Phylogeny and Haplotype Analysis of Fungi Within the *Fusarium incarnatum-equiseti* Species Complex

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ABSTRACT

Fusarium spp. are ranked among the top 10 most economically and scientifically important plant-pathogenic fungi in the world and are associated with plant diseases that include fruit decay of a number of crops. *Fusarium* isolates infecting bell pepper in Trinidad were identified based on sequence comparisons of the translation elongation factor gene (EF-1a) with sequences of *Fusarium incarnatum-equiseti* species complex (FIESC) verified in the *FUSARIUM*-ID database. Eighty-two isolates were identified as belonging to one of four phylogenetic species within the subclades FIESC-1, FIESC-15, FIESC-16, and FIESC-26, with the majority of isolates belonging to FIESC-15. A comparison of the level of DNA polymorphism and phylogenetic inference for sequences of the internal transcribed spacer region (ITS1-5.8S-ITS2) and EF-1a sequences for Trinidad and *FUSARIUM*-ID type species was carried out. The ITS sequences were less informative, had lower haplotype diversity and restricted haplotype distribution, and resulted in poor resolution and taxa placement in the consensus maximum-likelihood tree. EF-1a sequences enabled strongly supported phylogenetic inference with highly resolved branching patterns of the 30 phylogenetic species

within the FIESC and placement of representative Trinidad isolates. Therefore, global phylogeny was inferred from EF-1a sequences representing 11 countries, and separation into distinct *Incarnatum* and *Equiseti* clades was again evident. In total, 42 haplotypes were identified: 12 were shared and the remaining were unique haplotypes. The most diverse haplotype was represented by sequences from China, Indonesia, Malaysia, and Trinidad and consisted exclusively of *F. incarnatum* isolates. Spain had the highest haplotype diversity, perhaps because both *F. equiseti* and *F. incarnatum* sequences were represented; followed by the United States, which contributed both *F. equiseti* and *F. incarnatum* sequences to the data set; then by countries representing Southeast Asia (China, Indonesia, Malaysia, Thailand, and Philippines) and Trinidad; both of these regions were represented by only *F. incarnatum* sequences. Trinidad shared two haplotypes with China and one haplotype with the United States for only *F. incarnatum* isolates. The findings of this study are important for devising disease management strategies and for understanding the phylogenetic relationships among members of the FIESC.

Trinidad (10.6918° N, 61.2225° W) is the southern-most island in the West Indies and is located approximately seven miles off Venezuela's northeastern coast. The island is the larger of a twin island republic (Trinidad and Tobago) and is bordered by the Atlantic Ocean and Caribbean Sea. It was reported in 2011 that 540 km² of the total land area (5,131 km²) is arable as defined by the Food and Agriculture Organization. In Trinidad, bell pepper crops are grown year-round in fields located in the northeast and south. Depending on the availability of seed, the most commonly grown varieties are 'Anaconda', 'Aristotle', 'Canape', and 'Paladin', with 'King Henry' being the most popular variety grown in 2012 (Mohammed 2013; Ramdial and Rampersad 2014). Growers generally use seedlings prepared by agricultural suppliers and seedling companies. Bell pepper fruit in Trinidad are sold and consumed as fresh fruit, without any undergoing any processing. Between 2006 and 2010, the acreage under production and the quantity harvested fluctuated (e.g., 505,000 kg in 2008 to 149,000 kg in 2009, with increased production to 369,000 kg in 2010) (Mohammed 2013). In addition to several marketing channels available throughout the country, Trinidad exports bell pepper to Barbados, with an annual quota to be maintained due to an agricultural trade agreement for export of certified produce between the two countries. There has also been a steady increase in the importation of bell pepper from the United States between 2006 and

2010 (Mohammed 2013). The relatively high production cost faced by local growers, poor performance of the available varieties against diseases affecting pepper in Trinidad, yield loss (estimated to be between 20 to 40% in fields that were sampled) incurred due to disease, and changes in market value of locally produced pepper compared with imported pepper have resulted in abandoned fields, usually at midharvesting stage.

Symptoms of *Fusarium* infection in bell pepper are seen as a single, watery lesion with evidence of internal fruit rot, both of which reduce the marketability of the fruit. The lesion may begin at the calyx and extend downward but it can develop anywhere on the fruit. To date, the disease had not been reported prior to 2010, when the last survey of the main production areas in Trinidad was carried out (Ramdial and Rampersad 2014). Management of the disease is based on chemical control; however, there is no registered fungicide for the control of *Fusarium* fruit rot of bell pepper in Trinidad. There are also no resistant cultivars available and cultural methods such as field sanitation and rotation away from solanaceous crops for a minimum of 2 years have proven ineffective at controlling the disease. The relatively recent emergence of *Fusarium* fruit rot of bell pepper fruit in Trinidad may be explained by a combination of epidemic-associated fungus, host, and environmental factors; for example, (i) increased susceptibility of bell pepper cultivars to *Fusarium* disease; (ii) low efficacy of chemical control; (iii) production of small, dry asexual spores by the fungus which survive in dry weather conditions and which may cause infection via wind dispersal; and (iv) wetter conditions of the rainy season may result in higher moisture levels on flowers that encourage fungal growth and infection of the stigma or rain splash from the soil onto low-lying fruit.

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary tables are published online.

Fusarium spp. also produce a diverse range of mycotoxins, including trichothecenes (Woloshuk and Shim 2013), that contaminate agricultural products and render them unsuitable for human or animal consumption. There is also evidence to suggest that trichothecenes can promote or suppress virulence which may be host-specific or specific to the individual *Fusarium* spp. (Bai et al. 2002; Desmond et al. 2008; Ilgen et al. 2008; Desjardins et al. 1996; Proctor et al. 1995). *Fusarium* mycotoxins can be associated with both acute and chronic toxic effects, the extent of which may rely on the mycotoxin type, the level and duration of exposure, the species that is exposed, and the relative age of the affected organism (Antonissen et al. 2014). This underlines the importance of accurate identification of the pathogen and knowledge of phylogenetic relationships with mycotoxin producers.

Fusarium spp. are ranked among the most economically and scientifically important plant-pathogenic fungi in the world (Dean et al. 2012). The genus consists of filamentous ascomycete fungi (Sordariomycetes: Hypocreales: Nectriaceae) that are associated with several diseases, including wilts, blights, and necrosis of various horticultural, field, ornamental, and forest species in both agricultural and naturally occurring ecosystems (Ma et al. 2013). *Fusarium lactis* was recently reported as a common pathogen of pepper, in addition to

several other minor pathogens (e.g., *F. proliferatum*, *F. subglutinans*, and *F. oxysporum*) (Van Poucke et al. 2012).

The *Fusarium* genus includes close to 300 phylogenetic species; however, with respect to describing new species, many researchers have agreed on the need for a revision of classification because studies have shown that intra- and interspecific boundaries as currently defined within the genus may be inaccurate or poorly resolved (Aoki et al. 2014; Benyon et al. 2000; Moretti 2009; O'Donnell et al. 2004; Skovgaard et al. 2003; Taylor et al. 2000; Torp and Nirenberg 2004; Waalwijk et al. 1996). Phylogenetic analysis has primarily focused on inferring hypothetical evolutionary relationships of agriculturally or medically important isolates (O'Donnell et al. 2010). For DNA-based identification of fungi in general, the universal barcoding region is the internal transcribed sequence (ITS) of ribosomal DNA (rDNA), and it is also the most commonly sequenced DNA region for investigating the molecular ecology of a range of fungi (Peay et al. 2008; Stielow et al. 2015). For *Fusarium* spp., this region is too conserved and does not distinguish among members within a given species complex (O'Donnell et al. 2015). However, several recent publications still describe genetic diversity of various *Fusarium* spp. based on ITS sequences (Arif et al. 2014; Dubey et al. 2014; Soren et al. 2015; Zarrin et al. 2016). Protein-coding genes—for example, β -tubulin

TABLE 1. *Fusarium incarnatum-equiseti* species complex (FIESC) designation and haplotype distribution for FIESC and Trinidad isolates

Isolate ^a	Field location ^b	EF-1a FIESC designation ^c	EF-1a haplotype among FIESC and Trinidad isolates ^d	ITS haplotype among FIESC and Trinidad isolates
*TT-1	Aranguuez	FIESC 15c	EF-Hap_27	ITS-Hap1
*TT-2	Macoya	FIESC 15c	EF-Hap_27	ITS-Hap1
TT-3	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-4	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-5	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-6	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
*TT-7	Macoya	FIESC15a	EF-Hap_27	ITS-Hap1
TT-8	Aranguuez	FIESC 1a	EF-Hap_48	ITS-Hap12
TT-9	Maloney	FIESC15a	EF-Hap_31	ITS-Hap1
TT-10	Aranguuez	FIESC 16c	EF-Hap_28	ITS-Hap3
TT-11	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-12	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-13	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-14	Aranguuez	FIESC 16c	EF-Hap_28	ITS-Hap3
TT-15	Aranguuez	FIESC 16c	EF-Hap_28	ITS-Hap3
*TT-16	Aranguuez	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-17	Aranguuez	FIESC 16c	EF-Hap_28	ITS-Hap2
*TT-18	Maloney	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-19	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-20	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
*TT-21	Macoya	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-22	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-23	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-24	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-25	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-26	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-27	Aranguuez	FIESC15a	EF-Hap_27	ITS-Hap1
TT-28	Aranguuez	FIESC15a	EF-Hap_27	ITS-Hap1
TT-29	Aranguuez	FIESC15a	EF-Hap_27	ITS-Hap1
TT-30	Aranguuez	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-31	Aranguuez	FIESC15a	EF-Hap_27	ITS-Hap1
*TT-32	Aranguuez	FIESC 26b	EF-Hap_47	ITS-Hap2
TT-33	Aranguuez	FIESC15a	EF-Hap_27	ITS-Hap1
TT-34	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-35	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-36	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-37	Aranguuez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-38	Aranguuez	FIESC15a	EF-Hap_27	ITS-Hap1

(continued on next page)

^a Isolates with an asterisk (*) were selected as the representative isolates for inclusion in the phylogenetic analyses.

^b Field locations and fields that were surveyed: Aranguuez, five fields; Macoya, three fields; Maloney, three fields; Caura, one field; Penal, two fields; and Bonne Aventure, two fields. FIESC isolates were not detected in fields located in Caura and Penal.

^c Translation elongation factor (EF-1a) FIESC designations were assigned based on the 99.4% similarity threshold (O'Donnell et al. 2015) and FIESC type strains were mined from *FUSARIUM*-ID database.

^d Underscore () was used in the EF-1a haplotype designations to distinguish them from the ITS haplotypes.

(TUB), translation elongation factor 1-a (EF-1a), and RNA polymerase II subunits 1 and 2 (RPB1 and RPB2)—have been recommended because these regions are easily sequenced and sequences can be aligned across the entire genus (O'Donnell et al. 2000, 2010, 2015). The EF-1a gene sequence, with a sequence similarity threshold of 99.4%, is a suitable genetic marker for discriminating among *Fusarium* spp., and allows discrimination of genotypes at the subspecific level (Geiser et al. 2004; O'Donnell et al. 2015). Sequence data from only one of these loci (RPB1 and RPB2 or EF-1a) is required for reliable identification of unknown isolates and for phylogenetic analysis, provided that the similarity threshold is obtained (O'Donnell et al. 2015).

Genetic diversity and phylogenetic analyses inferred from multiple sequence comparisons of local and global isolates can reveal evolutionary relationships which may or may not coincide with geographical region or host species. A high degree of genetic diversity can also indicate greater ability to adapt to changing environmental conditions and may reflect increased biological fitness of these genotypes. The tropics are among the world's oldest, most diverse ecosystems and *Fusarium* spp., in such habitats, have been represented by pathogens that are coevolving with their plant host, as well as by newly introduced pathogens (Ploetz 2006). It is hypothesized that, although there will be shared genotypes between Trinidad and other tropical regions, there may also be a genetic

signature that is unique to the Trinidad isolates. The objectives of this study, therefore, were to (i) confirm the identity of *Fusarium* spp. infecting bell pepper in Trinidad; (ii) compare the level of DNA polymorphism of ITS and EF-1a sequences of type species and Trinidad isolates; (iii) infer the phylogenetic relationships of *F. incarnatum-equiseti* species complex (FIESC) type species, isolates from Trinidad, and isolates from other countries; and (iv) determine the haplotype diversity and distribution among FIESC isolates. Preliminary findings which identified the pathogen as *F. incarnatum* were published by Ramdial et al. (2016).

MATERIALS AND METHODS

Fungal isolate collection. Sixteen bell pepper fields were sampled at the harvesting stage in fields located in the main areas of year-round production. Symptomatic fruit were collected and surface disinfected by rinsing in 70% ethanol for 1 min, followed by another rinse in 0.6% sodium hypochlorite solution for 1 min. Samples were then washed three times in sterilized distilled water and dried on sterile tissue paper in a laminar flow hood placed at the center of ethanol-amended agar plates (2% water agar amended with 5% absolute ethanol, streptomycin at 50 mg/liter, and tetracycline at 50 mg/liter) (Sigma-Aldrich Co.). Plates were incubated for 5 days at 25°C in the dark. After incubation, a 4-mm³

TABLE 1. (continued from preceding page)

Isolate ^a	Field location ^b	EF-1a FIESC designation ^c	EF-1a haplotype among FIESC and Trinidad isolates ^d	ITS haplotype among FIESC and Trinidad isolates
TT-39	Aranguez	FIESC15a	EF-Hap_27	ITS-Hap1
TT-40	Aranguez	FIESC 1a	EF-Hap_48	ITS-Hap12
*TT-41	Macoya	FIESC 26b	EF-Hap_47	ITS-Hap2
*TT-42	Maloney	FIESC 26b	EF-Hap_47	ITS-Hap2
TT-43	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-44	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-45	Macoya	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-46	Macoya	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-47	Macoya	FIESC15a	EF-Hap_27	ITS-Hap1
TT-48	Macoya	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-49	Maloney	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-50	Maloney	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-51	Maloney	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-52	Maloney	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-53	Maloney	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-54	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-55	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-56	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-57	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-58	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-59	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-60	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-61	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-62	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap1
TT-63	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-64	Bonne aventure	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-65	Bonne aventure	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-66	Bonne aventure	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-67	Bonne aventure	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-68	Bonne aventure	FIESC15a	EF-Hap_27	ITS-Hap1
TT-69	Bonne aventure	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-70	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap1
TT-71	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-72	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-73	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-74	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-75	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-76	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-77	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-78	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-79	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-80	Aranguez	FIESC 15a	EF-Hap_27	ITS-Hap2
TT-81	Aranguez	FIESC 16c	EF-Hap_28	ITS-Hap2
TT-82	Bonne aventure	FIESC 15a	EF-Hap_27	ITS-Hap1

block of agar taken from the advancing mycelial edge of a 5-day-old culture was removed and placed in the center of a potato dextrose agar (PDA) plate (Oxoid Ltd.) supplemented with streptomycin at 50 mg/liter and tetracycline at 50 mg/liter. Cultures were incubated for 7 days at 25°C in the dark. Pure cultures were obtained by standard serial dilution and from hyphal tips and maintained on PDA slants at 4°C for temporary storage, and as conidial suspensions in 50% glycerol at -70°C for long-term storage.

Morphological characterization. Single-spore subcultures were grown on PDA and carnation leaf agar (CLA) (Leslie and Summerell 2006). Plates were incubated at 25°C for 7 days in alternating dark and light 12-h photoperiods. Colony and conidia characteristics were recorded and included colony growth rate after 5 days of growth on CLA, colony texture and pigmentation in aerial and reverse views, appearance and mean size of 20 well-developed macro- and microconidia, and number of septa. Four replicates for each isolate were used and the experiment was conducted twice.

Pathogenicity tests. A 10^5 conidia ml⁻¹ suspension (20 µl of each isolate) was used to inoculate three nonwounded and three wounded sites of five healthy, mature Aristotle pepper fruit. Negative control fruit were treated with sterile distilled water. Inoculated fruit were kept in a moist chamber at 26°C (± 2) for 5 days. Fruit were examined for the development of symptoms over this 5-day incubation period. Pathogenicity tests were conducted twice. The identity of the fungus associated with symptomatic fruit was determined based on EF-1a sequence comparisons as described in the section below.

DNA extraction, polymerase chain reaction amplification, and sequencing. Fresh mycelium was harvested from cultures grown in potato dextrose broth supplemented with streptomycin at 50 mg/liter and tetracycline at 50 mg/liter for 4 days at 28°C at 120 rpm in a rotary shaker. Fungal genomic DNA was prepared using the hexadecyltrimethylammonium bromide extraction method and a working DNA concentration of 10 ng/µl was used in polymerase chain reaction (PCR) amplification. The ITS region of rDNA (White et al. 1990) and the EF-1a gene were amplified using published cycling parameters (O'Donnell et al. 2004, 2015). PCR products were sequenced directly and in both directions (Amplicon Express). Nucleotide sequences were aligned using the MAFFT alignment program (<http://mafft.cbrc.jp/alignment/server/>). Sequences were then edited using Bioedit sequence alignment editor software, version 7.2.5 (<http://www.mbio.ncsu.edu/bioedit/page2.html>).

DNA polymorphism. There is evidence to suggest that the reason for the observed discordance in the classification schemes for *Fusarium* may be due to misidentification of isolates (Thrane 2001). Only verified sequences of isolates of FIESC retained in the *FUSARIUM*-ID database were mined and served as type strains within the FIESC for this study (O'Donnell et al. 2004, 2015). These sequences formed type strain sequence data set where $N_{EF-1a} = 74$ and $N_{ITS} = 92$ (Supplementary Table S1). The global sequence data set contained only published or verified sequences deposited in GenBank (Supplementary Table S2) together with representative Trinidad sequences where $N_{EF-1a} = 90$.

The relative degree of DNA polymorphism was determined for both the ITS and EF-1a gene sequences using DNA Sequence Polymorphism software (DNASP), version 5.10 (Librado and Rozas 2009; Rozas et al. 2003) and ARLEQUIN, version 3.1 (Excoffier et al. 2005) and was based on the following parameters: number of polymorphic sites (S), number of mutations (Eta), number of haplotypes (h), haplotype diversity (Hd), Watterson's estimator θ (S) and (π), nucleotide diversity (π), and the number of nucleotide differences (k). This data would also be used as an indicator of phylogenetic informativeness for each gene sequence data set.

Data sets. Three data sets were generated: (i) *FUSARIUM*-ID FIESC ITS sequences for all 30 type strains, (ii) *FUSARIUM*-ID

FIESC EF-1a sequences for the 30 type strains, and (iii) FIESC sequences mined from GenBank as a sampling of isolates from 10 different countries. There were no a priori assumptions made in mining the sequences, except that the sequences conformed to the recommendations of Geiser et al. (2004) and O'Donnell et al. (2015) and were correctly identified as *F. equiseti*, *F. incarnatum*, or *F. incarnatum/equiseti*. Three representative sequences of Trinidad isolates (representing the FIESC 15, FIESC 16, FIESC 26, and FIESC 1 subclades) were included in the phylogenetic analyses. All GenBank accession numbers and *FUSARIUM*-ID identifiers are reported.

Phylogenetic inference. Maximum-likelihood (ML) analyses were conducted for separate ITS and EF-1a sequence data sets. Phylogenetic inference based on the EF-1a datasets was carried out, using ML implemented in Genetic Algorithm for Rapid Likelihood Inference (GARLI) 2.1 (Cummings et al. 2008; Zwickl 2006) and in MEGA7 (Tamura et al. 2013). Selection of the best models of nucleotide substitution for the ML analyses was carried out and, based on the highest ML score, all sequence data sets were subjected to the GTR + I + G model. The rooted ML tree is shown and was generated from bootstrapping 1,000 replicates. The trees were rooted with *F. concolor* (Castellá and Cabañes 2014).

Haplotype analysis. Sequences of the global EF-1a sequence data set were aligned using MAFFT software. The alignment was imported into Bioedit v. 7.0.9. Sequences were again visually inspected, manually adjusted, and realigned in preparation for importing into molecular statistical programs. Detection of recombination was carried out using the Recombination Analysis Tool (Etherington et al. 2005) because recombination in the sequences would confound genealogical relationships and has the potential to result in overlapping haplotypes (Carbone and Kohn 2004).

A haplotype network to visualize the relationships among haplotypes for the global EF-1a sequence data set was calculated using PopART v. 1.7 (<http://popart.otago.ac.nz>) (Leigh and Bryant 2015). A TCS network, which is a parsimony-based method of analysis, was constructed for the aligned haplotype sequences (Clement et al. 2002; Templeton et al. 1992). Default settings of a 1:1 transition/transversion ratio and an ϵ value set at 0 were initially used. The ratio was determined using MEGA6 (Tamura et al. 2013) and the estimated transition/transversion bias (R) was found to be 1.49. The default setting was then changed in PopART and the networks were redrawn. This had no meaningful effect on the networks generated. Similarly, the values of ϵ were changed from 0 to 10, which resulted in longer calculation time. Exceeding this ϵ value resulted in an increase in the pattern complexity of interconnecting nodes of the different groupings, with greater difficulty in interpreting the networks.

RESULTS

Morphology and growth rate. In total, 82 isolates identified as belonging to the FIESC were characterized in this study based on morphological traits, pathogenicity tests, and sequencing of the EF-1a and ITS gene regions. For purified isolates grown on CLA, colonies were dense, with floccular aerial mycelium, and cream to salmon coloration in reverse. Microconidia were single celled, hyaline, nonseptate, and ovoid, and measured 10 to 12 by 3 to 4 µm. Macroconidia were four to five septate, slightly curved and tapered at apex, and measured 28 to 31 by 3 to 5 µm. Isolates were considered to be fast growing, with a mean growth rate on of 4.5 ± 0.2 mm/day.

Pathogenicity tests. Koch's postulates were fulfilled because the fungus was recovered from inoculated wounded fruit showing symptoms of disease similar to those observed in the field. A single watery lesion developed at wounded inoculation sites on each of the five fruits for each isolate. No lesions developed in the healthy

control fruit treated with sterile distilled water. EF-1a sequences of the recovered isolates were identified as *F. incarnatum* (Desm.) Sacc. 1886, which belongs to the FIESC.

DNA polymorphism. Comparisons of chromatograms and sequences of the Trinidad isolates were carried out against DNA sequences, alignments, and corrected ABI sequence chromatograms in the *FUSARIUM*-ID database (<http://isolate.fusariumdb.org/blast.php>) in addition to sequences in GenBank (<http://www.ncbi.nlm.nih.gov/>) according to the recommendations of Geiser et al. (2004), O'Donnell et al. (2010, 2015), and web guides for using *FUSARIUM*-ID (<http://isolate.fusariumdb.org/guide.php>).

The percent sequence similarity for Trinidad isolates ($N = 82$) was determined after comparison with sequences of the *FUSARIUM*-ID database, and the values ranged from 99.3 to 100% sequence similarity, well within the threshold reported by O'Donnell et al. (2015). The pathogen was identified as *F. incarnatum* (Desm.) Sacc. 1886, which belongs to the FIESC. The majority of the isolates were *F. incarnatum* species type and were identified as FIESC-15 (representative sequence submitted to GenBank, KR003731), with fewer isolates belonging to type strains FIESC-16 and FIESC-26 (Table 1).

The ITS sequences had a much lower number of polymorphic sites than the EF-1a sequences (Tables 2 and 3). Concomitantly, this gene region also had a lower number of mutations. Nucleotide diversity was lower for the ITS sequences than for the EF-1a sequences. This implies that the mean number of nucleotide substitutions per site between any two randomly selected DNA sequences of the EF-1a sequences was greater than that of the ITS sequences.

Phylogenetic inference. Phylogeny was poorly resolved in the ML ITS tree generated using type strain sequences. Bootstrap score (bs) support was weak (bs > 50%) and there was very low confidence in placement of sequences with mostly polytomic branching patterns. The ITS sequences were not variable enough to allow for resolution of the isolates into discrete subclades and did not identify Trinidad isolates to the subclade level (ML tree not shown).

Branches were well supported for the EF-1a ML phylogenetic tree constructed for type strain sequences and were largely separated according to type strain and phylogenetic species (Fig. 1A and B). The analysis involved 145 nucleotide sequences. Trinidad isolates were positioned within the *F. incarnatum* grouping (FIESC-15, -16, and -26) and only one Trinidad isolate was found to be most similar to other *F. equiseti* sequences (FIESC-1). Therefore, there were four phylogenetic species identified in the Trinidad data set. The branches were supported by high bootstrap scores (bs > 75%). There was also clear separation of *F. equiseti* and *F. incarnatum* species into two distinct clusters (bs > 50%). The ML tree generated by GARLI had similar topology (tree not shown).

The phylogenetic relationships among global isolates of the FIESC complex were inferred by ML analysis of EF-1a sequences because the ITS sequences in the data set were too invariable for accurate resolution of phylogeny. There was no evidence of recombination within the EF-1a sequences. There was an apparent

separation of isolates identified as *F. equiseti* and those as *F. incarnatum* based on the metadata profile of the sequences mined from GenBank. Trinidad isolates clustered with the other *F. incarnatum* sequences in the data set. The ML tree reconstructed using GARLI had a similar topology when branching patterns and branch support were compared (tree not shown). There was evidence of clustering based on type strain because *F. equiseti* isolates from Spain formed a separate, moderately supported cluster (bs = 68%) which represented the FIESC-5 type strain. Isolates from Northern Europe (i.e., Denmark and Norway) and the United States were also grouped into a separate cluster with moderate support (bs = 99%), and this represented the FIESC-25 type strain.

Within the *F. incarnatum* cluster, isolates from Spain, Philippines, and China formed a highly supported and distinct group (bs = 99%) along with three Trinidad isolates, and this cluster represented the FIESC-26 species. Another moderately supported branch was shared among the remaining five Trinidad isolates and isolates from China, the United States, Indonesia, and Malaysia, which represented the FIESC-15 and -16 phylogenetic species.

Haplotype distribution: FIESC sequence data set. For the FIESC type strains data set, the ITS sequences exhibited a haplotype diversity of 0.814 whereas the EF-1a sequences exhibited a haplotype diversity of 0.977 and the haplotypes from the EF-1a sequences were more diverse than those identified for the ITS sequences. As such, haplotype diversity was higher for the EF-1a sequences than the ITS sequences. With respect to the ITS sequences, there were 12 haplotypes in the entire 92-sequence data set, 3 of which had Trinidad isolate membership (Table 1). Haplotype 1 exhibited the highest frequency within the ITS sequences, being generated from 22 sequences (both Trinidad sequences and *Fusarium* type strain sequences). On the other hand, Haplotypes 4-5, -9, -11, and -12 exhibited the lowest frequencies among the ITS sequences, and were generated from only one sequence each. In comparison, for the type strain data set, 48 haplotypes were obtained for the EF-1a sequences, each generated from either the *Fusarium* type strain sequences or the Trinidad sequences. Haplotype 27 exhibited the highest frequency among the EF-1a sequences (Table 1). Haplotypes 1-2, 4, 6-7, 8, 10-16, 19, 21-23, 25, 29, 31-34, 36-45, and 48 exhibited the lowest frequencies among the EF-1a sequences, being generated from only one sequence each.

Haplotype distribution: Global sequence data set. For the global EF-1a sequence data set, there were 42 haplotypes detected based on sequence analysis of 79 isolates, which represented 11 different countries (Table 4). An analysis of haplotypes revealed that 50% of the total number of haplotypes detected were generated from insertion/deletion type mutations (InDels) using a multiallelic model in DNASP (Table 5). There were 46 singleton variable sites detected and these largely accounted for the remaining haplotypes obtained. Haplotype and nucleotide diversities are generally considered to be low where H_d and π are less than 0.5. Analysis of the global EF-1a sequences revealed that both indices were high

TABLE 2. DNA polymorphism data for *Fusarium incarnatum-equiseti* species complex (FIESC) *FUSARIUM*-ID type strains and Trinidad isolates based on internal transcribed spacer (ITS) and translation elongation factor (EF-1a) gene sequence comparisons

DNA polymorphism parameter	EF-1a	ITS
Number of sequences	74	92
Selected region analyzed	1-445	1-508
Number of polymorphic sites, S	130	22
Number of mutations, Eta	171	23
Number of haplotypes, h	48	11
Haplotype diversity, H_d	0.977	0.814
Nucleotide diversity, π	0.051	0.004
θ (S)	0.081	0.009
θ (π)	0.055	0.004
Number of nucleotide differences, k	20.535	1.493

TABLE 3. DNA polymorphism data for global isolates of the *Fusarium incarnatum-equiseti* species complex (FIESC) based on translation elongation factor (EF-1a) gene sequence comparisons

DNA polymorphism parameter	EF-1a
Number of sequences	79
Selected region analyzed	1-395
Number of polymorphic sites, S	151
Number of mutations, Eta	180
Number of haplotypes, h	42
Haplotype diversity, H_d	0.958
Nucleotide diversity, π	0.073
θ (S)	0.116
θ (π)	0.081
Number of nucleotide differences, k	25.325

($Hd = 0.958$ and $\pi = 0.073$). Tests of the standard neutral model for a demographically stable population—Tajima's D (Tajima 1989), Fu and Li's F^* , and Fu and Li's D^* (Fu 1997; Fu and Li 1993)—were carried out on the EF-1a sequences and it was found that statistics of

neutrality were negative and not significant at $P \geq 0.10$, perhaps due to the intronic regions present in the EF-1a sequence.

A consensus maximum-parsimony (MP) tree was inferred for the haplotype sequences generated and the topological tree is presented

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Fig. 1. Phylogenetic relationships of *Fusarium incarnatum-equiseti* species complex (FIESC) isolates based on translation elongation factor (EF-1a) gene sequences. The rooted maximum-likelihood (ML) tree (drawn to scale with branch lengths measured in the number of substitutions per site) with the highest log likelihood (-2375.7202) is shown; bootstrap support was calculated from 1,000 replicates and values $>50\%$ are indicated above the branches. **A**, The ML tree is collapsed for the *F. equiseti* cluster and **B**, the ML tree is collapsed for the *F. incarnatum* cluster.

(Fig. 2A). MP analysis was selected rather than ML (which was used to infer the phylogenetic trees) because the TCS haplotype network was generated using parsimony-based statistics and would enable comparisons of groupings with high bootstrap support. High bootstrap scores (>90%) supported haplotype placement which corresponded to distinct groupings of *F. incarnatum* and *F. equiseti* sequences. The patterns of haplotype network corresponded to the

MP topological tree even though the tree was bifurcating and the network appeared to be reticulate at the interior and star-like at the tips. There was no one discernible central haplotype from which most other haplotypes diverged (Fig. 2B). Several haplotypes (many of which were from Spain) were unique and were represented by only one sequence (Table 4). Sequences from Spain were represented in 12 haplotypes, nearly half the total number of

Fig. 1. Continued from previous page.

haplotypes detected in this aspect of this study. There was some evidence of geographic structure in the distribution of haplotypes. Twelve haplotypes were shared and two of these were represented by country-specific sequences (Spain and the United States). Spain had the highest number of unique and shared haplotypes compared with the other geographical locations. The most diverse haplotype was Hap_16, which was represented by sequences from China, Indonesia, Malaysia, and Trinidad and which consisted exclusively of *F. incarnatum* isolates. Sequences of isolates from Northern Europe (Denmark, Norway, and Sweden) belonged exclusively to two haplotypes (Hap_13 and Hap_14), which consisted of only *F. equiseti* isolates. Hap_13 was also the most frequent haplotype, with 12 representative sequences. Interestingly, Trinidad and China sequences shared the same two haplotypes (Hap_16 and Hap_33), which consisted of *F. incarnatum* isolates. Trinidad sequences also shared one haplotype with the United States (Hap_21), which also consisted of *F. incarnatum* isolates.

When the distribution of haplotypes was plotted on to a world map (Fig. 2C), there was some correlation between haplotype diversity and geographical imprint. Spain had the highest haplotype diversity, perhaps because both *F. equiseti* and *F. incarnatum* sequences were represented; followed by the United States, which contributed both *F. equiseti* and *F. incarnatum* sequences to the data set; then by countries representing Southeast Asia (China, Indonesia, Malaysia, Thailand, and Philippines) and Trinidad (both regions were represented by only *F. incarnatum* sequences).

DISCUSSION

The objectives of this study were to (i) determine the identity of *Fusarium* spp. infecting bell pepper in Trinidad, (ii) compare the level of DNA polymorphism of ITS and EF-1a sequences, (iii) determine the haplotype diversity and distribution, and (iv) infer the phylogenetic relationships of FIESC isolates. Separate sequence data sets were prepared and analyzed for FIESC type strains representing 30 phylogenetic species obtained from the *FUSARIUM*-ID database, and for 79 FIESC isolates from 11 different countries.

The EF-1a sequences outperformed the ITS sequences in providing superior taxa resolution for members of the FIESC. It is known that for some fungal groups of Ascomycetes (e.g., *Trichoderma* and *Fusarium*), EF-1a sequences are recommended for species delineation (O'Donnell et al. 2015). When the distance to the nearest neighbor for fungal species, especially for Ascomycetes (e.g., *Penicillium* and *Fusarium*), are examined for ITS sequences, very low genetic distance values are obtained and, hence, poor resolution and taxon placement in phylogenetic inference (Vitale et al. 2011). For *Fusarium* spp., ITS sequences are also unable to resolve recent lineages (O'Donnell et al. 2013). In general, slowly evolving genes (e.g., EF-1a and RPB2) are more suitable for inferring deep phylogenies, while gene sequences undergoing faster evolutionary rates (e.g., TUB2) perhaps capture more recent evolutionary and speciation events. Sections of protein-coding genes (e.g., EF-1a gene) theoretically satisfy both requirements because these sequences consist of variable intronic sequences and conserved exonic sequences (Stielow et al. 2015). According to the results of this study and others, it is important to determine the level of DNA polymorphism in sequences to be used for intraspecies identification and for phylogenetic inference because this information has a direct impact on confidence of identity and resolution of phylogenetic placement and phylogenetic signal, and affects genetic diversity indices, including haplotype diversity and distribution (O'Donnell et al. 2015).

The low level of genetic diversity found for FIESC isolates infecting bell pepper in Trinidad based on EF-1a sequences is apparently not uncommon because Castellá and Cabañes (2014) also reported a low level of phylogenetic species diversity for isolates associated with symptomless infection of wheat in Spain. However, the results of this study indicate that Spain is host to the

largest number of EF-1a haplotypes and the most diverse EF-1a haplotypes infecting a wide range of plant species.

Based on ML analysis, there was evidence of clustering based on geography as *F. equiseti* isolates from Spain which formed a separate, well-supported cluster. Similarly, isolates from Northern Europe (i.e., Denmark and Norway) and the United States were grouped into a separate cluster with moderate support. These two distinct groupings were also reported by Marín et al. (2012) as type II *F. equiseti* for Spain isolates and type I *F. equiseti* for the Northern Europe isolates.

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Fig. 2. A. Maximum-parsimony (MP) analysis of haplotypes. The consistency index is 0.764, the retention index is 0.932, and the composite index is 0.772 for all sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated. There were 154 positions in the final dataset. The tree presented is unrooted. **B.** TCS haplotype network generated for global translation elongation factor (EF-1a) sequences representing 11 countries. The size of the circle indicates the relative frequency of sequences belonging to a particular haplotype (smallest circle = 1 sequence to largest circle = 13 sequences). Hatch marks along the network branches indicate the number of mutations. Each color represents a different geographic location. **C.** Distribution of haplotypes and haplotype diversity according to geographic location. Each color represents a different geographic location.

The majority of *F. incarnatum* sequences in the data set were from isolates collected in warm temperate and tropical regions or climates. This finding is in keeping with the global distribution of *F. incarnatum* according to the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) fungal database (<https://nt.ars-grin.gov/fungalatabases/>). Conversely, the distribution of *F. equiseti* is described as cosmopolitan on the same fungal database. Geography and climate are reported to be two important epidemic-associated factors that influence the occurrence

and establishment of *Fusarium* spp. as plant pathogens (Castellá and Cabañes 2014). Interestingly, isolates associated with human infections in Thailand were positioned with other sequences of plant-infecting isolates which may indicate little host specificity among members of the species complex. The USDA-ARS fungal database has a record of 269 *F. incarnatum*–host combinations and 280 *F. equiseti*–host combinations (including synonyms of both fungal species). *Fusarium* spp. demonstrate a high level of diversity in their morphological, physiological, and cultural characteristics

Fig. 2. Continued from previous page.

(Booth 1971; Leslie and Summerell 2006). This variation may reflect the ability of different *Fusarium* spp. to survive in a wide range of ecological niches as pathogens, endophytes, and soil saprophytes in temperate and tropical climates (Nelson et al. 1983).

Many of the haplotypes were produced as a result of InDels, which are informative when working with highly similar sequences, especially those of a species complex such as FIESC. However, suitable algorithms are still to be developed that take such InDels into account and allow for meaningful biological interpretation (Salzburger et al. 2011). In naturally occurring field populations,

TABLE 4. Haplotype frequency (Freq) and distribution for global isolates based on translation elongation factor gene sequences

Haplotype ^a	Freq	Sequences
Hap_1	1	JF496568, Spain
Hap_2	1	JF496573, Spain
Hap_3	1	JF496572, Spain
Hap_4	1	JF496569, Spain
Hap_5	4	JF496571, Spain; JF496577, Spain; KF962942, Spain; JF496570, Spain
Hap_6	1	KF962941, Spain
Hap_7	1	KF962940, Spain
Hap_8	4	JF496576, Spain; KF962943, Spain; JF496575, Spain; KF962944, Spain
Hap_9	1	GQ505601, United States
Hap_10	2	GQ505605, United States; GQ505654, United States
Hap_11	2	JF496574, Spain; KF962945, Spain
Hap_12	1	GQ505663, United States
Hap_13	1	GQ505594, United States; GQ505599, United States; GQ505644, United States; GQ505653, United States; AJ543563, Denmark; AJ543561, Denmark; AJ543562, Denmark; AJ543563, Denmark; AJ543564, Denmark; AJ543565, Norway; AJ543566, Norway; AJ543557, Sweden
Hap_14	3	AJ543558, Denmark; AJ543559, Denmark; AJ543560, Norway
Hap_15	1	AJ543571, Malta
Hap_16	8	KF208617, China; KF208619, China; HM770722, Indonesia; KF993969, Malaysia; HM770724, Indonesia; TTEF16, Trinidad; TTEF18, Trinidad; TTEF21, Trinidad
Hap_17	1	KF208618, China
Hap_18	1	KF267263, China
Hap_19	1	JX268971, United States
Hap_20	1	HM770723, China
Hap_21	4	GQ915510, United States; JX268996, United States; TTEF1, Trinidad; TTEF7, Trinidad
Hap_22	1	KF255481, Thailand
Hap_23	1	KF255466, Thailand
Hap_24	1	KF993970, Malaysia
Hap_25	1	KF993968, Malaysia
Hap_26	3	KF255480, Thailand; KF255476, Thailand; JX269001, United States
Hap_27	1	KM893089, Malaysia
Hap_28	1	KF962946, Spain
Hap_29	1	KF962947, Spain
Hap_30	2	KF962948, Spain; KF962950, Spain
Hap_31	1	KF962949, Spain
Hap_32	1	JF715934, Philippines
Hap_33	3	HM770725, China; KF993967, China; TTEF41, Trinidad
Hap_34	1	KF962951, Spain
Hap_35	2	KF962952, Spain; JF715935, Philippines
Hap_36	1	KF962953, Spain
Hap_37	1	KF962954, Spain
Hap_38	1	KF962955, Spain
Hap_39	1	KF962956, Spain
Hap_40	1	KF962957, Spain
Hap_41	1	TTEF32, Trinidad
Hap_42	1	TTEF42, Trinidad

^a Haplotype: underscore was used in the haplotype designations to differentiate them from the haplotypes obtained for the *Fusarium incarnatum-equiseti* species complex subclade species analysis.

there will be a mixture of ancestral haplotypes and surviving descendants; however, the relationship between the two may be reticulate and not bifurcate, due to nondichotomous historical events (e.g., recombination, incomplete lineage sorting, horizontal gene transfer, and deep coalescence) (Posada and Crandall 2001). These events would leave different “signatures” in the data and result in different patterns in a given network. In fact, Ma et al. (2013) suggested that horizontal gene transfer played a critical role in the evolution of *Fusarium* genomes. The coalescent theory proposes that high-frequency haplotypes may have been present in the population for a long time and, therefore, the less-frequent haplotypes may represent more recent mutation events. This is supported by the findings of this study, where several of the terminal haplotypes located at the tips in the network were produced from a single mutation event indicated by a single hatch mark along the branch. These network tips were also unique haplotypes represented by one sequence and it is likely that sufficient time had not elapsed to accumulate mutations that are host or geographic associated to an extent where the sequence contributed significantly to nucleotide divergence (Carbone and Kohn 2004). The terminal haplotypes located at the network tips as well as unique haplotypes may represent more recent lineages rather than older ones. The older haplotypes would be positioned toward the interior of the network. Newer haplotypes would also have a more restricted geographical association and the older haplotypes have a broader geographical distribution if there is suspected restricted gene flow (Carbone and Kohn 2004). However, in a reticulate network, there can be both older and newer haplotypes positioned at the internal nodes of the network. There is no straightforward interpretation of this type of network, with resulting difficulty in understanding its biological relevance (e.g., the pattern may represent real evolutionary events but it is not clear which ones may, in fact, be a mathematical anomaly) (Morrison 2005). As such, these haplotype networks are considered to be an exploratory tool to understanding the phylogenetic hypothesis that describes the relationships among closely related species such as those of the FIESC.

Conservative exploration of the haplotype network and the haplotype distribution on the world map illustrate that the *F. equiseti* strains were separated from the *F. incarnatum* strains by a number of mutational steps with separation of isolates according to geographical location (e.g., Spain, the United States, Denmark, and Norway). It is proposed that both direct and indirect influences on the dissemination of the pathogen may explain the haplotype network, where there may have been recent anthropogenic movement of the pathogen in contaminated plant material and dispersal of inoculum that originated from other hosts, followed by rapid host adaptation with a potential for wide spatial distribution. In addition, hundreds of millions of tons of Saharan dust is carried off the coast of Africa and other areas in interior Africa to the Caribbean and South America each year, usually during the hurricane season. The dust is seen as a continuous Saharan Air Layer (NASA Earth Observatory, <http://earthobservatory.nasa.gov/IOTD/view.php?id=81864&src=eoai-iodt>) which provides a medium for movement of over 300 microbes that are pathogenic to plants, animals, and humans, the

TABLE 5. Analysis of insertion/deletion mutation (InDel) data for haplotype sequences

InDel index	Data
Total number of InDel sites analyzed	30
Total number of (InDel and non-InDel) sites analyzed	348 + 30 = 378
Total number of InDels events analyzed, <i>I</i>	21
Average InDel length event	1.81
Average InDel length	1.65
Number of InDel Haplotypes	27
InDel Haplotype Diversity	0.928
InDel Diversity, <i>k(i)</i>	3.094
InDel Diversity per site, π	0.008

majority of which are plant-pathogenic fungi and include *Fusarium* spp. (Garrison et al. 2006) (A. Ramsubhag, personal communication). *Fusarium* spp. are well adapted to long-distance atmospheric transport because their asexual spores are resistant to desiccation, ultraviolet radiation, and other harsh environmental conditions.

There have been reports of other *Fusarium* spp. infecting bell pepper in Belgium, Canada, the Netherlands, and the United Kingdom; for example, *F. oxysporum*, *F. proliferatum*, *F. solani*, and *F. lactis* species complexes have been shown to produce mycotoxins in bell pepper fruit (Van Poucke et al. 2012; Yang et al. 2011). However, this is the first report of FIESC infecting bell pepper fruit. Because members of the FIESC are known mycotoxin producers, the level of exposure after consumption of infected bell pepper fruit must be determined. Van Poucke et al. (2012) purported that, for *F. lactis* infecting bell pepper fruit in Belgium, the affected part of the fruit may be removed and consumed because there was negligible movement of mycotoxins into healthy flesh and, therefore, a low level of mycotoxins and risk of exposure. Future research will have to include (i) a more detailed assessment involving sequence analysis of multiple genes and development of microsatellites, (ii) screening FIESC (Trinidad) isolates for the production of mycotoxins in bell pepper, and (iii) genetic chemotyping of isolates based on toxin biosynthesis gene sequence analysis.

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