Differentiation of Eleven *Fusarium* spp. Isolated from Sugar Beet, Using Restriction Fragment Analysis of a Polymerase Chain Reaction–Amplified Translation Elongation Factor 1 α Gene Fragment

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Accepted for publication 31 March 2009.

ABSTRACT

Nitschke, E., Nihlgard, M., and Varrelmann, M. 2009. Differentiation of eleven *Fusarium* spp. isolated from sugar beet, applying restriction fragment analysis of polymerase chain reaction–amplified translation elongation factor 1 α gene fragment. *Phytopathology* 99:921–929.

Sugar beet in Europe is commonly grown in wheat and maize crop rotations and subsequently pile-stored for several weeks. Beet is threatened by the colonization of saprophytic as well as pathogenic *Fusarium* spp. A tool for reliable identification based on sequence information of the translation elongation factor 1 α (TEF-1 α) gene was developed for the numerous *Fusarium* spp. being isolated from sugar beets. In all, 65 isolates from different species (*Fusarium avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. solani*, *F. tricinctum*, and *F. venenatum*) were

obtained from sugar beet at different developmental stages from locations worldwide. Database sequences for additional species (*F. sporotrichioides*, *F. poae*, *F. torulosum*, *F. hostae*, *F. sambucinum*, *F. subglutinans*, and *F. verticillioides*), isolated from sugar beets in previous studies, were included in the analysis. Molecular sequence analysis of the partial TEF-1 α gene fragment revealed sufficient variability to differentiate between the *Fusarium* spp., resulting in species-dependent separation of the isolates analyzed. This interspecific divergence could be translated into a polymerase chain reaction restriction fragment length polymorphism assay using only two subsequent restriction digests for the differentiation of 17 of 18 species.

Additional keywords: diagnosis, fungi, PCR-RFLP.

The genus *Fusarium* contains a variety of different taxa with various morphological characters. The soilborne plant-pathogenic *Fusarium* spp. are among the most economically important plant pathogens with regard to their persistence in soil, organic matter, or plant residues (10). Quantitative yield reduction (40) and fungal production of mycotoxins affecting food quality are of particular concern in *Fusarium* spp.-infected plants (58).

Sugar beet is attacked by various phytopathogenic and saprophytic fungi throughout the entire cultivation period and particularly during storage. Several early studies detected infections with a number of *Fusarium* spp. at different developmental stages of sugar beet (1,8,27,35,36) and in harvested sugar beet during pile storage (7,9). In addition to *Fusarium oxysporum* f. sp. *betae*, which induces phloem necrosis, chlorosis, wilting, and death of foliage (*Fusarium* yellows) as well as rot of taproots (cortex root rot) (36,61), the isolated *Fusarium* spp. identified by their morphological characters included a broad spectrum of different species such as *F. culmorum*, *F. equiseti*, *F. avenaceum*, *F. graminearum*, *F. acuminatum*, *F. solani*, *F. sporotrichioides*, *F. subglutinans*, and *F. moniliforme*.

Infestations caused by *F. oxysporum* f. sp. *betae* represent an increasing problem in the United States. At least eight states are affected by *Fusarium* yellows in sugar beet-producing areas, resulting in considerable yield reductions (20,23,44,54). Recently, for Minnesota and Wyoming, Hanson (22) reported *F. graminearum* as an additional causal agent of *Fusarium* yellowing, formerly associated only with *F. oxysporum* f. sp. *betae* infec-

tions. Possible implications of *F. graminearum* isolated from sugar beet on crops grown in the same rotation were pinpointed by investigations of Estrada et al. (16) and Burlakoti et al. (13), who demonstrated the cross-pathogenicity of *F. graminearum* isolates from sugar beet to cereals and potato. Furthermore, the general ability of mycotoxin production of these cross-pathogenic isolates was shown (11,13).

In addition to *Fusarium* spp. infestations of sugar beet, Bosch and Mirocha (7) confirmed that several species produced mycotoxins resulting in zearalenon accumulation in sugar beet and fiber samples derived thereof. Because different *Fusarium* spp. possess variable toxigenic potential, accurate identification is necessary.

Traditionally, the identification of *Fusarium* spp. is based on morphology, including distinctive features such as the shape, size, and septation of conidia, chlamydospores; and the evaluation of additional characteristics of the general mycelium growth rate and mycelium color on agar cultures (31). In general, members of the genus *Fusarium* are commonly accepted to be difficult to identify at the species level relying only on microscopic traits.

In the last decade, molecular-based techniques have made a significant impact on the speed and reliability of fungal species identification for diagnostic as well as phylogenetics and taxonomic purposes (32,46,47,49,50,56,60). Established DNA-based methods for *Fusarium* spp. detection and identification include species-specific polymerase chain reaction (PCR) assays (6,25,26,30,52), as well as the use of conserved DNA regions for comparative sequencing and phylogenetic analysis (18,49) and development of PCR-restriction fragment length polymorphism (RFLP) assays. The application of species-specific PCR is reliable in well characterized host–pathogen interactions but of limited use when multiple species need to be differentiated. Thus, a method that rapidly distinguishes several *Fusarium* spp. is

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doi:10.1094/PHYTO-99-8-0921

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needed. The sequence analysis of ribosomal RNA (rDNA) (3,21,28,38), β -tubulin (49), or translation elongation factor 1 α (TEF-1 α) (28,29,49) allows a more reliable identification but is more time and labor intense. In particular, the TEF-1 α gene was shown to be a useful genetic region for phylogenetic and taxonomic studies in *Fusarium* spp. Therefore, the TEF-1 α has been used as a single-locus identification tool in the assembled FUSARIUM-ID v.1.0 database (18).

The development of PCR-RFLP assays offers further possibilities for simplified, fast, and routine determination and diagnosis, especially where time and expense may preclude sequencing efforts. Duggal et al. (14) observed polymorphisms between and within *Fusarium* spp. in the internal transcribed spacer (ITS) region. However, Turner et al. (59) demonstrated that sequence divergence in the ITS region was too small and insufficient for the resolution of closely related species such as *F. tricinctum* and *F. avenaceum* by PCR-RFLP analysis. Other areas showed promise; for example, the intergenic spacer of rDNA (IGS) allowed a clear differentiation, as demonstrated by Edel et al. (15). Therein, 18 different species could be discriminated by comparisons and classifications of haplotype formation by using a minimum of four different restriction enzyme digests. Further analyses were able to relate IGS-PCR-RFLP analysis to host specificity and mycotoxin-producing ability based on five to six subsequent restriction enzyme digests (33,34). A number of IGS-PCR-RFLP analyses predominantly have been used to charac-

terize intraspecific relationships in *F. culmorum* (37), *F. moniliforme* (24), and *F. oxysporum* (39). Interestingly, the TEF-1 α gene fragment was only recently subjected to PCR-RFLP analysis for successful differentiation of the three *F. oxysporum* clades (6), thereby proving the potential to use TEF-1 α gene fragment variability for intraspecific discrimination in RFLP analyses.

No fast and reliable tool currently is available for the accurate identification of the numerous species frequently isolated from sugar beet. The objective of this study was the development of a simple diagnostic technique for the routine identification of *Fusarium* spp. frequently occurring in sugar beet fields. Of particular interest is the discrimination of common soil saprophytes and maize- and cereal-associated species due to the importance of stalk crops in sugar beet crop rotations. Based on the sequence information of the TEF-1 α gene, an RFLP assay of a single fungal-derived PCR product was developed, differentiating, in total, 18 *Fusarium* spp. assembled of 11 species isolated from sugar beet and 7 closely related species represented by their TEF-1 α nucleotide sequences deposited in databases.

MATERIALS AND METHODS

***Fusarium* isolates and microscopic analysis.** The *Fusarium* isolates analyzed in this study are listed in Table 1 with their geographic origin. The German isolates were collected, identified, and stored at the Department of Phytopathology, Institute of

TABLE 1. *Fusarium* isolates originating from sugar beet used in this study^a

Species	Isolate ^b	Origin	Sequence comparison to FUSARIUM-ID v. 1.0 ^c	
			Most-related accession	Sequence similarity (%)
<i>Fusarium avenaceum</i>	ave-161	Germany	ID566	99
	ave-661 (DSM21724)	Sweden	ID566	99
<i>F. cerealis</i>	cer-129	Germany	ID367	99
	cer-121 (DSM21715)	Germany	ID367	100
	cer-158	Germany	ID367	99
<i>F. culmorum</i>	cul-646 (DSM21713)	Germany	ID369	100
	cul-01	Sweden	ID369	100
	cul-19	Sweden	ID369	99
	cul-92	Sweden	ID369	99
	cul-131	Sweden	ID369	99
	cul-141	Germany	ID369	99
	cul-142	Germany	ID369	100
	cul-164	Germany	ID369	99
	cul-653	Sweden	ID369	99
	cul-26 (DSM 21714)	Germany	ID369	99
<i>F. equiseti</i>	equ-44	Italy	ID38	96
	equ-60	France	ID38	99
	equ-90	Sweden	ID39	98
	equ-138	United States	ID38	99
	equ-122	United States	ID38	100
	equ-113	United States	ID38	94
	equ-115	Sweden	ID39	98
	equ-149	Germany	ID39	98
	equ-150	Germany	ID39	98
	equ-151 (DSM21802)	Germany	ID39	98
	equ-160	Germany	ID39	98
	equ-157	Germany	ID39	97
	equ-159 (DSM21725)	Germany	ID39	98
	equ-174 (DSM21726)	Italy	ID38	99
<i>F. graminearum</i>	gra-109	United States	ID12	99
	gra-36	Germany	ID12	99
	gra-163 (DSM21727)	Germany	ID12	99
	gra-152 (DSM21803)	Germany	ID12	99

(continued on next page)

^a Sugar beet-derived isolates of this study, their most related database accessions, and additional database accessions of related *Fusarium* spp. are subject of all translation elongation factor-1 α investigations.

^b Representatives of each species were deposited in the open collection of the German collection of microorganisms and cell cultures as indicated by their DSM deposition number.

^c Most related sequence from FUSARIUM-ID.

^d Sequences of related species (1) as well as representatives of the three clades of *Fusarium solani* (2) from FUSARIUM-ID.

^e Sequences of related species from GenBank.

Sugar Beet Research. The isolates derived from sugar beet outside Germany were collected by the Plant Pathology Unit at Syngenta Seeds, Landskrona, Sweden. To isolate *Fusarium* spp. from sugar beet tissue, the following isolation procedure was used. Throughout the growing season, freshly harvested sugar beet plants displaying root rot symptoms were washed thoroughly and a piece of beet tissue of 0.3 to 0.5 cm³ was removed from the center of the beet using a surface-sterilized knife. Following surface sterilization with 70% ethanol for 30 s and subsequent washing in sterile double-distilled water, tissue was placed on potato dextrose agar (PDA) supplemented with streptomycin (150 mg liter⁻¹). All isolates are held as long-term cultures at the Department of Phytopathology. For microscopic and nucleic-acid-based analysis, monoconidial cultures of the isolates were established on small nutrient agar (SNA) (41). Microscopic characterization of conidia and conidiophores of the analyzed isolates was carried out according to the methods of Leslie and Summerell (30) to confirm the molecular data acquired. Additional isolates were obtained from two fungal collections, the Centraalbureau voor Schimmelcultures (CBS, The Netherlands) and the German collection of microorganisms and cell cultures (DSMZ, Germany) and included as references (Table 2).

Fungal DNA extraction and PCR amplification. Monoconidial isolates were grown on PDA at 22°C under white light for 10 days. DNA was extracted from mycelium using the DNeasy kit (Qiagen, Germany) according to the manufacturer's

instructions. To amplify the TEF-1 α gene fragment with an approximate length of 700 bp, primers EF1 and EF2 (46) were used. A standard PCR reaction was carried out with 35 cycles of denaturing at 95°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min. The amplification reaction was verified by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

Molecular cloning of PCR products and sequencing. The TEF-1 α PCR products were purified and cloned into the pGemTeasy vector (Promega, Germany) according to the manufacturer's instructions. Sequencing of clones in both directions was carried out commercially (MWG Biotech, Germany) using standard primers flanking the multiple cloning site of the cloning vector.

Sequence analysis. Sequences were edited using VectorNTI (Invitrogen, Germany). Sequence alignment of the TEF-1 α PCR product sequence excluding the primer sequences and generation of dendrograms was carried out by ClustalX (57). The sequence alignment included a total of 65 isolates representing 11 species (Table 1). To expand the available dataset and test the reliability of the phylogenetic analyses and PCR-RFLP patterns, database accessions of *Fusarium* isolates were integrated into the investigations as listed in Table 1. FUSARIUM-ID-derived sequences were labeled by their ID in combination with an abbreviation for the species (e.g., hos-ID48 for *F. hostae*, database entry ID48). TEF-1 α sequences of *F. subglutinans* (sub-ID77), *F.*

TABLE 1. (continued from preceding page)

Species	Isolate ^b	Origin	Sequence comparison to FUSARIUM-ID v. 1.0 ^c	
			Most-related accession	Sequence similarity (%)
<i>F. oxysporum</i>	oxy-13	Germany	ID479	99
<i>F. oxysporum</i>	oxy-08	France	ID169	100
	oxy-685 (DSM21728)	Germany	ID479	99
	oxy-108	United States	ID164	99
	oxy-112	United States	ID169	99
	oxy-114	United States	ID169	100
	oxy-57	Poland	ID150	99
	oxy-165 (DSM21719)	Germany	ID145	100
	oxy-166 (DSM21730)	Germany	ID137	100
	oxy-47 (DSM21731)	Germany	ID135	96
	oxy-173	Italy	ID133	99
	oxy-169 (DSM21732)	Italy	ID479	99
	oxy-175 (DSM21733)	Italy	ID479	99
	oxy-176 (DSM21734)	Italy	ID169	100
<i>F. proliferatum</i>	pro-55 (DSM21735)	Sweden	ID432	98
<i>F. redolens</i>	red-11	Germany	ID360	99
	red-143	Germany	ID358	99
	red-145	Germany	ID360	100
	red-171 (DSM21736)	Germany	ID358	99
	red-23	Germany	ID358	99
	red-148 (DSM21737)	Germany	ID358	100
	red-20	France	ID360	100
<i>F. solani</i>	sol-17 (DSM21738)	Italy	ID307	96
	sol-111	United States	ID301	97
	sol-61	Italy	ID307	96
<i>F. tricinctum</i>	tri-154	Germany	ID32	97
	tri-156 (DSM21783)	Germany	ID32	97
	tri-167 (DSM21740)	Germany	ID32	97
	tri-38	Germany	ID32	97
<i>F. venenatum</i>	ven-03 (DSM21739)	France	ID21	99
	ven-64	United States	ID21	96
	ven-162	Germany	ID21	100
<i>F. hostae</i>	hos-ID48 ^{d1}	...	...	...
<i>F. poae</i>	poa-ID23 ^{d1}	...	...	...
<i>F. sambucinum</i>	sam-ID22 ^{d1}	...	...	...
<i>F. solani</i>	sol-ID123 ^{d2}	...	...	...
	sol-ID111 ^{d2}	...	...	...
	sol-ID125 ^{d2}	...	...	...
<i>F. sporotrichioides</i>	spo-ID177 ^{d1}	...	...	...
<i>F. subglutinans</i>	sub-ID77 ^{d1}	...	...	...
<i>F. torulosum</i>	tor-AJ543615 ^c	...	...	...
<i>F. verticillioides</i>	ver-ID50 ^{d1}	...	...	...

sporotrichioides (spo-ID177), *F. poae* (poa-ID23), and *F. verticilloides* (ver-ID50) were supplemented based on the isolated *Fusarium* spp. spectrum of Bosch and Mirocha (7). Moreover, due to common strong morphological similarities, database TEF-1 α sequences of *Fusarium* spp. potentially to be confused with species isolated from sugar beet were included. These were, notably, *F. hostae* (hos-ID48), due to its close relationship with *F. redolens* (5,19), as well as *F. sambucinum* (sam-ID22) and *F. torulosum* (GenBank accession no. AJ543616), due to their relationship with *F. venenatum* (42,43). Additionally, TEF-1 α sequences were supplemented to represent the three clades of *F. solani* (45). Differentiation of *F. oxysporum* clades (49) was according to Bogale et al. (6). Dendrograms were visualized using TreeView (51), showing the phylogenetic relationships between the nucleotide sequences. In order to evaluate the support for the observed branching topologies for maximum parsimony, bootstrap analysis was performed and values were calculated from 1,000 replicates, indicated at each node. All the newly obtained sequences have been submitted to the EMBL/GenBank sequence database (FY939658 to FY939722).

RFLP analysis of PCR products. Unpurified TEF-1 α PCR product (20 μ l) was digested with 5 units of different restriction enzymes, including *Bst*UI, *Bsp*HI, *Xho*I, *Mae*II, and *Alu*I (Fermentas, Germany), using the buffers supplied by the manufacturer in a total volume of 25 μ l at 37°C overnight to ensure complete digestion. Restriction fragments were resolved by electrophoresis on 3% agarose gels and visualized using a UV transilluminator as described above. Determination of restriction patterns were made by comparison to a parallel-resolved DNA marker (50 bp ladder; Fermentas). In silico analyses of digestion patterns of TEF-1 α gene fragments for different restriction enzymes were performed using VectorNTI.

RESULTS

Isolation and morphological analysis. Analysis of fungal species from sugar beet collected in Germany and worldwide displaying root rot symptoms resulted in a subset of 65 isolates. In all, 34 isolates from Germany, 22 from different European countries, and 9 from the United States were identified as *Fusarium* spp. Identification to the species level was achieved by detailed microscopic analysis of typical *Fusarium* structures and enabled a grouping of the isolates into 11 different *Fusarium* spp.

TABLE 2. Overview on reference *Fusarium* isolates of culture collections used for proof and verification of polymerase chain reaction restriction fragment length polymorphism analyses

Species	Accession of reference isolate ^a	Host
<i>Fusarium avenaceum</i>	DSM62161	<i>Dianthus caryophyllus</i>
<i>F. cerealis</i>	CBS134.80	<i>Zea mays</i>
	CBS 135.80	<i>Beta vulgaris</i>
<i>F. culmorum</i>	DSM62191	<i>Triticum aestivum</i>
	DSM6223	<i>Corylus avellana</i>
	CBS250.52	<i>Secale cereale</i>
<i>F. equiseti</i>	DSM62202	<i>C. avellana</i>
<i>F. graminearum</i>	CBS389.62	<i>T. aestivum</i>
<i>F. poae</i>	DSM62376	<i>Avena sativa</i>
<i>F. proliferatum</i>	DSM62267	<i>Z. mays</i>
<i>F. redolens</i>	DSM62379	<i>Dianthus caryophyllus</i>
	DSM62380	<i>Asparagus officinalis</i>
<i>F. sambucinum</i>	DSM62433	<i>Beta vulgaris</i>
<i>F. sporotrichioides</i>	DSM62376	<i>Avena sativa</i>
<i>F. tricinctum</i>	CBS144.78	<i>T. aestivum</i>
<i>F. verticilloides</i>	DSM62267	<i>Z. mays</i>

^a DSM-labeled isolates were provided by the German collection of micro-organisms and cell cultures and CBS-labeled cultures were retrieved from the culture collection of The Centraalbureau voor Schimmelcultures, The Netherlands.

representing a broad spectrum of common soilborne pathogenic and saprophytically living species (Table 1).

Phylogenetical analysis of *Fusarium* isolates. To test whether the TEF-1 α gene would be feasible for differential detection and subsequent molecular identification, initially, DNA samples from all isolates were used for PCR amplification of an $\approx$ 700-bp TEF-1 α gene fragment. No amplification products were obtained using DNA samples from other common soilborne and sugar-beet-colonizing fungi (*Pythium ultimum*, *Aphanomyces cochlioides*, *Phoma betae*, and *Rhizoctonia solani*), proving the *Fusarium* specificity of the EF1/EF2 primer pair (data not shown). The TEF-1 α PCR products were T-vector cloned and subsequently sequenced. The TEF-1 α fragment sequences, including primer sequences, were subjected to a *Fusarium* database (FUSARIUM-ID v. 1.0) comparison using the BLAST-N search utility (2). The most closely related sequences, as well as their percentage of sequence homology to the TEF-1 α sequence of each isolate resulting from this search, are displayed in Table 1. In all cases, the results of TEF-1 α sequence comparison to the FUSARIUM-ID database confirmed the preliminary identification based on the microscopic analysis. The high degree of nucleotide sequence similarity (94 to 100%) for all analyzed isolates indicated that the intraspecific divergence was limited for the 11 different species despite the worldwide geographical origin of the isolates included. Overall, out of the *Fusarium* spp. isolated from sugar beet and supplementary species represented by database sequences, 18 different species were included in the TEF-1 α gene fragment sequence comparison (Table 1). ClustalX multiple sequence comparisons of the TEF-1 α gene fragments of all isolates in this study revealed stretches of high similarity next to stretches of high divergence (data not shown). The resulting dendrogram (Fig. 1A) showed that the analyzed TEF-1 α gene region possessed sufficient variability to differentiate between the 18 investigated *Fusarium* spp., resulting in a clear, well-structured, and species-dependent separation of the analyzed isolates forming nine main branches. The main dendrogram revealed a good overall resolution of all analyzed species resulting either in branches containing only isolates from one species (branch 4, 6, 8, and 9) or branches grouping between two and four species. Known related species clustered using this method; for example, the branch of *F. redolens* and *F. hostae* (branch 1) was separated from the cluster formed by *F. oxysporum*, *F. proliferatum*, *F. subglutinans*, and *F. verticilloides* (branch 2); the group of *F. culmorum*, *F. cerealis*, and *F. graminearum* (branch 5); or the branch containing *F. sambucinum* and *F. venenatum* (branch 7) as well as *F. avenaceum*, *F. tricinctum*, and *F. torulosum* (branch 3). The overall alignment did not clearly resolve TEF-1 α sequence divergence of isolates belonging to one single or more closely related species. Therefore, sequences of individual branches (supported by bootstrap values of >900) were each used for a separate ClustalX multiple sequence comparison. These analyses resulted in a clear species-specific resolution for each subbranch (Fig. 1B to G). Every isolate-derived sequence in this study clustered with sequences of the predicted species, revealing enough polymorphism to distinguish and identify even isolates of closely related species. Branch 1 was built up by the isolates of *F. redolens* and *F. hostae*, wherein the isolate of *F. hostae* clearly outgrouped from the isolates of *F. redolens* (Fig. 1B). Similarly branch 3, 5, and 7 showed a species-specific separation of *F. torulosum*, *F. tricinctum*, and *F. avenaceum* (Fig. 1D); *F. cerealis*, *F. culmorum* and, *F. graminearum* (Fig. 1E); as well as *F. venenatum* and *F. sambucinum* (Fig. 1F), respectively. In branch 7, the U.S. isolate ven-64 of *F. venenatum* separated noticeably from the German *F. venenatum* isolates by its TEF-1 α nucleotide composition as indicated by the scale bar (Fig. 1F). Branch 2 consisted of the species *F. oxysporum*, *F. verticilloides*, *F. subglutinans*, and *F. proliferatum*. *F. verticilloides*, *F. subglutinans*, and *F. proliferatum* were distinguishable and well separated from

Fig. 1. A, Unrooted tree showing the phylogenetic relationships between translation elongation factor 1 α (TEF-1 α) sequences of all *Fusarium* isolates of this study plus *Fusarium* database sequences displayed in Table 1. Clusters of *Fusarium* isolates at the end of each main branch (1 to 9) forming main groups of closely related *Fusarium* spp. or isolates of one species were removed because of the low resolution of this view. B to G, Rooted phylogenetic trees of TEF-1 α sequences clustering into main branches 1 to 9 in A. Sequences were aligned and neighbor-joining trees constructed using ClustalX. Boot strap values were calculated from 1,000 replicates and are indicated at each node. The scale bar indicates 0.1 and 0.01% substitution per nucleotide, respectively.

the isolates of *F. oxysporum* (Fig. 1C). The latter species differentiated into several subbranches which could be assigned to the three clades of *F. oxysporum* defined by O'Donnell (49) (Fig. 1C, parenthesis). However, investigations of pathogenicity still need to be performed to differentiate these sugar-beet-derived isolates into pathogenic and nonpathogenic ones because this question was not addressed using the TEF-1 α sequence analysis. TEF-1 α sequences representing isolates of sugar-beet-pathogenic *F. oxysporum* f. sp. *betae* were not available from the FUSARIUM-ID or GenBank database, preventing the disclosure of any further information concerning the *F. oxysporum* intraspecific relationship of the sugar-beet-derived *F. oxysporum* isolates of this study out of the analyzed TEF-1 α gene region. Phylogenetic relationships of sugar-beet-derived *F. solani* isolates (branch 9) were analyzed in detail due to the inclusion of representative sequences for the three clades of *F. solani* (Fig. 1G). However, the sugar-beet-derived sequences clearly outgrouped and obviously were not related to these clades.

Development of a TEF-1 α gene-fragment-based PCR-RFLP assay for differentiation of *Fusarium* spp. Based on the reliable species-specific discrimination of the TEF-1 α gene fragment, the sequences were used for the development of a PCR-RFLP assay to simplify *Fusarium* spp. determination. Initially, TEF-1 α sequences of all sugar-beet-derived *Fusarium* isolates, including all the database references as listed in Table 1, were subjected to in silico RFLP analyses. Testing of several frequent cutting restriction enzymes revealed the suitability of *Bst*UI to provide characteristic band patterns to differentiate between groups of closely related species (Table 3). The formation of these groups corresponded to the clustering of the main dendrogram in Figure 1A. The species *F. poae*, *F. solani*, and *F. graminearum* were characterized by a species-specific *Bst*UI-related haplotype and thereby distinguishable from all other analyzed species by this single restriction digest (Table 3). However, as expected, in most cases, closely related species gave identical restriction patterns. This overlapping was seen within groups formed in the main dendrogram (Fig. 1A) (e.g., branch 1, formed by *F. hostae* and *F. redolens*, or branch 3, consisting of *F. torulosum*, *F. avenaceum*, and *F. tricinatum*). Few species clustering into different branches also produced identical or very similar *Bst*UI haplotypes (e.g.,

branch 4 and 7 [*F. sporotrichioides*, *F. venenatum*, and *F. sambucinum*] or branch 2- and 8-derived isolates of *F. oxysporum* and *F. equiseti*) (Table 3). Whereas, for most species, a single *Bst*UI-related restriction pattern was conserved in all tested isolates. Multiple restriction patterns were generated from the isolates of *F. oxysporum* and *F. equiseti* (Table 3). There was no correlation between the *Bst*UI-related haplotype formation of *F. oxysporum* isolates and the clustering into different clades. Although, for analyzed *F. oxysporum* sequences, *Bst*UI fragmentation was consistent and reproducible for sugar-beet-derived as well as database-derived sequences, the *F. solani*-related *Bst*UI fragmentation pattern was only valid and specific for sugar-beet-derived sequences and their closest related database sequences. In silico analyses of database sequences for *F. solani* isolates belonging to different formae speciales, mating type, or clade were characterized by highly divergent *Bst*UI patterns (data not shown). However, despite different geographical origins, the sugar-beet-derived *F. solani* sequences exhibited a specific *Bst*UI restriction pattern different from all other analyzed *Fusarium* spp.

TEF-1 α PCR products from the 11 different sugar-beet-derived *Fusarium* spp. were subsequently *Bst*UI digested and a clearly resolved fragmentation of sizes as small as 40 bp was achieved by electrophoresis on 3% agarose gels (Fig. 2). In addition, reference isolates of different *Fusarium* spp., which originated from several diverse hosts, were included in this analysis (Table 2). Generally, the single *Bst*UI-digested TEF-1 α PCR fragments resulted in clearly distinguishable restriction patterns enabling separation of cereal-associated species such as *F. culmorum*, *F. cerealis*, *F. graminearum*, and *F. tricinatum* from species such as *F. equiseti*, as well as a differentiation of *F. redolens* from the wilting pathogen *F. oxysporum* (Fig. 2). The final species determination depended on a second restriction digest according to the pregrouping results of *Bst*UI digestion. The combination of *Bst*UI/*Bsp*HI, *Bst*UI/*Xho*I, *Bst*UI/*Bsr*BI, *Bst*UI/*Mae*II, and *Bst*UI/*Alu*I in silico restriction digests resolved *F. cerealis*/*F. culmorum*, *F. torulosum*/*F. avenaceum*/*F. tricinatum*, *F. redolens*/*F. hostae*, *F. sporotrichioides*/*F. sambucinum*/*F. venenatum*, and *F. equiseti* *F. subglutinans*/*F. verticillioides*/*F. oxysporum*, respectively (Table 3). This possibility of species-specific differentiation was proven for 10 of the 11 different sugar-beet-derived *Fusarium* spp. (Figs. 2

TABLE 3. Analysis of translation elongation factor-1 α gene fragment sequence of 18 *Fusarium* spp. enabling species-specific differentiation by in silico polymerase chain reaction restriction fragment length polymorphism analysis using two subsequent different restriction digests

Branch ^a	Species	No. of analyzed isolates ^b	Approximate fragment length	Banding pattern, primary digest ^c	Secondary enzyme ^c	Banding pattern, secondary digest ^d
1	<i>Fusarium redolens</i>	9 (2)	730	290, 260, 150, 30	<i>Bsr</i> BI	400, 300, 30
	<i>F. hostae</i> ^e	1 (1)	730	290, 260, 150, 30		400, 330
2	<i>F. proliferatum</i> ^f	2 (1)	710	320, 260, 130	<i>Alu</i> I	380, 190, 100, 40
	<i>F. subglutinans</i> ^e	1 (1)	710	320, 260, 130		300, 200, 100, 70, 40
	<i>F. verticillioides</i> ^e	1 (1)	710	320, 260, 130		330, 190, 100, 50, 40
	<i>F. oxysporum</i> ^f	22 (8)	710	710 or 450, 260 or 320, 260, 130		380, 190, 100, 40 or 480, 190, 40
3	<i>F. torulosum</i> ^e	1 (1)	710	290, 250, 170	<i>Xho</i> I	340, 310, 60
	<i>F. avenaceum</i>	3 (1)	710	290, 250, 170		370, 340
	<i>F. tricinatum</i>	5 (1)	710	290, 250, 170		400, 310
4	<i>F. sporotrichioides</i> ^e	1 (1)	700	280, 240, 180	<i>Mae</i> II	390, 270, 40
5	<i>F. cerealis</i>	4 (1)	700	280, 240, 120, 60	<i>Bsp</i> HI	620, 80
	<i>F. culmorum</i>	11 (1)	700	280, 240, 120, 60		700
	<i>F. graminearum</i> ^g	5 (1)	710	410, 240, 60		
6	<i>F. poae</i> ^{e,g}	1 (1)	700	300, 240, 160		
7	<i>F. sambucinum</i> ^e	1 (1)	700	280, 240, 180	<i>Mae</i> II	370, 280, 30, 20
	<i>F. venenatum</i>	4 (1)	700	280, 240, 180		380, 270, 30, 20
8	<i>F. equiseti</i>	16 (2)	710	710 or 430, 280	<i>Alu</i> I	480, 190, 40
9	<i>F. solani</i> ^g	8 (5)	730	290, 170, 150, 90, 30		

^a Main branch in Figure 1A.

^b Numbers in parentheses indicate number of database sequences of the total amount of analyzed isolates.

^c Banding patterns of *Bst*UI primary and secondary digests. Fragment sizes were rounded to the nearest 10 bp.

^d Secondary restriction enzyme required for differentiation.

^e Isolates were only subjected to translation elongation factor-1 α in silico analysis by database sequences.

^f No further differentiation between *F. proliferatum* and *F. oxysporum* was achieved by second digest.

^g Isolates could be differentiated from all other analyzed species using just the *Bst*UI digest.

and 3). The specific combination of two subsequent restriction digests resulted in all but two cases in characteristic band patterns differentiating the investigated *Fusarium* spp. Difficulties in a clear discrimination of the restriction pattern of *Bst*UI- and *Alu*I-digested TEF-1 α PCR products from *F. oxysporum* and *F. equiseti* isolates were due to the very slight differences in the size of the restriction fragments in *Bst*UI digests and overlapping haplotype formation in *Alu*I digests (Fig. 3). However, we were unable to obtain a better resolution by using an additional third restriction digest. Similarly, no restriction enzyme combination (in silico simulations applied 10 different enzymes) was able to differentiate TEF-1 α sequences from *F. oxysporum* and *F. proliferatum*. Although the isolates of *F. oxysporum* and *F. proliferatum* possessed 7% nucleotide sequence divergence in the analyzed TEF-1 α fragment region, it was not sufficient for species-specific discrimination, contrary to the possibility of differentiating 4, 4.6, and 2.3% sequence divergence between *F. sambucinum*/*F. venenatum*, *F. torulosum*/*F. avenaceum*/*F. tricinctum*, and *F. redolens*/*F. hostae*, respectively. However, in addition to the use of reference isolates (Table 2), a detailed validation of the determined PCR-RFLP patterns of the different *Fusarium* spp. included additional 400 sugar-beet-derived *Fusarium* isolates. In almost every case, the PCR-RFLP-based result was in accordance with the morphological identification (data not shown).

DISCUSSION

In this study, molecular-based identification and differentiation of 65 isolates belonging to 11 *Fusarium* spp. isolated from sugar beet and additional 7 closely related species represented by TEF-1 α nucleotide sequence database entries and culture collection reference isolates were conducted. In addition to the identification based on morphological data, for the first time we were able to provide evidence for the presence of these species in sugar beet using DNA-based identification methods.

The *Fusarium* spectrum isolated from sugar beet plantlets or freshly harvested beet worldwide consisted of 11 different species. This demonstrates that, in addition to *F. oxysporum* f. sp. *betae* and *F. graminearum* as reported previously (see introduction), sugar beet is already infested at harvest by many other *Fusarium* spp. The different *Fusarium* spp. partly coincided with the spectrum of species isolated from stored beet by Bosch and Mirocha (7), thereby implicating sugar beet as a host for *Fusarium* spp. infections already in vegetative development. Further studies will be necessary to prove the ability of these pathogenic species and the environmental conditions required to actively

infect and colonize sugar beet and separate them from the saprophytes. Subsequent investigations also have to determine whether mycotoxins are produced in beet tissue by the different species because cross pathogenicity and mycotoxin production of sugar-beet-derived *Fusarium* isolates was already proven (11–13).

Considering the time-consuming and complex morphology-based identification of *Fusarium* spp., the need for a rapid and reliable identification assay for simultaneous determination covering a broad and diverse range of *Fusarium* spp. was evident. Compared with the use of species-specific PCR (see introduction), where one reaction for each species plus appropriate controls had to be carried out, the time expense for cloning and sequencing of the TEF-1 α gene fragment was comparable or even lower. Especially in cases where, as in the study presented here, the interest is focused on a broad and even unknown species spectrum, the sequencing of conserved housekeeping genes such as TEF-1 α allows a reliable identification within a similar time period (18,28,44,49). Fungal isolation from the host was required to produce a pure culture as opposed to a direct molecular detection from host tissue extracted DNA to eliminate complications from mixed infections. This limits the possible application for *Fusarium* spp. in planta diagnosis but, on the other hand, eliminated difficulties in molecular detection due to sugar-beet-derived inhibitory compounds (e.g., large amounts of polysaccharides in nucleic acid extracts). However, the isolation of *Fusarium* spp. from plant tissue proved to be very effective regarding the ease with which growth on solid media took place without any competition of bacterial growth. This offers the advantage of discriminating and subsequently identifying a large number of different *Fusarium* spp., starting from a single PCR product followed by sequencing of parallel obtained clones. As was shown by the partial TEF-1 α gene analysis conducted for the 65 isolates of this study, the sequence divergence was large enough to discriminate between that broad range of morphologically and even phylogenetically closely related *Fusarium* spp. The clustering of the dendrogram was supported by the supplemented database sequences of all species in this study, as well as *F. verticillioides*, *F. subglutinans*, *F. sporotrichioides*, *F. poae*, *F. torulosum*, *F. sambucinum*, and *F. hostae*. The pattern of TEF-1 α -based grouping of different species to well-supported clusters corresponded to close species relationships as described previously in a study on phylotoxigenic relationship (29). A phylogenetically related similarity of *F. tricinctum* and *F. avenaceum* was supported by ITS, IGS, mtSSU, and β -tubulin sequence analysis (62). In addition to these reports, the species description of *F. hostae* (19) supported the formation of *F. hostae* and *F. redolens* as a closely related clade, with clear distinction to the species complexes of *Gibberella fujikuroi*, *F. solani*, and *F.*

Fig. 2. Different restriction patterns of polymerase chain reaction-amplified translation elongation factor-1 α gene fragments of 11 *Fusarium* spp. obtained from sugar beet following digestion with *Bst*UI and analyzed in 3% agarose gel. Lane M, 50-kb DNA ladder; lane 1, *Fusarium solani*; lane 2, *F. graminearum*; lane 3, *F. redolens*; lane 4, *F. venenatum*; lane 5, *F. culmorum*; lane 6, *F. cerealis*; lane 7, *F. avenaceum*; lane 8, *F. tricinctum*; lanes 9 and 10, *F. equiseti*; lanes 11 to 13, *F. oxysporum*; lane 14, *F. proliferatum*.

Fig. 3. Restriction patterns of differentially digested polymerase chain reaction-amplified translation elongation factor-1 α gene fragments analyzed in 3% agarose gel. Lane M, 50-kb DNA ladder; lane 1, *Fusarium avenaceum* (*Xho*I); lane 2, *F. tricinctum* (*Xho*I); lane 3, *F. culmorum* (*Bsp*HI); lane 4, *F. cerealis* (*Bsp*HI); lanes 5 and 6, *F. oxysporum* (*Alu*I); lane 7, *F. proliferatum* (*Alu*I); lane 8, *F. equiseti* (*Alu*I).

oxysporum by molecular analyses of TEF-1 α and β -tubulin. The reliability and stability of our sequence analysis were supported by additional validation analyses. First, we added *Fusarium* database sequences to the analyzed *Fusarium* spp., as well as phylogenetically related species such as *F. hostae*, *F. sambucinum*, and *F. torulosum* or species such as *F. sporotrichioides* and *F. subglutinans* previously isolated from sugar beet. The additional analysis of sequences represented in the FUSARIUM-ID database for all the species isolated from sugar beet proved the reliability and indicated its potential use as an identification assay or diagnostic tool in other crops. The implementation of *Fusarium* reference isolates originating from different hosts (Table 2) already supports this application. However, a further validation of only in-silico-represented species would be desirable and would support differentiation results (e.g., for *F. redolens* and *F. hostae* in regard to discrimination difficulties using ITS-RFLP (5). Furthermore, it could also be used to confirm the putative identity of novel *Fusarium* spp. from sugar beet such as that recently reported by Rivera et al. (53).

The excellent TEF-1 α -based differentiation of the analyzed *Fusarium* spp. prompted us to develop a PCR-RFLP assay as an advanced, technically simpler, and reliable tool for the species-specific determination and discrimination of *Fusarium* isolates. Interestingly, species-specific haplotypes were achieved for *F. graminearum* and *F. solani*, despite both species having been described as species complexes (45,48). Although the *F. graminearum*-related *Bst*UI restriction pattern of TEF-1 α was consistent over the described seven phylogenetical lineages (50) of *F. graminearum* (with the exception of lineage 5) (data not shown), the *F. solani*-related *Bst*UI restriction pattern was only valid for the cluster of sugar-beet-derived and most related sequences. The supplement of TEF-1 α sequences as representatives for the three *F. solani* clades (45) enabled no assignment of sugar-beet-derived *F. solani* isolates to these clades due to clear separation as distinct branches. Regarding the small number of investigated isolates in this study and the observation of ≈ 50 species within the *F. solani* species complex (44), it is only possible to conclude that the chosen restriction enzyme *Bst*UI was suitable for the differentiation of *F. solani* isolates of this study from all other analyzed *Fusarium* spp. Investigations based on conserved 28S rDNA (21) and PCR-RFLP analysis of the more variable ITS region (15,55) revealed *F. solani* as a polymorphic species; therefore, further detailed investigations are needed to determine the intraspecific differentiation of *F. solani* as well as the relation to pathogenicity for the isolates in this study.

The formation of three PCR-RFLP haplotypes in the species *F. oxysporum* indicated a high degree of intraspecific variability, possibly caused by the formation of a species complex (4). Representatives of all three *F. oxysporum* clades (49) were included and revealed a distribution of sugar-beet-derived *F. oxysporum* isolates over all three clades. Any further conclusions regarding a relation of the sugar-beet-derived *F. oxysporum* isolates to sugar-beet-pathogenic *F. oxysporum* f. sp. *betae* were not possible, because no TEF-1 α sequences for *F. oxysporum* f. sp. *betae* isolates were available. As detected for TEF-1 α in our study, Llorrens et al. (33) obtained intraspecific variability in IGS analysis and suggested the feasibility of converting this into differentiations at an intraspecific level. The potential to relate traits like pathogenicity of the isolates to the TEF-1 α haplotypes, as shown for banana-derived isolates of *F. oxysporum* (39), requires further investigations applying pathogenicity tests on sugar beet.

As outlined by Geiser (17), intron-rich portions of coding sequences are ideal markers for fungal phylogenetics. The TEF-1 α gene proved to be a suitable genetic region for the differentiation of *Fusarium* spp. due to sufficient sequence variability in the intron sequences of the analyzed partial gene fragment (49). All restriction recognition sites chosen for differential digest were located in the intron sequences of the TEF-1 α gene. Available

PCR-RFLP assays based on ribosomal DNA sequences (15,33,34) clustered analyzed species according to the haplotype formation by using four to six different restriction digests to reveal species-specific resolution of *Fusarium* spp. Compared with these approaches, the TEF-1 α -based PCR-RFLP assay of our study required only two different digests whereby the restriction enzyme used for the second digest specifically depended on the restriction pattern of the *Bst*UI digest.

In conclusion, the isolation of 11 different *Fusarium* spp. from sugar beet enabled us to develop a DNA-based identification tool discriminating these species as well as related ones. The results revealed the suitability of this method for a fast and reliable identification of *Fusarium* spp. using two different restriction digests of one single PCR product. Further analyses are required to confirm its reliability in crops grown in rotation with sugar beet, especially cereals and maize. At least, the dataset extension with reference isolates derived from a diverse set of hosts (Table 2) strongly support those applications.

ACKNOWLEDGMENTS

We thank L. Hanson and R. Pilot for critical reading of the manuscript.

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