Q Super *Taq*

TABLE 7 Primers and probes used for the TaqMan experiments

Gene ID	Target gene	Target *forma specialis*	Primer name	Sequence (5′–3′)	Product size (nt)
21	Fom effector 7	*niveum*	fp7589	CCGGTACCCCAGCTTTATGT	116
			fp7590	CAGCAACGTTCTGAAAGCGT	
			probe_3	HEX-TGCAGGTTGGCAGGCCCCTG-BHQ1	
100	HPEG	*niveum*	fp7591	CACCAACAACTATGCGGCAC	138
			fp7592	GCAATTGACCCAGCTGCAAT	
			probe_4	FAM-AGTCGCCGGCCACCACATTGA-BHQ1	
EF1α	*Elongation factor 1 α*	All strains	fp7710	CGCTGAGCTCGGTAAGGG	97
			fp7711	CCAGAGAGCAATATCGATGGTGA	
			probe_7	TAMRA-ACGCCTGGGTTCTTGACAAGCTCA-BHQ2	

buffer, 0.25 U of Sphaero-Q Super *Taq* (Gorinchem, Netherlands), 3 pmol of each primer, 1 pmol of each probe, dNTPs (0.2 mM each), 0.1× ROX reference dye (Thermo Fisher Scientific), and 1 μl of template DNA. Four simultaneous amplifications were performed for each sample to confirm the reproducibility of the results. A negative-control sample consisted of sterile Milli-Q substituted for the DNA template. The PCR program was set as follows: 2 min at 94°C and 40 cycles of 30 s at 94°C, 48 s at 60°C, and 12 s at 60°C (data collection).

Disease assays. Pathogenicity tests were performed using the root dip method (65). In short, conidia were isolated from 5-day-old cultures in NO_3 medium (0.17% yeast nitrogen base, 3% sucrose, 100 mM KNO_3) by filtering through Miracloth (Merck; pore size of 22 to 25 μm). Spores were centrifuged, resuspended in sterile Milli-Q water, counted, and brought to a final concentration of 10^7/ml. When the first true leaves were emerging (after ~10 days), 5 to 8 seedlings per treatment were uprooted, inoculated, individually potted, and kept at 20°C (*F. oxysporum* f. sp. *radicis-cucumerinum*) or 25°C (all other *formae speciales*) in the greenhouse. The following plant cultivars were used: *Cucumis sativus* cv. Paraiso, *Cucumis melo* cv. Cha-T, and *Citrullus lanatus* cv. Black Diamond. Two weeks after inoculation, disease was scored using a disease index from 0 to 4 as described in detail by van Dam et al. (33).

Accession number(s). Whole-genome shotgun projects for the newly sequenced strains of *F. oxysporum* have been deposited at GenBank under BioProject no. PRJNA389501. Raw sequence data have been deposited into the Sequence Read Archive under accession number SRP109253. All publicly available genome sequences that were used were obtained from GenBank. Their NCBI accession numbers can be found in Table 5.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01868-17.

SUPPLEMENTAL FILE 1, PDF file, 4.4 MB.

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