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Genetic variability among isolates of *Fusarium oxysporum* from sugar beet

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Fusarium yellows, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *betae* (Fob), can lead to significant yield losses in sugar beet. This fungus is variable in pathogenicity, morphology, host range and symptom production, and is not a well characterized pathogen on sugar beet. From 1998 to 2003, 86 isolates of *F. oxysporum* and 20 other *Fusarium* species from sugar beet, along with four *F. oxysporum* isolates from dry bean and five from spinach, were obtained from diseased plants and characterized for pathogenicity to sugar beet. A group of sugar beet *Fusarium* isolates from different geographic areas (including nonpathogenic and pathogenic *F. oxysporum*, *F. solani*, *F. proliferatum* and *F. avenaceum*), *F. oxysporum* from dry bean and spinach, and *Fusarium* DNA from Europe were chosen for phylogenetic analysis. Sequence data from β- tubulin, EF1α and ITS DNA were used to examine whether *Fusarium* diversity is related to geographic origin and pathogenicity. Parsimony and Bayesian MCMC analyses of individual and combined datasets revealed no clades based on geographic origin and a single clade consisting exclusively of pathogens. The presence of FOB and nonpathogenic isolates in clades predominately made up of *Fusarium* species from sugar beet and other hosts indicates that *F. oxysporum* f. sp. *betae* is not monophyletic.

Keywords: *Beta vulgaris*, fusarium yellows, genetic diversity, pathogenicity

Introduction

Sugar beet (*Beta vulgaris*) is grown worldwide and produces one-third of the world's sugar supply (Draycott, 2006). Fusarium wilt or fusarium yellows of sugar beet is caused by *Fusarium oxysporum*. f. sp. *betae* (Fob) (Stewart, 1931; Hanson & Jacobsen, 2009) and can result in significant reduction in sugar concentration, root yield and juice purity (Hanson & Jacobsen, 2009). Symptoms of this disease include interveinal yellowing, chlorosis, wilting and necrosis of the leaves and a grey to brown discoloration in the root tissue (Stewart, 1931; Ruppel, 1991). The disease is seen most often in areas of fields that are low and compacted, especially during periods of high temperatures (Hanson & Jacobsen, 2009). *Fusarium oxysporum* is a significant pathogen on many important agricultural crops (Nelson *et al.*, 1983) and isolates are categorized into 'formae speciales' according to their ability to cause disease on specific host plants or specific symptoms on a specific host (Armstrong & Armstrong, 1981). For example, isolates of *F. oxysporum* f. sp. *radicis-betae* that cause root tip rot on sugar beet have been reported to be distinct from the isolates of *F. oxysporum* f. sp. *betae* that cause fusarium yellows with no external root symptoms (Martyn *et al.*, 1989). Isolates within a 'forma specialis' can be separated further by determining their interaction with a set of host cultivars (i.e. a race-type interaction) (Gordon & Martyn, 1997). Current research has shown that different cultivars express differing disease symptoms when inoculated with the same isolates (Hanson *et al.*, 2009); however, the existence of races within FOB has not been shown.

Classification by 'formae speciales' and races is difficult for isolates of *F. oxysporum* able to infect sugar beet, because some isolates also infect other hosts, such as spinach (*Spinacia oleraceae*) (Armstrong & Armstrong, 1976), various weed species (MacDonald & Leach, 1976) and dry bean (*Phaseolus vulgaris*) (Wickliffe, 2001), and this cross pathogenicity goes against the definition of a 'forma specialis'. *Fusarium oxysporum* can

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also be categorized using vegetative compatibility grouping (VCG) as a metric of similarity (Harveson & Rush, 1997; Koenig *et al.*, 1997). Based on research in other fungal species, isolates that have identical alleles at loci determining heterokaryon incompatibility are vegetatively compatible (Leslie *et al.*, 2006). It was once assumed that isolates within a 'forma specialis' or VCG were monophyletic, having evolved from a common ancestor. However, more recent work has shown 'formae speciales' are more often polyphyletic with more than one distinct lineage (Gordon *et al.*, 1989), suggesting multiple origins of pathogenicity (Koenig *et al.*, 1997).

Many additional methods have been used to characterize genetic diversity and evolutionary origin of a particular 'forma specialis'. These include restriction fragment length polymorphism (RFLP) (Koenig *et al.*, 1997), random amplified polymorphic DNA (RAPD) (Windels, 1992), amplified fragment length polymorphism (AFLP) (Leslie *et al.*, 2006), and comparison of intron sequences from conserved gene regions (Jiménez-Gasco *et al.*, 2002). Gene genealogies make it possible to reconstruct the evolutionary history within a 'forma specialis', differentiate among taxa or species, or determine variability within a species (Jiménez-Gasco *et al.*, 2002; Leslie *et al.*, 2006). DNA sequences from coding regions may contain insufficient variation to resolve a gene genealogy, therefore sequences derived from rapidly evolving non-coding DNA, such as intergenic regions, introns within genes or spacer elements within the rDNA repeat, are likely to be more appropriate for phylogeny reconstruction (Leslie *et al.*, 2006).

There are no effective fungicides for managing this pathogen of sugar beet, and crop rotation is often ineffective because isolates pathogenic on beet can live on the other crops within the rotation without the appearance of symptoms, increasing levels of inoculum (Gordon *et al.*, 1989). Therefore, host resistance should be the most expedient means for disease management. To facilitate breeding for host resistance, basic understanding of the variability that exists within the pathogen population and how the variability pertains to pathogenicity is required.

In this study, an exon region of the β-tubulin gene, an intron region of translation elongation factor 1α (EF1α), and the internal transcribed spacer (ITS) regions of the rDNA 5·8S rRNA gene were used in combination to describe variation among *F. oxysporum* isolated from diseased sugar beet, dry bean and spinach. The goal of this study was to better understand phylogenetic relationships among *F. oxysporum* isolates collected from sugar beet, and determine if this diversity correlates with pathogenicity or geographic origin.

Materials and methods

Collection of fungal isolates

Sugar beet plants exhibiting symptoms of fusarium yellows were collected from commercial fields in Colorado, Oregon and Nebraska from 1998 to 2004. Isolates were obtained from the crown, vascular region of the tap root, and the root tip. Tissue was removed from diseased sugar beet using a sterile cork borer (7 mm diameter), surface-disinfected in 0·6% sodium hypochlorite for 1 min (Windels, 1992), rinsed once with sterile distilled water, blotted dry with a sterile paper towel, and transferred to Petri dishes containing potato dextrose agar (PDA; Becton, Dickinson and Co.). Plates were incubated on the laboratory bench for 5 days at 25 ± 2°C. Fungi were transferred to freshly prepared PDA to eliminate contamination. Single spore isolates were either prepared from cultures of *Fusarium* species (Nelson *et al.*, 1983; Leslie *et al.*, 2006) or isolates were subcultured onto 2% (w/v) water agar (Bacto Agar; Becton, Dickinson and Co.) for hyphal tip transfer (Nelson *et al.*, 1983) to obtain pure cultures. Additional isolates of *F. oxysporum* from sugar beet, spinach and dry bean as well as *Fusarium* DNA (isolated and extracted from diseased sugar beets in Europe) were received from other researchers (Table 1).

Isolate identification and storage

Fusarium isolates were identified to species according to the methods of Nelson *et al.* (1983). Spore production and spore type were assessed using a wet mount from sporodochia produced on sterile carnation leaves (Nelson *et al.*, 1983) or by scraping spores from mycelia on the carnation leaves for those isolates that did not produce sporodochia.

Fusarium species were maintained by serial transfer on PDA for further analysis. For long-term storage, mycelium from *Fusarium* isolates was stored desiccated on sterile glass fibre filter paper at −20°C (Hanson & Hill, 2004).

Pathogenicity screening

One hundred and fifteen *Fusarium* isolates cultured from diseased sugar beet, dry bean and spinach were tested for pathogenicity on a fusarium yellows susceptible sugar beet germplasm, 'FC 716' (Panella *et al.*, 1995), in the greenhouse using a method modified from the procedures of Hanson & Hill (2004). Sugar beet plants were grown in pasteurized (3 h at 72°C and 0·4 Bar) Metro-Mix 200 potting mix (The Scotts Company) at approximately 28°C, kept under 16 h of daylight, and watered daily to maintain vigorous growth. After 5 weeks, plants were removed from the soil, rinsed under running tap water, and roots were placed in a spore suspension (mix of macroconidia and microconidia), at approximately 1×10^5 spores mL^{-1} in sterilized, reverse-osmosis (RO) water for 8 min with intermittent agitation. Control plant roots were placed in sterile, RO water. Individual beets were replanted into planting cones (3·8 cm diameter × 21 cm deep; Steuwe and Sons, Inc.) containing moist, pasteurized potting mix. Cones were placed in

Table 1 Geographic origin and pathogenicity on sugar beet seedling (cv. FC716) of *Fusarium oxysporum*, *F. proliferatum* and *F. solani* cultured from sugar beet, bean and spinach

Isolate[a]	Donor's Designation	Geographic Origin	Location	Species	Host	Provided by[b]	Year of Isolation	Pathogenicity[c]
F5	F5	Texas		*oxysporum*	Sugarbeet	R.M. Harveson		NP
F88	F88	Texas		*oxysporum*	Sugarbeet	R.M. Harveson	1993	NP
F113	F113	Texas		*oxysporum*	Sugarbeet	R.M. Harveson		NP
F120	F120	Texas		*oxysporum*	Sugarbeet	R.M. Harveson	1993	NP
F125	F125	Texas		*oxysporum*	Sugarbeet	R.M. Harveson	1993	NP
F127	F127	Texas		*oxysporum*	Sugarbeet	R.M. Harveson	1993	NP
F1	F1	Texas		*oxysporum*	Sugarbeet	R.M. Harveson	1994	NP
F55	F55	Texas		*oxysporum*	Sugarbeet	R.M. Harveson	1994	LP
F172	F172	Wyoming		*oxysporum*	Sugarbeet	R.M. Harveson	1995	NP
FOB13	F180	Oregon		*oxysporum*	Sugarbeet	R.M. Harveson	1994	P
F17		Oregon	Salem	*oxysporum*	Sugarbeet	SBRU	2001	P
F19		Oregon	Salem	*oxysporum*	Sugarbeet	SBRU	2001	P
F32		Oregon	Salem	*oxysporum*	Sugarbeet	SBRU	2001	P
F38		Oregon	Salem	*oxysporum*	Sugarbeet	SBRU	2001	P
F42		Oregon	Salem	*oxysporum*	Sugarbeet	SBRU	2001	P
F46		Oregon	Salem	*oxysporum*	Sugarbeet	SBRU	2001	P
F174	F174	California		*oxysporum*	Sugarbeet	R.M. Harveson	1995	LP
FOB21	Flynn	Montana		*oxysporum*	Sugarbeet	B.J. Jacobsen	1998	P
H7	H7	Montana	Hardin	*oxysporum*	Sugarbeet	B.J. Jacobsen	2004	P
H8	H8	Montana		*oxysporum*	Sugarbeet	B.J. Jacobsen	2004	P
Fo22	Fusarium #1	Minnesota	Sabin	*oxysporum*	Sugarbeet	J.J. Weiland	1998	NP
Fo23	Fusarium #2	Minnesota	Sabin	*oxysporum*	Sugarbeet	J.J. Weiland	1998	NP
Fo25	Fusarium #4	Minnesota	Sabin	*oxysporum*	Sugarbeet	J.J. Weiland	1998	NP
Fo26	Fusarium #5	Minnesota	Sabin	*oxysporum*	Sugarbeet	J.J. Weiland	1998	NP
Fo27	Fusarium #6	Minnesota	Sabin	*oxysporum*	Sugarbeet	J.J. Weiland	1998	NP
Fo29	Fusarium #8	Minnesota	Sabin	*oxysporum*	Sugarbeet	J.J. Weiland	1998	NP
Fo17	Fo17	Minnesota		*oxysporum*	Sugarbeet	C. Windels	2004	P
Fo37	Fo37	Minnesota		*oxysporum*	Sugarbeet	C. Windels	2004	P
Fo204b		Colorado	Peckham	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	NP
FOB216a	FOB216a	Colorado	Crook	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
FOB216b	FOB216b	Colorado	Crook	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
FOB216c	FOB216c	Colorado	Crook	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
FOB216d	FOB216d	Colorado	Crook	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
FOB220a	FOB220a	Colorado	Iliff	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
Fo220d		Colorado	Iliff	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	NP
FOB257a	FOB257a	Colorado	Brush	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
FOB257c	FOB257c	Colorado	Brush	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
FOB266a	FOB266a	Colorado	Padroni	*oxysporum*	Sugarbeet	H.F. Schwartz	1998	P
F28		Colorado		*oxysporum*	Sugarbeet	SBRU	2001	LP
F49		Colorado		*oxysporum*	Sugarbeet	SBRU	2001	P
B13	B13	Colorado		*oxysporum*	Dry bean	H.F. Schwartz	1988	NP
B52	B52	Colorado	Sedgwick	*oxysporum*	Dry bean	H.F. Schwartz	1988	NP
B291	B291	Colorado		*oxysporum*	Dry bean	H.F. Schwartz	1988	NP
FOP144b	FOP144b	Nebraska		*oxysporum*	Dry bean	H.F. Schwartz	1998	NP
FUS001	FUS001	Washington	Skagit Co	*oxysporum*	Spinach	L. du Toit	2002	P
FUS003	FUS003	Washington	Skagit Co	*oxysporum*	Spinach	L. du Toit	2002	P
FUS004	FUS004	Washington	Skagit Co	*oxysporum*	Spinach	L. du Toit	2002	P
Spinach A	Spinach A	Washington	Skagit Co	*oxysporum*	Spinach	L. du Toit	2003	P
Spinach B	Spinach B	Washington	Skagit Co	*oxysporum*	Spinach	L. du Toit	2003	P
F20		Oregon		*avenaceum*	Sugarbeet	SBRU	2001	LP
F37		Nebraska	Scottsbluff	*solani*	Sugarbeet	SBRU	2001	N
F81	F81	Texas		*solani*	Sugarbeet	R.M. Harveson		N
FOB273a	FOB273a	Colorado	Ogallala	*proliferatum*	Sugarbeet	H.F. Schwartz	1998	NP
F44	F44	Texas		*proliferatum*	Sugarbeet	R.M. Harveson	1992	NP
F126	F126	Texas		*proliferatum*	Sugarbeet	R.M. Harveson	1993	NP
F140	F140	Texas		*proliferatum*	Sugarbeet	R.M. Harveson	1994	NP
F156	F156	Texas		*proliferatum*	Sugarbeet	R.M. Harveson	1994	NP
F182	F182	Oregon		*proliferatum*	Sugarbeet	R.M. Harveson	1995	LP
e20	e20	Italy		*solani*	Sugarbeet	B. Holtschulte	2003	no data

Table 1 *Continued.*

Isolate[a]	Donor's Designation	Geographic Origin	Location	Species	Host	Provided by[b]	Year of Isolation	Pathogenicity[c]
e4	e4	France		*oxysporum*	Sugarbeet	B. Holtschulte	2003	no data
e10	e10	France		*oxysporum*	Sugarbeet	B. Holtschulte	2003	no data
e11	e11	Germany		*oxysporum*	Sugarbeet	B. Holtschulte	2003	no data
e12	e12	Germany		*oxysporum*	Sugarbeet	B. Holtschulte	2003	no data
e13	e13	Italy		*oxysporum*	Sugarbeet	B. Holtschulte	2003	no data
e14	e14	Italy		*oxysporum*	Sugarbeet	B. Holtschulte	2003	no data
e15	e15	Italy		*oxysporum*	Sugarbeet	B. Holtschulte	2003	no data

[a]Name as designated in the collection of the USDA/ARS Sugarbeet Research Unit.
[b]Isolates provided by R. M. Harveson (Harveson & Rush, 1997); B. J. Jacobsen, Montana State University, Bozeman, MT; J. J. Weiland, USDA-ARS Fargo, ND; H. F. Schwartz, Colorado State University, Fort Collins, CO; C. Windels, University of Minnesota, Crookston, MN; SBRU, USDA-ARS Sugarbeet Research Unit, Fort Collins, CO.
[c]Those isolates that were significantly different from the water control at $P = 0{\cdot}05$ (Dunnett's one-tailed *t*-test) in all experiments are considered pathogenic (P); isolates not significantly different from controls in all experiments are nonpathogenic (NP); an isolate was considered to have low pathogenicity (LP) if it was significantly different than the control in some experiments but not others.

cone trays in a completely randomized block design. Plants were held for 2 days at approximately 22°C to reduce transplant shock, and then moved to a greenhouse set at 28°C and 16 h of daylight. Plants were fertilized every 2 weeks with a liquid 15-30-15 fertilizer and watered daily.

Five to 10 sugar beet plants were inoculated with each isolate and a water control. Although most tests had 10 plants per isolate (12/20), the tests were run over a number of years and, in order to have sufficient plants at the same growth stage, sometimes fewer plants were available. Each isolate was screened in a minimum of two experiments, i.e. replicated sequentially. A group of isolates plus the water-inoculated control was considered an experiment. Individual plants were rated weekly for fusarium yellows symptoms for 6 weeks using a 0–4 rating scale (Hanson & Hill, 2004), where 0 = no disease, plants healthy; 1 = plants slightly to extremely stunted, leaves wilted and yellowing; 2 = leaves chlorotic and necrotic on edges; 3 = total leaf death and tap root becoming dried and brown; and 4 = plant death. After 6 weeks, roots were removed and sampled to re-isolate and confirm the presence of *Fusarium* species used for inoculation.

Pathogenicity determination

A disease index (DI) was calculated from the disease ratings for each isolate in all experiments. The 0–4 rating scale described above was used on the final (and most severe) rating to determine the individual plant DI. Individual plants were used as replications in the experiments, and the DI of a single isolate or the water control was the mean score of all of the plants inoculated with that single isolate or the water control. DI values were analyzed using PROC MIXED (SAS) with Dunnett's one-tailed *t*-test (Dunnett, 1955), to determine if there were significant differences among isolates in each experiment. A sampling of isolates from different geographic locations and pathogenicity levels were chosen for phylogenetic analysis (Table 1).

DNA extraction

The genomic DNA extraction method for the isolates collected in 1998–2000 was adapted from the methods of Nelson *et al.* (1997). Briefly, 7 mm diameter mycelial plugs were transferred to 50 mL of potato dextrose broth (PDB) (Becton, Dickinson and Co). Cultures were grown for 5 days at 25 ± 2°C on a rotary shaker at 100 r.p.m. with 8 h of light per day. Fungal tissue was collected on a double layer of sterile cheesecloth, rinsed with sterile water, and lyophilized at −50°C for 48 h. Tissue was ground in liquid nitrogen and incubated in a lysis buffer containing 25 mM Tris-HCl pH 8·0, 50 mM NaCl, 25 mM ethylene diaminetetraacetic acid (EDTA) and 1% (w/v) sodium dodecyl sulphate (SDS). DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated using −20°C isopropanol (1:1). Precipitated DNA was washed with 70% ethanol, dried and suspended in 1× TE buffer (Tris-hydrochloride buffer – 10 mM Tris-HCl pH 8·0, 0·1 mM EDTA). Isolates collected from 2001 to 2004 were grown in PDB, lyophilized, and ground as above; DNA was extracted using the Easy-DNA kit (Invitrogen Co.) according to manufacturer's recommendations. DNA was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies).

DNA amplification and sequencing

DNA sequence was selected to include the following: a highly conserved exon region (β-tubulin); a conserved region containing at least one intron (translation elongation factor 1α, EF1α); and a region that was highly variable (nuclear rDNA region containing the two internal

transcribed spacer (ITS) regions and the 5·8S rRNA). Primers used were described initially by Koenraadt *et al.* (1992) and White *et al.* (1990).

Polymerase chain reaction (PCR) amplification for the target genes, β-tubulin and ITS, were based upon the methods of Koenraadt *et al.* (1992). PCR and amplification conditions with EF1α were based upon the conditions of Carbone & Kohn (1999). For all primer pairs, sterile, RO water was used as a control in place of fungal DNA to test for contamination. All reactions were repeated at least twice to ensure reproducibility. PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf). PCR products were purified using a QIAquick Spin kit (Qiagen, Inc.), and sequenced by Macrogen (Macrogen, Inc.) or Macrogen USA (Macrogen Corp.).

Phylogenetic analysis

Sequences from β-tubulin were edited, and then aligned using Sequencher v4·1 software (Gene Codes Corp.). The ITS and EF1α sequences were aligned with CLUSTAL X v1·83 (Thompson *et al.*, 1997), followed by visual inspection and manual adjustment. Ambiguously aligned regions were not included in phylogenetic analyses. Parsimony bootstrap consensus trees were reconstructed for individual datasets and the combined dataset using the heuristic search option of PAUP*4·0b10 (Swofford, 1999), with 1000 random stepwise addition replicates, the tree-bisection-reconnection branch-swapping procedure, and MULTREES off (TBR-M strategy of DeBry & Olmstead, 2000). Trees were rooted using isolate F20, *F. avenaceum*, as the outgroup (Table 1). The ability of the β-tubulin, EF1α and ITS datasets to be combined was evaluated using the partition homogeneity test (Farris *et al.*, 1995) implemented in PAUP with 1000 homogeneity replicates and MAXTREES set to 1000.

Bayesian MCMC phylogenetic analysis using MrBayes 3·0 (Ronquist & Huelsenbeck, 2003) was conducted using the combined dataset. Two chains were run simultaneously for $1{\cdot}5 \times 10^7$ generations. Results from each independent run were compared to ensure convergence of the two runs onto a single stationary distribution of trees. 1×10^7 generations were discarded as burn-in, after which 50 000 trees were sampled from each replicate run to determine the optimal consensus tree and calculate the posterior probabilities of the clades.

Results

Species identification

In the process of identification and recording morphological characters, several isolates that were previously thought to be *F. oxysporum* were determined to be *F. proliferatum*. In addition, one isolate from Texas was also re-identified as *F. solani* (Table 1). Identification based on morphological characteristics of the *F. proliferatum* and *F. solani* isolates was confirmed by comparing sequence homology of the isolates with sequences of *F. proliferatum* and *F. solani* isolates in the GenBank (BLASTn analysis) (data not shown).

Pathogenicity testing and determination

If the initial *F*-test showed no significant differences among isolates and the water control, all isolates in that experiment were considered nonpathogenic (NP – i.e. not significantly different from the control) (Table 1). If there were significant differences, a Dunnett's one-tailed *t*-test (Dunnett, 1955) was used to compare each isolate with the control, using a Dunnett- or Dunnett-Hsu-adjusted *P* value (Dunnett, 1955; Hsu, 1992), with $P = 0{\cdot}05$ considered the threshold for significance. Those isolates which had a significantly higher DI than the control every time tested (at least twice) were considered pathogenic (P) (Table 1). Those isolates which were significantly different from the control in some experiments but not others, were considered to be of lower aggressiveness (LP) (Table 1).

Of 73 *Fusarium* species that were received or collected from sugar beet in 1998, 50 were identified as *F. oxysporum* and screened for pathogenicity on sugar beet. Sixteen of the isolates were pathogenic (data not shown). *Fusarium oxysporum* isolates from R. M. Harveson, previously reported pathogenic on sugar beet (Harveson & Rush, 1997), were nonpathogenic on sugar beet, FC716. Four *F. oxysporum* cultures isolated from dry bean in 1998 (B13, B52, B291, FOP144b) were determined to be nonpathogenic on sugar beet (Table 1). In 2001, 62 *Fusarium* cultures were isolated from sugar beet. Of these isolates, 36 were identified as *F. oxysporum* and tested for pathogenicity. Eight isolates were pathogenic to sugar beet (data not shown). Twenty isolates of other *Fusarium* species were also screened and one isolate each of four, *F. acuminatum*, *F. avenaceum*, *F. solani* and *F. verticillioides* caused fusarium yellows-like symptoms on sugar beet (Hanson & Hill, 2004). In 2002 and 2003, five *F. oxysporum* isolates collected from spinach were tested, and all were pathogenic on sugar beet seedlings (Table 1).

Phylogenetic analysis

Phylogenetic trees from parsimony bootstrap analysis of individual datasets (β-tubulin, ITS and EF1α) showed progressively increasing resolution. ITS had the least resolution and EF1α the most resolution (Fig. 1). All three datasets consisted of three large groups of isolates (A–C) and three small groups (D–F) (Fig. 1). Parsimony analysis of the combined dataset revealed the same major and minor groups as the individual data sets (Fig. 2a, right tree). Group C is resolved into smaller groups as with EF1α alone (Figs 1 & 2a). As with β-tubulin and EF1α, groups D, E and F were found at the base of the tree (Figs 1 & 2a). Support values for the groups were comparable to the values for the individual datasets. In spite of the overall similarities in the tree structure between the combined analysis and the individual analyses, the partition homogeneity test indicated that the individual datasets held incongruent phylogenetic information and therefore

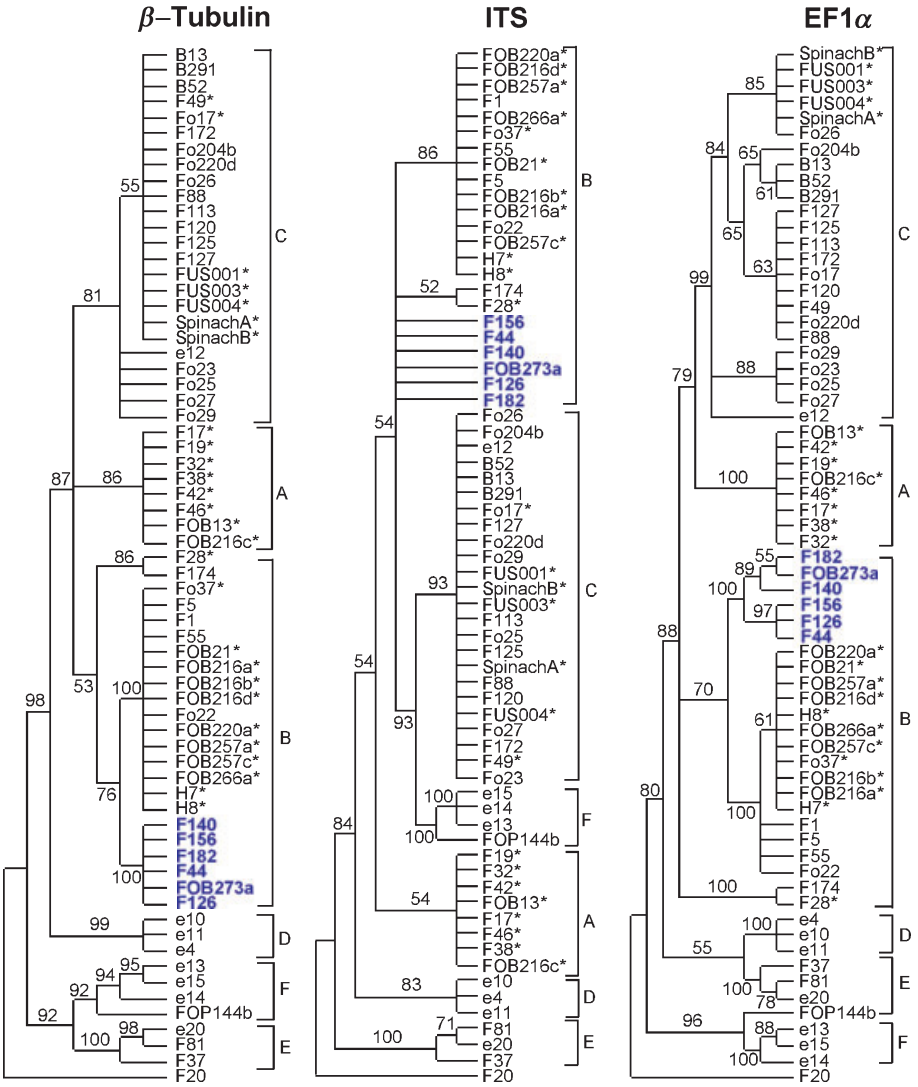

Figure 1 Parsimony bootstrap consensus trees for clades A–F β-tubulin, ITS and EF1α sequences from both pathogenic and nonpathogenic *Fusarium oxysporum* cultured from sugar beet, dry bean and spinach, and isolates of *F. proliferatum* and *F. solani*. Isolates labelled *are considered pathogenic on sugar beet. *Fusarium proliferatum* isolates are in bold (blue). An isolate of *F. avenaceum* was used as the outgroup. Branches with bootstrap support values below 50% have been collapsed.

should not be combined under the parsimony criteria. However, Cunningham (1997) found that under certain criterion, data sets that were found to be incongruent could be analyzed as a combined dataset and still improve phylogenetic accuracy.

Because of the incongruent results in the parsimony test, Bayesian MCMC phylogenetic analysis (which does not require homogeneity of data partitions) of the combined dataset was performed (Fig. 2b). The consensus tree from this analysis resulted in the same groupings, A–F, as all parsimony analyses and had similar topology with the parsimony consensus tree of the combined dataset (Fig. 2a). Clade A contained Fob isolates from Oregon and one Fob from Colorado (FOB216c). Clade B contained nonpathogenic *F. oxysporum* from Texas (F5, F1 and F55) and Minnesota (Fo22); pathogenic *F. oxysporum* from Colorado (FOB216a, FOB216b, FOB216d, FOB257a, FOB257c, FOB220a and FOB266a) and Montana (FOB21, H7 and H8); and a pathogenic *F. oxysporum* from Minnesota (Fo37) (Fig. 2b). Within this group, a pathogenic *F. oxysporum* from Colorado (F28) and a nonpathogenic *F. oxysporum* from California (F174) cluster closely together.

Within clade C, groups were resolved like the parsimony tree from EF1α (Figs 1 & 2b). In clade C, *F. oxysporum* from dry bean grouped with nonpathogenic Fo204b from sugar beet (support value of 98); the five pathogenic spinach *F. oxysporum* isolates and nonpathogenic Fo26 from sugar beet grouped with a support value of 100%; and pathogenic *F. oxysporum* isolates F49 and Fo17 grouped with the nonpathogenic *F. oxysporum* from sugar beet with 88% support (Fig. 2b).

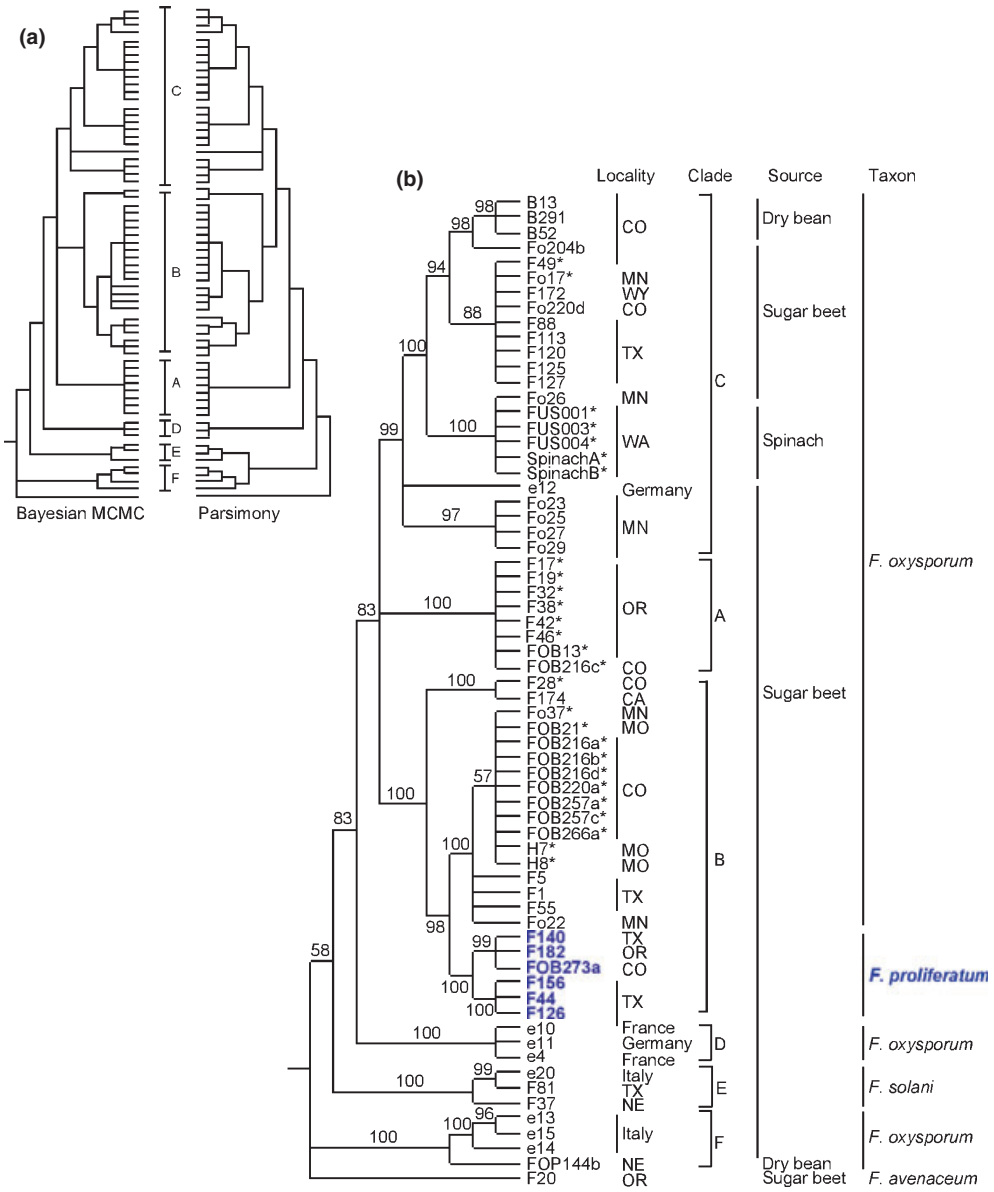

Figure 2 (a) Comparison of the consensus trees from both the Bayesian MCMC (left tree) and parsimony (right tree) analyses of the combined dataset (β-tubulin, ITS and EF1α). Both analyses resulted in the same clades (A–F) and had similar topology; (b) Bayesian analysis of the combined dataset from β-tubulin, ITS and EF1α for both pathogenic and nonpathogenic *Fusarium oxysporum* cultured from sugar beet, dry bean and spinach, and isolates of *F. proliferatum* and *F. solani*. Isolates labelled * are considered pathogenic on sugar beet. *Fusarium proliferatum* isolates are in bold (blue). An isolate of *F. avenaceum* was used as the outgroup. Bayesian posterior-probabilities (×100) are indicated at internodes. Branches and support values below 50 have been collapsed.

Groups D (European *F. oxysporum* isolates e4, e10 and e11), E (*F. solani* isolates e20, F81 and F37), and F (European *F. oxysporum* isolates e13, e14, e15 and dry bean isolate, FOP144b) fall outside of the three large groups (A, B and C in Fig. 2b).

The *F. proliferatum* isolates form a group within clade B (100% support), and are resolved into two sub groups: F140, FOB273a and F182 (99% support); F156, F44 and F126 (100% support). The *F. proliferatum* and *F. solani* (clade E) isolates form groups separated phylogenetically from the *F. oxysporum* isolates in all analyses, although the group formed by the *F. proliferatum* isolates falls within a large clade of *F. oxysporum* (clade B – Figs 1 & 2a,b).

Discussion

The hypotheses that phylogenetic relationships among *F. oxysporum* isolates from sugar beet would correlate with pathogenicity and/or geographic origin was not

supported. Clade A consisted primarily of pathogenic isolates from Oregon but also contained a single pathogenic *F. oxysporum* from Colorado. Phylogenetic analyses of β- tubulin, EF1α and ITS sequences consistently revealed three major and minor groups of isolates, most of which contained at least one *F. oxysporum* sequence from sugar beet. Parsimony analysis of EF1α and the combined dataset analyses by parsimony and Bayesian MCMC resulted in the most resolved clades with the highest support values. The pathogens and nonpathogens in clade B could be separated, but only when EF1α sequences or the combined dataset was analyzed (Figs 1 & 2b). In addition, four nonpathogenic isolates from Minnesota (group C) cluster within the β-tubulin, EF1α analyses, and with analysis of the combined dataset. This might be explained by the small number of isolates sampled from these locations. The pool of isolates should be expanded in subsequent studies to include more locations and similar numbers of isolates from each location, to allow for a more accurate picture of associations between pathogenicity and/or geographic origin and phylogeny.

The three *F. solani* isolates evaluated form a clade which falls outside of the major clades of *F. oxysporum* isolates in all analyses. The *F. proliferatum* isolates also grouped together but are located within the major clades of *F. oxysporum* isolates (Fig. 2b). The identification of *F. proliferatum* isolates by morphology was supported by the results of the sequence BLASTn analysis. Nonetheless, the phylogenetic analysis shows that some isolates of *F. oxysporum* are more closely related to the *F. proliferatum* isolates than they are other isolates of Fob. It has been shown that the *Fusarium oxysporum* complex is phylogenetically diverse and the 'formae speciales' are not always correlated with phylogenetic analyses (Baayen *et al.*, 2000).

There were three isolates described as tip rotting in the study (from Harveson & Rush, 1998), F44, F127 and F156. None of them were pathogenic here, and the isolate labelled F156 was re-identified as *F. proliferatum*. Because the isolates included did not show the expected phenotype, their classification is uncertain. This is an area that would benefit from a study of a larger number of isolates with a well-defined tip rot phenotype.

Research using conserved gene regions has indicated that some *F. oxysporum* 'formae speciales' are monophyletic (Jiménez-Gasco *et al.*, 2002), while other studies have identified polyphyletic groups (Koenig *et al.*, 1997). The identification in this study of *F. oxysporum* isolates from sugar beet being located in clades with, or closely related to, *F. oxysporum* isolates from dry bean and spinach or other *Fusarium* species, suggests that *F. oxysporum* from sugar beet is most likely polyphyletic.

Another potential cause of the variability among *F. oxysporum* cultured from sugar beet is the broad host range of this species. Isolates of *F. oxysporum* f. sp. *betae* and *spinaceae* were cross-pathogenic on other hosts (Armstrong & Armstrong, 1976; MacDonald & Leach, 1976). An isolate of *F. oxysporum* f. sp. *phaseoli* caused disease on sugar beet, while three isolates of *F. oxysporum* f. sp. *betae* were pathogenic to dry bean (Wickliffe, 2001). The phylogenetic analyses here show that *F. oxysporum* from dry bean and spinach consistently clustered with *F. oxysporum* from sugar beet. Evidence for cross-pathogenicity also has been observed in greenhouse studies where the five *F. oxysporum* isolates from spinach used in this study were pathogenic on sugar beet germplasm FC716 (unpublished data).

Genetic resistance is the primary means of controlling fusarium yellows (Hanson & Jacobsen, 2009). Although genetic resistance may provide control in some localities, growers have reported that control of fusarium yellows may be lost when supposedly resistant sugar beet cultivars are grown in different parts of the country (S. Godby, Western Sugar Cooperative, 2100 E. Overland Drive, Scottsbluff, NE 69361, USA, personal communication). Variability in resistance in different geographic areas could indicate races within Fob. Races occur in a number of 'formae speciales' of *F. oxysporum* (Gordon & Martyn, 1997; Jiménez-Gasco *et al.*, 2002); however, they have not been reported within Fob. The only proposed means of identifying races in Fob has been based on cross-pathogenicity between isolates from sugar beet and spinach. Armstrong & Armstrong (1976) suggested that Fob and *F. oxysporum* f. sp. *spinaciae* (Fos) be combined into Fos, with the designation of two races based on host specificity. Another possibility to consider is that environmental conditions, both in the field or greenhouse, may contribute variability in pathogenicity response. It has been observed that temperature, plant age and inoculum amount can influence the scoring of pathogenicity of Fob isolates.

Ongoing greenhouse tests suggest that a number of sugar beet isolates (FOB220a, F19, FOB13, Fo37, H7 and FOB21) cause a differential response on several commercial sugar beet cultivars. While some cultivars show a broad spectrum resistance to most Fob isolates tested, and others show susceptibility to most isolates, a number of cultivars vary significantly in their response to different isolates (Hanson *et al.*, 2009). This evidence suggests that *F. oxysporum* f. sp. *betae* might exhibit cultivar specific pathogenicity, and therefore might be divided into races. However, Ruppel (1991) also found a large portion of *F. oxysporum* isolated from beet were nonpathogenic when tested on numerous sugar beet cultivars. Taking this into consideration, because isolates used in this study were only tested on one sugar beet cultivar, some isolates could be erroneously classified as nonpathogenic. By testing the isolates on multiple cultivars it could be determined if isolates group by pathogenicity in the phylogenetic trees, but at the level of races, which would not have been apparent under the current experimental conditions.

The polyphyletic nature of *F. oxysporum* isolates from sugar beet and the cross pathogenicity of some FOB to other crops will make the resistance breeding efforts more difficult. There will potentially be more diverse pathogenicity factors involved, making it essential to pyramid resistance to account for the potential races so that the

isolates do not overcome the resistance easily. There is a need by plant breeders to understand whether the resistance source they are working with confers resistance to a race of Fob or provides a durable, horizontal resistance to all or most Fob isolates. This information will be needed to determine effective resistance breeding strategies and the best strategies to screen germplasm in the greenhouse or field.

Inoculum levels of *F. oxysporum* can build up in the soil due to the wide host range of Fob and the fact that sugar beet is a good symptomless host for numerous nonpathogenic isolates (Armstrong & Armstrong, 1976; Ruppel, 1991; Hanson & Hill, 2004). It is speculated that this environment allows such locally adapted genotypes, which outnumber pathogenic isolates (Ruppel, 1991; Hanson & Hill, 2004), to develop pathogenicity overtime. The evidence is consistent with this hypothesis as shown by the presence of closely related nonpathogenic and pathogenic isolates from different geographic locations within the same clades.

This study represents one of the first molecular phylogenetic studies of *F. oxysporum* from sugar beet as compared to Nitschke *et al.* (2009), which compared multiple species using only EF1α. This work emphasizes the importance of developing genetic markers to gain a better understanding of the evolutionary structure of Fob. Future objectives should include examining sequence data from isolates from other states and those causing different symptoms on sugar beet such as root rot (Martyn *et al.*, 1989). Single nucleotide polymorphisms (SNPs) could be used to develop markers for identifying pathogenic *F. oxysporum* isolates and possibly used to predict the degree of pathogenicity of *F. oxysporum* isolates on sugar beet. Also of interest is further examination of the host range for *F. oxysporum* f. sp. *betae*. Efficacy of some resistant cultivars varies by geographic location, which may be a result of race-specific interactions between sugar beet and Fob. Further development of genetic markers associated with host specific pathogenicity (virulence) will allow plant breeders to select for sources of resistance and produce varieties for growers based on the race(s) of *Fusarium* in their field. It would also be interesting to examine the sequences amplified from β-tubulin, EF1α and ITS from clades containing strictly pathogenic or nonpathogenic *F. oxysporum* isolates, to determine if there are SNPs associated with the phenotype.

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