Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* Inferred from Mitochondrial Small Subunit, Elongation Factor 1-α, and Nuclear Ribosomal Intergenic Spacer Sequence Data

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ABSTRACT

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Fusarium oxysporum f. sp. *lactucae*, causal agent of Fusarium wilt of lettuce, is a serious pathogen recently reported in Arizona. Sequence analysis of the mitochondrial small subunit (mtSSU), translation elongation factor 1-α (EF-1α) gene, and the nuclear ribosomal DNA intergenic spacer (IGS) region was conducted to resolve relationships among f. sp. *lactucae* isolates, *F. oxysporum* isolates from other hosts, and local nonpathogenic isolates. Analysis of mtSSU sequences provided limited phylogenetic resolution and did not differentiate the *lactucae* isolates from 13 other *F. oxysporum* isolates. Analysis of EF-1α sequences resulted in moderate resolution, grouping seven formae speciales with the *lactucae* isolates. Analysis of the IGS region revealed numerous sequence polymorphisms among *F. oxysporum* formae speciales consisting of insertions, deletions, and single nucleotide transitions and substitutions. Repeat sequence analysis revealed several duplicated subrepeat units that were distributed across much of the region. Based on analysis of the IGS sequence data, *lactucae* race 1 isolates resolved as a monophyletic group with three other formae speciales of *F. oxysporum*. In all analyses, *lactucae* race 2 isolates composed a separate lineage that was phylogenetically distinct and distantly related to the *lactucae* race 1 isolates.

Fusarium wilt of lettuce was first reported in 1967 when it was responsible for significant losses in lettuce in Japan. The causal organism was determined to be *Fusarium oxysporum* f. sp. *lactucae* Schlechtend.:Fr. Matuo and Motohashi (22) and pathogenicity on lettuce was confirmed. In the United States, the disease was first reported in the San Joaquin valley of California in 1993, where it was responsible for lettuce losses in three fields near the town of Huron (18). The disease subsequently was reported in Arizona in 2001 in 5 fields near the town of Yuma and in 11 additional fields during the 2002–03 cropping season (21). The occurrence of the disease in both Arizona and California is particularly serious due to its damaging nature and the importance of lettuce production to both agricultural economies. Worldwide, the disease has been reported in three other countries: Taiwan (1996), Iran (1999), and Italy (2001) (12,17,25).

Strains of *F. oxysporum* exhibit considerable physiological variation. The fungus is found worldwide and commonly is isolated from nearly all soil types. Both pathogenic and nonpathogenic forms are able to colonize many plants and crop residues, as well as being capable of rapidly recolonizing fumigated soils (14). Pathogenic forms often display a high degree of host specificity, and numerous formae speciales have been described. Within each forma specialis, races have been identified based on the differential host cultivars on which the races cause disease (4). Regarding the f. sp. *lactucae*, three races have been identified to date with races 1, 2, and, most recently, 3 present in Japan (10). Only race 1 has been reported in the United States (10,23).

Distinguishing formae speciales in the *F. oxysporum* spp. complex most often requires pathogenicity tests. Although isolates within a forma specialis are related by their pathogenicity to a given host, genetic heterogeneity within certain formae speciales often has revealed a polyphyletic origin (14). This heterogeneity has important implications on the evolution of pathogenic forms within the species, and on the development of resistant host cultivars. Therefore, it is critical to be able to quickly and accurately differentiate genetic diversity. Many molecular methods have been used to differentiate between nonpathogenic and pathogenic forms of *F. oxysporum*. Mitochondrial haplotype analysis and sequence analysis of the mitochondrial small subunit (mtSSU), translation elongation factor 1-α (EF-1α) gene, and nuclear ribosomal (r)DNA intergenic spacer (IGS) region have been used to determine diversity within certain formae speciales such as *melonis*, *cucumerinum*, *vasinfectum*, *cubense*, and *lycopersici* (3,27,33). The IGS region contains greater sequence variation than the other genomic regions and, thus, may have greater utility in phylogenetic analyses. However, analyses to date have been limited to restriction fragment length polymorphisms or partial sequences, or have focused on human pathogens (2,3,11,28).

The objective of the present study was to determine phylogenetic relationships among *F. oxysporum* f. sp. *lactucae* isolates, native nonpathogenic *F. oxysporum* isolates from Arizona, and selected formae speciales based on mtSSU, EF-1α, and full-length IGS sequences. These data will be valuable in understanding the origin and genetic diversity of the pathogen in Arizona, and in the identification of pathogen-specific molecular markers that may be used in the development of DNA-based methods of detection.

MATERIALS AND METHODS

Fungal isolates. Sources of the isolates used in this study are listed in Table 1. *F. oxysporum* isolates from Arizona were re-

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covered from infected lettuce tissue and from field soil using standard phytopathological isolation techniques and standard soil dilution techniques, respectively. For recovery from lettuce, tissue was surfaced sterilized in 10% NaOCl and 5-mm^3 pieces were cut from the advancing margins of the disease symptom and plated on Komada's medium (19). Putative *F. oxysporum* colonies were subcultured on identification media (Komada's, potato dextrose agar [PDA, Difco brand; Becton, Dickinson and Co., Franklin Lakes, NJ], and 0.5% KCl medium) (6) and their identity was confirmed by morphological characterization. For recovery from soil, 10 g of each soil sample ($\approx$200 to 300 g total for each field soil sample) were suspended in 200 ml of 1% sodium hexametaphosphate and stirred for 8 min. The suspension (20 ml) was added to 80 ml of 0.1% water agar and stirred for 5 min. Five plates of Komada's selective medium each were inoculated with 1 ml of the suspension per soil sample. Following 7 days of incubation at 25°C, putative *F. oxysporum* colonies then were isolated in pure cultures and identification was performed as described previously.

Also included were f. sp. *lactucae* race 1 isolates from California (HL1 [ATTC 76616] and HL2) and race 1 and race 2 isolates from Japan (S-1, and FK09701 and FK9501, respectively). Additional formae speciales of *F. oxysporum* were selected based on their pathogenicity on host species belonging to the same plant family as lettuce (Asteraceae) as well as those from phylogenetically diverse plant families.

Pathogenicity tests. Putative *F. oxysporum* f. sp. *lactucae* isolated from diseased lettuce tissue and field soil, and isolates obtained from collaborators in California and Japan, were tested for pathogenicity on three major commercial lettuce cultivars (Beacon, Lighthouse, and Winterhaven). The roots of 10-day-old seedlings were washed in running tap water and $\approx$1 cm of root tip

TABLE 1. Isolates used in this study and associated sequences with GenBank accession number noted[v]

Strain[w]	Species name[x]	Geographic origin[y]	GenBank accession[z]		
			mtSSU	EF-1α	IGS
BMP1300	*Fusarium oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831907	DQ837658	DQ831864
BMP1301	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831908	DQ837659	DQ831865
BMP1306	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831909	DQ837660	DQ831866
BMP1307	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831910	DQ837661	DQ831867
BMP1308	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831911	DQ837662	DQ831868
BMP1323	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831912	DQ837663	DQ831869
BMP1324	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831913	DQ837664	DQ831870
BMP1326	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831914	DQ837665	DQ831871
BMP1331	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831915	DQ837666	DQ831872
BMP1333	*F. oxysporum* f. sp. *lactucae*[p]	Wellton, AZ	DQ831916	DQ837667	DQ831873
HL-1	*F. oxysporum* f. sp. *lactucae*[p]	Fresno, CA	DQ831918	DQ837668	DQ831874
HL-2	*F. oxysporum* f. sp. *lactucae*[p]	Fresno, CA	DQ831919	DQ837669	DQ831875
BMP1880	*F. oxysporum* f. sp. *lactucae*[p] (soil)	Wellton, AZ	DQ831917	DQ837670	DQ831879
BMP1363	*F. oxysporum* f. sp. *lactucae*[p] (soil)	Wellton, AZ	DQ831921	DQ837671	DQ831876
BMP1370	*F. oxysporum* f. sp. *lactucae*[p] (soil)	Wellton, AZ	DQ831922	DQ837672	DQ831877
BMP1375	*F. oxysporum* f. sp. *lactucae*[p] (soil)	Wellton, AZ	DQ831920	DQ837673	DQ831878
BMP1389	*F. oxysporum*[np] (soil)	Yuma, AZ	DQ831926	DQ837676	DQ831881
BMP1397	*F. oxysporum*[np] (soil)	Yuma, AZ	DQ831924	DQ837677	DQ831882
BMP1388	*F. oxysporum*[np] (soil)	Yuma, AZ	DQ831925	DQ837675	DQ831883
BMP1385	*F. oxysporum*[np] (soil)	Wellton, AZ	DQ831923	DQ837674	DQ831880
S-1	*F. oxysporum* f. sp. *lactucae*[p]	Japan	DQ831906	DQ837657	DQ831863
F9501	*F. oxysporum* f. sp. *lactucae*[p]	Japan	DQ831944	DQ837693	DQ831893
FK09701	*F. oxysporum* f. sp. *lactucae*[p]	Japan	DQ831945	DQ837694	DQ831892
FOV14	*F. oxysporum* f. sp. *vasinfectum*[nd]	California	DQ831942	DQ837695	DQ831885
TX388	*F. oxysporum* f. sp. *melonis*[nd]	Texas	DQ831943	DQ837696	DQ831887
FOLR2	*F. oxysporum* f. sp. *lycopersici*[nd]	California	DQ831941	DQ837692	DQ831894
FOA50	*F. oxysporum* f. sp. *asparagi*[nd]	Australia	DQ831940	DQ837691	DQ831886
NRRL22536	*F. oxysporum* f. sp. *callistephi*[nd]	Germany	DQ831928	DQ837679	DQ831897
NRRL22535	*F. oxysporum* f. sp. *batatas*[nd]	Germany	DQ831927	DQ837678	DQ831895
NRRL26871	*F. oxysporum* f. sp. *spinaciae*[nd]	Japan	DQ831938	DQ837687	DQ831888
NRRL26445	*F. oxysporum* f. sp. *phaseoli*[nd]	South Carolina	DQ831934	DQ837686	DQ831900
NRRL28934	*F. oxysporum* f. sp. *opuntiarum*[nd]	Netherlands	DQ831939	DQ837689	DQ831884
NRRL22546	*F. oxysporum* f. sp. *medicaginis*[nd]	SE Asia	DQ831937	DQ837690	DQ831901
NRRL22538	*F. oxysporum* f. sp. *cepae*[nd]	Germany	DQ831929	DQ837681	DQ831891
NRRL25231	*F. oxysporum* f. sp. *vasinfectum*[nd]	North Carolina	DQ831931	DQ837680	DQ831896
NRRL26412	*F. oxysporum* f. sp. *heliotropa*[nd]	Maryland	DQ831933	DQ837685	DQ831903
NRRL26411	*F. oxysporum* f. sp. *fabae*[nd]	Maryland	DQ831936	DQ837684	DQ831902
NRRL26227	*F. oxysporum* f. sp. *rhois*[np]	Israel	DQ831932	DQ837683	DQ831898
NRRL22545	*F. oxysporum* f. sp. *matthiolae*[nd]	Germany	DQ831930	DQ837682	DQ831899
NRRL22550	*F. oxysporum* f. sp. *perniciosum*[nd]	N/A	**AF008472**	**AF008506**	ARS
NRRL26035	*F. oxysporum* f. sp. *canariensis*[nd]	N/A	**AF008451**	**AF008485**	ARS
NRRL26029	*F. oxysporum* f. sp. *cubense*[nd]	N/A	**AF008459**	**AF008493**	ARS
NRRL28378	*F. commune*[nd]	Netherlands	AF250560	AF246832	ARS
BMP1462	*F. subglutinans*[nd]	Wellton, AZ	DQ831946	DQ837698	DQ831904

[v] All mitochondrial small subunit (mtSSU), elongation factor 1-α (EF-1α), and intergenic spacer (IGS) sequences were determined in this study, with the exception of those with accession numbers in bold.

[w] All strains preceded by BMP are from B. M. Pryor, Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson; all strains preceded by NRRL are from K. O'Donnell (ARS culture collection), National Center for Agricultural Utilization Research, Peoria; FOA50 is from E. Nigh, division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson; and all other strains are from T. Gordon, Department of Plant Pathology, University of California, Davis.

[x] Superscripts: p = pathogenic on lettuce, np = not pathogenic on lettuce, and nd = pathogenicity on lettuce not determined.

[y] N/A = geographic origin of the strain is not available. The *lactucae* isolates recovered from soil are noted (soil).

[z] ARS = IGS sequences of these isolates were obtained through courtesy of Kerry O'Donnell.

was removed. The wounded roots were dipped in a spore suspension containing 1×10^6 spores/ml for 10 min. For each fungal isolate–lettuce cultivar combination, eight inoculated seedlings were transplanted to 20-cm-wide plastic pots containing a sterile mix of one part peat, one part vermiculite, and two parts sand (1:1:2). The experiment was set up in the greenhouse (24 to 29°C, relative humidity = 50 to 60%) in a completely randomized design. Each plant was rated for disease 20 days after inoculation using a scale of 0 to 3, where 0 = no disease symptoms, 1 = plant stunted compared with control, 2 = plant severely stunted, and 3 = plant dead. Disease incidence was calculated for each isolate–cultivar combination. In addition, a disease index was calculated for each isolate–cultivar combination using the formula $(n_1) + (n_2 \times 2) + (n_3 \times 3)/(n_0 + n_1 + n_2 + n_3)$, where n_0 is the number of plants in the disease category 0, n_1 is the number of plants in the disease category 1, n_2 = number of plants in the disease category 2, and n_3 represents the number of plants in the disease category 3 (10). Disease index data was analyzed by one-way analysis of variance (SigmaStat, ver. 3.1; Jandel Scientific, San Rafael, CA) to determine differences in virulence among isolates on each cultivar. The experiment was repeated once.

DNA isolation and sequence amplification. Five-day-old cultures of the test fungi grown on PDA were flooded with 5 ml of sterile water and the surface of the culture was scrapped with a spatula to obtain spores and mycelia in suspension. The spore or mycelia suspension (2 ml) was used to inoculate 100 ml of liquid

TABLE 2. Primers pairs for genomic regions amplified and sequenced in this study

Locus[z]	Primer sequence	Reference
Mitochondrial small subunit		
NMS1[a]	5′-CAGCAGTGAGGAATATTGGTCAATG-3′	Li et al. 1994 (20)
NMS2[b]	5′-GCGGATCATCGAATTAAATAACAT-3′	Li et al. 1994 (20)
Translation elongation factor 1-α		
EF-1H[a]	5′-ATGGGTAAGGAAGACAAGAC-3′	O'Donnell et al. 1998 (27)
EF-2T[b]	5′-GGAAGTACCAGTGATCATGTT-3′	O'Donnell et al. 1998 (27)
Intergenic spacer region		
CNL12[a]	5′-CTGAACGCCTCTAAGTCAG-3′	Anderson and Stasovski 1992 (1)
CNS1[b]	5′-GAGACAAGCATATGACTACTG-3′	White et al. 1990 (35)
U46.67[b]	5′-AATACAAGCACGCCGACAC-3′	Appel and Gordon 1996 (2)
RU46.67[a]	5′-GTGTCGGCGTGCTTGTATT-3′	This study
CN61[a]	5′-GGTTCAATTTGATGTCGGCT-3′	This study
RCN61[b]	5′-AGCCGACATCAAATTGACC-3′	This study
RU3[a]	5′-GTGTGAAATTGGAAAGTCGG-3′	This study
RRU3[b]	5′-CCGACTTTCCAATTTCACAC-3′	This study
CN34[b]	5′-CCAACACATGGGTGGTACCG-3′	This study
IGSF4[a]	5′-CCAGACTTCCACTGCGTGTC-3′	This study
CNS12[b]	5′-GCACGCCAGGACTGCCTCGT-3′	This study

[z] Superscripts: a = forward primers and b = reverse primers.
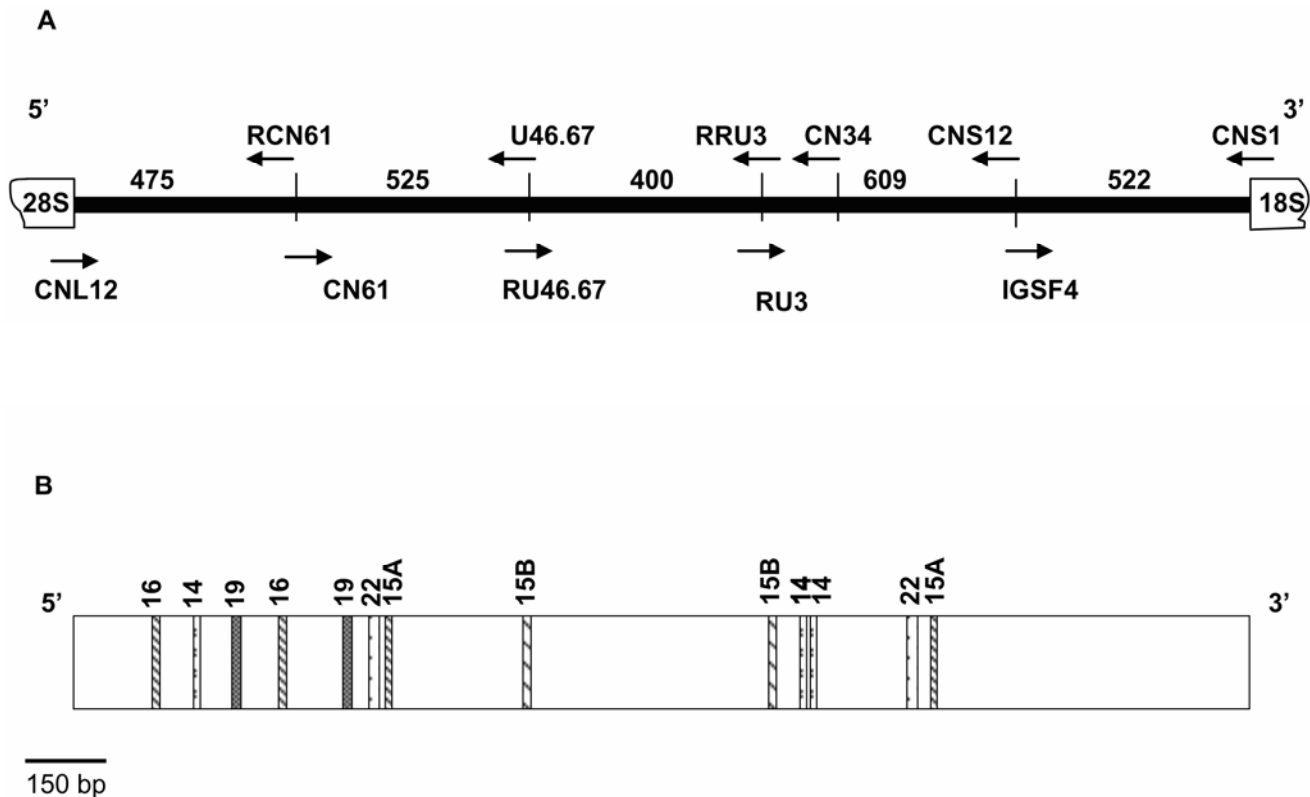

Fig. 1. A, Diagrammatic representation of the ribosomal DNA intergenic spacer (IGS) region within one tandem repeat. Except for primers CNL12, CNS1, and U47.67, all other primers were designed in this study. The numbers on the figure is the estimated size in base pairs of the amplification product that could be obtained using each flanking primer set. **B,** Pattern of distribution of repeat sequences within the IGS of *Fusarium oxysporum* f. sp. *lactucae* using the program DNA strider allowing for no mismatch of base pairs and no overlap of repeat sequence.

growth medium (1.2 g of DL-asparagine, 0.2 g of NaCl, 1.2 g of $K_2HPO_4\cdot3H_2O$, 0.25 g of $MgSO_4$, 0.5 g of yeast extract, and 20.7 g of D-glucose in 1 liter of water) and was incubated at room temperature for 5 days on a rotary shaker (120 rpm). The mycelia were harvested by filtration through miracloth (Calbiochem; EMD Biosciences Inc., San Diego, CA), frozen, and lyophilized. Total DNA was prepared from a sample of 50 mg of lyophilized mycelia using the BIO 101 Fast DNA kit (Qbiogene, Irvine, CA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed to amplify the mtSSU, EF-1α gene, and IGS rDNA using specific primers (Table 2). Each PCR reaction contained 0.2 mM deoxynucleotide triphosphates, 2.5 mM Amplitaq $MgCl_2$, 0.8 μM each primer, 1× Amplitaq reaction buffer, and 2 units of Amplitaq DNA polymerase (Applied Biosciences, Foster City, CA). PCR reactions were carried out in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) using the following steps for 35 cycles: 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min. For primers U46.67, RU46.67, and RRU3, the PCR annealing temperature was set at 63°C. The amplified products were purified with the aid of a QIAquick PCR purification kit (Qiagen Inc., Stanford, CA). The cleaned PCR products were sequenced at the DNA Sequencing Facility, University of Arizona. Due to its large size (≈2,600 bp), the complete sequence of the IGS region required the design of additional internal primers, and sequencing was performed in both directions to confirm the nucleotide sequence (Table 2). The map of the amplification and sequencing primers for the IGS region are presented in Figure 1A. All IGS as well as the mtSSU and EF-1α sequences have been submitted to GenBank (Table 1) and their alignments have been submitted to TreeBASE (SN2653-10318).

TABLE 3. Disease incidence and disease index of *Fusarium oxysporum* isolates on three iceberg lettuce cultivars (Beacon, Lighthouse, and Winterhaven) commonly used in Arizona

| Isolate | Disease incidence (%), disease index[y] | | | Path.[z] |
	Beacon	Lighthouse	Winterhaven	
HL2	100, 2.6 ab	100, 3.0 a	100, 2.8 a	P
HL1	100, 2.6 ab	100, 2.6 ab	100, 2.6 a	P
JCP007	100, 3.0 a	100, 2.6 ab	100, 2.6 a	P
BMP1323	100, 2.8 ab	100, 2.9 a	100, 2.6 a	P
BMP1324	100, 3.0 a	87.5, 1.8 b	100, 2.5 a	P
BMP1326	100, 3.0 a	100, 2.5 ab	100, 2.6 a	P
BMP1331	100, 2.9 a	100, 2.8 a	100, 2.9 a	P
BMP1300	100, 2.2 b	100, 2.2 ab	100, 2.6 a	P
BMP1301	100, 2.6 ab	100, 2.6 ab	100, 2.6 a	P
BMP1306	100, 2.5 ab	100, 2.9 a	100, 2.8 a	P
BMP1308	100, 2.5 ab	100, 2.6 ab	100, 3.0 a	P
BMP1307	100, 3.0 a	100, 3.0 a	100, 2.5 a	P
BMP1333	100, 3.0 a	100, 2.1 ab	100, 3.0 a	P
BMP1880	100, 3.0 a	100, 3.0 a	100, 3.0 a	P
BMP1375	100, 3.0 a	100, 3.0 a	100, 3.0 a	P
BMP1363	100, 2.8 ab	100, 3.0 a	100, 3.0 a	P
BMP1370	100, 2.9 a	100, 3.0 a	100, 3.0 a	P
F9501	100, 3.0 a	100, 2.9 a	100, 3.0 a	P
FK9701	100, 2.9 a	100, 2.9 a	100, 3.0 a	P
S-1	100, 3.0 a	100, 2.9 a	100, 2.6 a	P
BMP1389	0, 0	0, 0	0, 0	NP
BMP1397	0, 0	0, 0	0, 0	NP
BMP1388	0, 0	0, 0	0, 0	NP
BMP1385	0, 0	0, 0	0, 0	NP
NRRL26227	0, 0	0, 0	0, 0	NP
0.5% KCl	0, 0	0, 0	0, 0	NP

[y] Each treatment consisted of eight inoculated plants per cultivar. The disease incidence/severity was assessed with a 0-to-3 scale where 0 = no disease, 1 = plants stunted, 2 = plants severely stunted, and 3 = plants dead. The disease index was calculated using the formula $(n_1) + (n_2 \times 2) + (n_3 \times 3)/(n_0 + n_1 + n_2 + n_3)$, where n_0, n_1, n_2, and n_3 represent the number of plants with scores of 0, 1, 2, and 3, respectively. Disease index values followed by a different letter were significantly different ($P \leq 0.05$) based upon Tukey's test.

[z] Path. = determination of pathogenicity: P = pathogenic and NP = nonpathogenic.

Phylogenetic analysis. The sequences were aligned using the PILEUP program of the GCG Sequence Analysis Software Package (ver. 10.2; Accelrys, Inc., San Diego, CA). Manual adjustments of sequence alignments were performed with the aid of the data editor program of MacClade phylogenetic software (ver. 4.0; Sinauer Associates, Inc., Sunderland, MA). Phylogenetic analyses were performed using the Phylogenetic Analysis Using Parsimony package (PAUP, ver. 4.0b10; Sinauer Associates). Heuristic searches for the most-parsimonious trees were conducted with random stepwise addition (1,000 replications) and branch swapping algorithm using tree bisection-reconnection (TBR). For the parsimony criterion, analysis of data sets were performed with gaps set as either missing data or as a fifth state. For each analysis, clade stability was assessed using 1,000 bootstrap replications. For maximum likelihood analysis, heuristic searches were conducted using best fit models selected by Akaike Information Criterion using Modeltest (ver. 3.07). Clade stability was assessed using 500 bootstrap replicates with rearrangements limited to 10,000 per replicate to reduce computational time. Two *Fusarium* spp., *F. subglutinans* and *F. commune*, were used as outgroups in all analyses. Analysis of repetitive sequence subunits found in the IGS was performed using the program DNA Strider (ver. 1.3f16; Institute for Marine Biosciences, National Research Council, Halifax, NS, Canada).

Combined data analysis. The data sets were concatenated and partitioned by genetic locus. The Mickevich-Farris incongruence metric (I_{MF}) was used to measure the total amount of incongruence between data partitions (24). The partition-homogeneity test (P-H, PAUP ver. 4.0b10; Sinauer Associates) was used to obtain P values corresponding to each I_{MF} index to determine data partition combinability. The data partitions were analyzed pairwise and in total using maximum parsimony and maximum-likelihood criterion as described above.

Test of hypothesis. The parsimony criterion (9) also was used to test congruency of data sets against hypotheses for groupings drawn from results from mtSSU, EF-1α, and IGS sequence data. For each data set, a strict consensus tree was generated and used as a topological hypothesis. Heuristic searches were performed on alternative data sets under the constraint of each hypothesis using TBR branch swapping and random sequence addition (1,000 reps) algorithms applied in PAUP. All equally most parsimonious trees generated from each tree reconstruction condition were compared with the best trees generated without any constraint by Kishino-Hasegawa (KH), Templeton, and Winning-sites (WS) tests implemented in PAUP. The KH test (13,15) estimates the standard error and confidence intervals for the difference in log-likelihoods between two topologically distinct phylogenetic trees representing hypotheses that might explain particular aligned sequence data sets. The Templeton test under the maximum parsimony criterion evaluates the topological congruence between trees produced from unconstrained searches and trees obtained in constrained monophyly of particular groupings (16). The WS test uses a sign-rank test with a one-tail binomial probability to determine significant differences among pairwise comparisons of alternative hypotheses (32). Thus, the results of the three tests should give similar interpretation of the data under study.

RESULTS

Pathogenicity tests. All *F. oxysporum* isolates recovered from diseased lettuce roots ($n = 43$) and those supplied by collaborators (race 1 and race 2) were pathogenic on all of the three lettuce cultivars used and were confirmed to be *F. oxysporum* f. sp. *lactucae* (Table 1). Among the *F. oxysporum* isolates recovered from soil in Arizona ($n = 38$), ≈65% were pathogenic on all three lettuce cultivars used and were confirmed to be *F. oxysporum* f. sp. *lactucae*. For each isolate–cultivar combination, disease incidence was generally 0 or 100% (Table 3). Thus, the determination of pathogeni-

city was unambiguous and the utility of the three cultivars for confirmation of pathogenicity for f. sp. *lactucae* race 1 and race 2 isolates was evident. The disease index values for each isolates confirmed as f. sp. *lactucae* ranged from 1.8 to 3.0, depending on pairing of isolate and lettuce cultivar (Table 3). There was no significant difference ($P = 0.071$) in disease index among isolates on cv. Winterhaven (Table 3). However, there were significant differences ($P < 0.001$) in disease index among isolates on cvs. Beacon and Lighthouse (Table 3). When the experiment was repeated, results were very similar, with the exception of BMP1324 on cv. Lighthouse, in which case the disease incidence increased to 100% and the disease index increased to 2.2. Of the *F. oxysporum* isolates recovered from soil, four of the pathogenic and four of the nonpathogenic isolates were selected for inclusion in subsequent phylogenetic analyses (Table 1).

DNA amplification. The mtSSU primers NMS1 and NMS2 amplified fragments ranging in size between 730 and 739 bp from all isolates. The EF-1α primers EF-1H and EF-2T amplified fragments between of 709 and 721 bp from all isolates. The IGS primers CNL12 and CNS1 generated a product between 2,547 and 2,600 bp from all isolates. Internal primers used to generate

smaller fragments of the IGS for sequencing generated products that ranged between 400 and 609 bp.

mtSSU phylogeny. Alignment of the mtSSU sequences resulted in a data set of 683 characters, of which 15 (2.2%) were parsimony informative. Parsimony analysis resulted in one most-parsimonious tree (MPT) with three distinct clades (steps = 49, consistency index [CI] = 0.918, retention index [RI] = 0.946) (Fig. 2). The major clade 3 included all *lactucae* race 1 isolates, 13 other non-*lactucae* isolates, and nonpathogenic isolates with strong bootstrap support (96%). Clade 2, grouping together formae speciales (f. spp.) *spinaciae*, *vasinfectum* 2 (FOV14), and *melonis*, had a bootstrap support of 58%. The f. sp. *medicaginis* did not cluster with either of the previous two clades. The two race 2 isolates (F9501 and FK09701) grouped together in clade 1 along with the f. spp. *perniciosum*, *cubense*, and *canariensis* with a bootstrap support of 86%. When gaps were coded as a fifth character state, the same phylogeny was obtained with similar bootstrap support values (data not shown). Maximum likelihood analysis resulted in a tree nearly identical (slight differences in branch lengths) to that obtained in parsimony analysis with similar bootstrap values (Fig. 2).
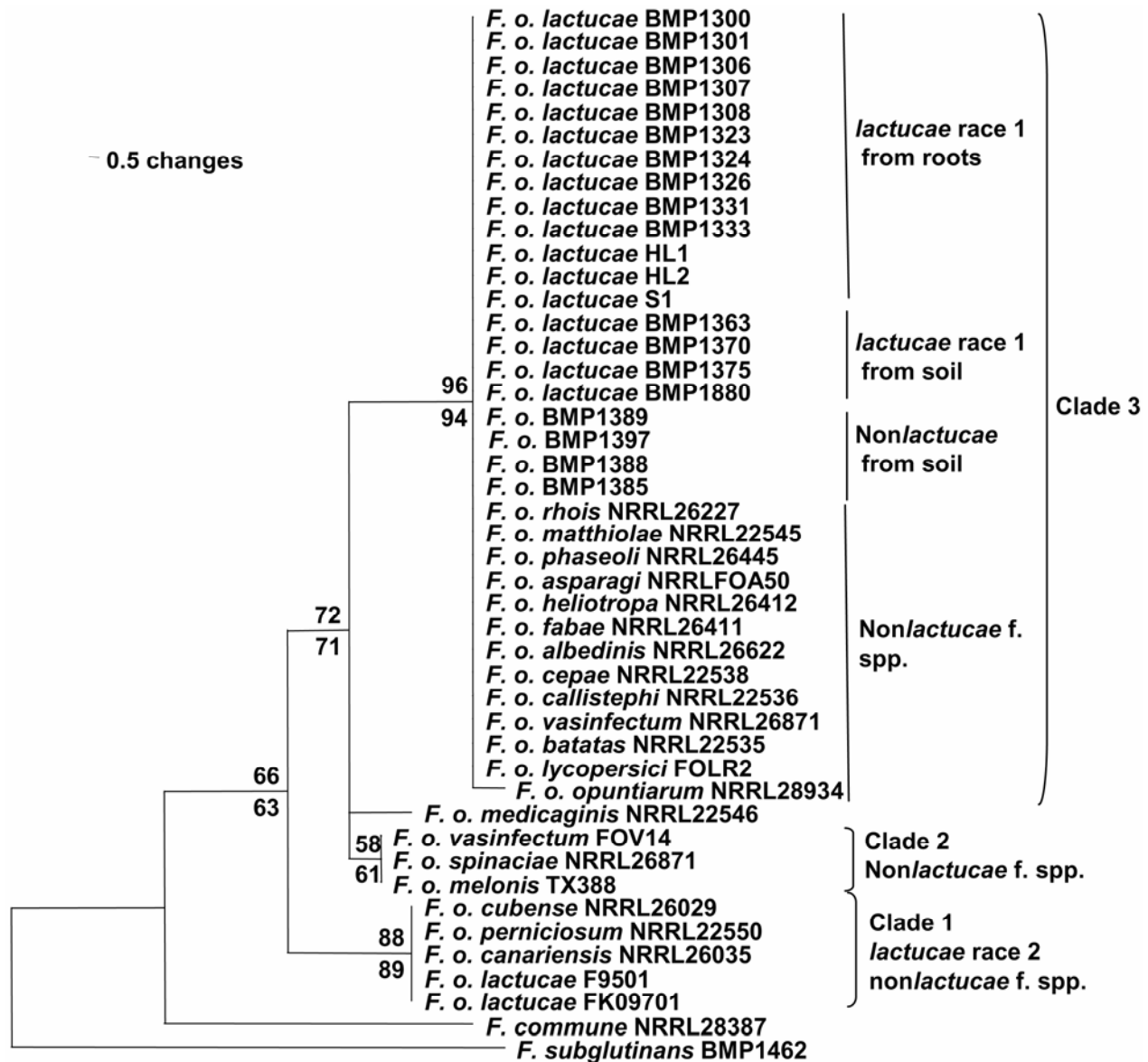

Fig. 2. Single most parsimonious tree generated with mitochondrial small subunit gene sequence data. *Fusarium subglutinans* and *F. commune* are included as outgroups. Bootstrap values are indicated as percentages above the nodes for maximum parsimony analysis (1,000 replications) and below the nodes for maximum likelihood analysis (500 replications). The length of the tree is 49 steps, and the values for consistency index and retention index are 0.918 and 0.946, respectively.

EF-1α phylogeny. Alignment of the EF-1α sequences generated a data set of 636 characters, of which 25 (4.0%) characters were parsimony informative. Parsimony analysis yielded one MPT with four clades (steps = 63, CI = 0.968, RI = 0.978) (Fig. 3). The *lactucae* race 1 isolates grouped together with seven other formae speciales (*rhois*, *matthiolae*, *cepae*, *phaseoli*, *albedinis*, *heliotropa*, and *fabae*), forming the major clade (clade 4) supported by a bootstrap value of 63%. The nonpathogenic isolates from soil (BMP 1385, BMP1388, BMP1389, and BMP1397) formed a distinct clade (clade 3) with f. spp. *asparagi*, *callistephi*, *batatas*, *vasinfectum* 1 (NRRL25231), and *lycopersici*. The f. spp. *opuntiarum* and *medicaginis* were not included in either of the previous two clades, although f. sp. *oputiarum* clustered with the two as part of the largest clade represented in the mtSSU tree. Clade 2 consisted of f. spp. *spinaciae*, *vasinfectum* 2, and *melonis*, with a bootstrap support value of 87%. Isolates F9501 and FK09701, both belonging to race 2, again clustered with the f. spp. *periciosum*, *cubense*, and *canariensis* as clade 1, with bootstrap support of 96%. Analysis with gaps treated as a fifth character state revealed the same phylogeny with similar bootstrap support values (data not shown). Maximum likelihood analysis resulted in a tree nearly identical to that obtained in parsimony analysis with similar bootstrap values (Fig. 3).

IGS phylogeny. Assembly of the IGS contigs and their alignment resulted in a data set of 2,226 characters, of which 195 (8.8%) were parsimony informative. Parsimony analysis generated 40 MPT in which the *lactucae* race 1 isolates and f. spp. *rhois*, *matthiolae*, and *phaseoli* formed a monophyletic group as clade 6 (steps = 657, CI = 0.822, RI = 0.810) (Fig. 4). Bootstrap support value for this group was 92%. The f. spp. *asparagi*, *callistephi*, *batatas*, *lycopersici*, and *vasinfectum* 1 grouped together, with strong bootstrap support of 96% (clade 5). Clade 4 was made up of f. spp. *albedinis*, *heliotropa*, *fabae*, *cepae*, and *medicaginis*, with a bootstrap support of 52%. Three of the nonpathogenic isolates (BMP1389, BMP 1397, and BMP 1388) from soil formed a distinct clade separated from the fourth nonpathogenic soil isolate, BMP1385 (clade 3). Clade 2 was composed of the f. spp. *periciosum*, *cubense*, and *canariensis*, with bootstrap support of 99%. In contrast to groupings by mtSSU and EF-1α sequences, clade 1 was made up solely of the *lactucae* isolates belonging to race 2 (F9501 and FK09701). The f. spp. *opuntiarum*, *spinaciae*, *melonis*, *medicaginis*, and *vasinfectum* 2 and soil isolate BMP1385 were resolved as individual lineages. The main differences between the 40 MPT trees were in the positions of the *lactucae* race 1 isolates from soil that oscillated between the subclade with *lactucae* race 1 isolates from roots and its present position on the tree. In addi-
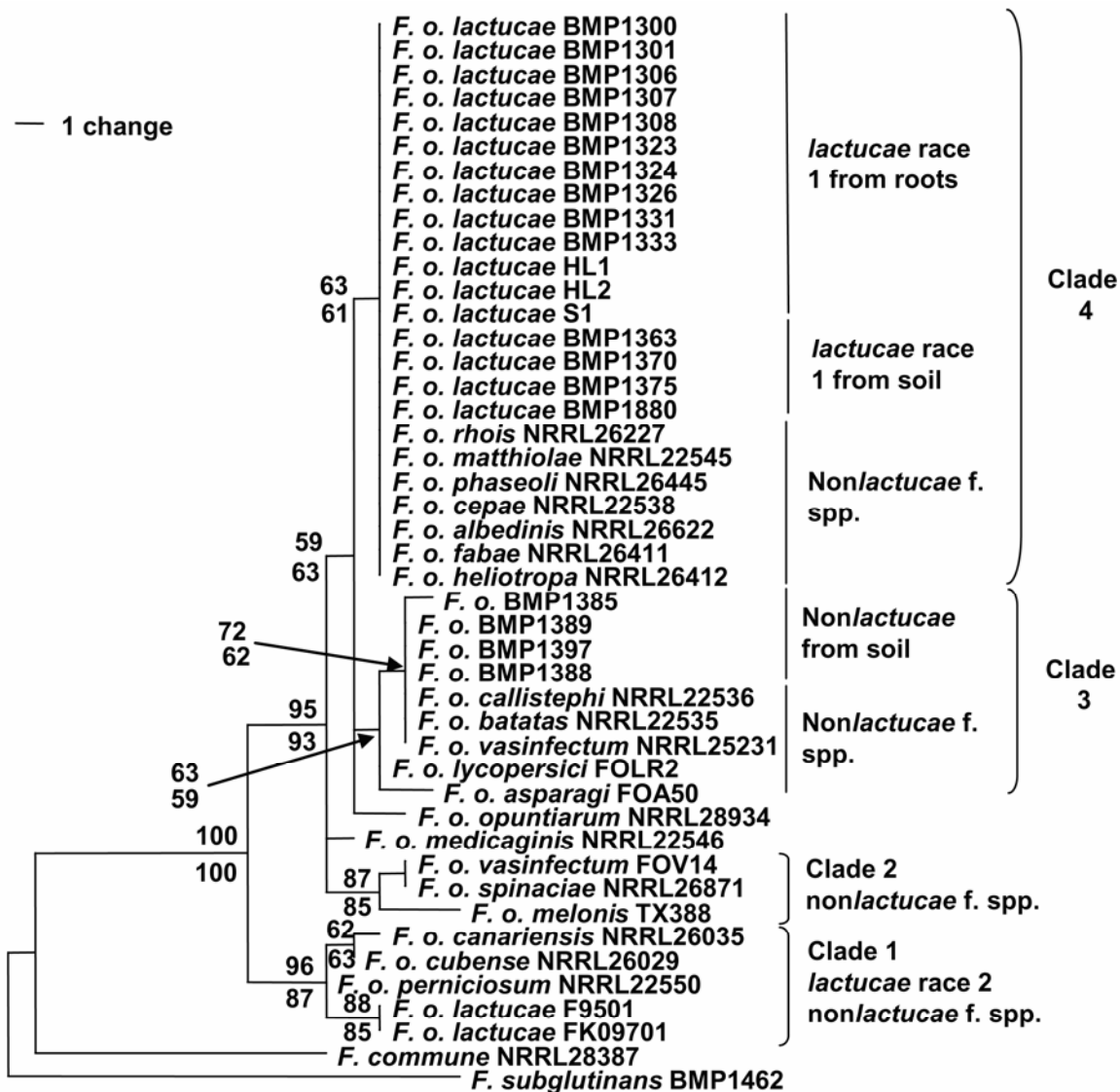

Fig. 3. Single most parsimonious tree generated with elongation factor 1-α gene sequence data. *Fusarium subglutinans* and *F. commune* are included as outgroups. Bootstrap values are indicated as percentages above the nodes for maximum parsimony analysis (1,000 replications) and below the nodes for maximum likelihood analysis (500 replications). The length of the tree is 63 steps, and the values for consistency index and retention index are 0.968 and 0.978, respectively.

tion, f. sp. *medicaginis* oscillated between clade 3 and forming an independent lineage. When gaps were coded as a fifth character state, the same phylogeny was obtained with similar bootstrap support values (data not shown). Maximum likelihood analysis resulted in a tree nearly identical to that presented in Figure 4 obtained in parsimony analysis (slight differences in relationships within clades 6 and 2) with similar bootsrap values (Fig. 4).

Analysis of the complete IGS sequence revealed numerous polymorphisms among the isolates. All *lactucae* race 1 isolates from tissue had identical IGS sequences. Three pathogenic race 1 isolates recovered from soil differed from pathogenic race 1 isolates recovered from lettuce roots by single nucleotide polymorphisms at three positions. Sequence comparison of the nonpathogenic *F. oxysporum* obtained from soil to sequences of *lactucae* race 1 isolates (both soil and root) revealed the presence of an 11-bp deletion in the nonpathogen sequences, as well as six insertions, nine transitions and, three transversions. The f. spp. *medicaginis*, *fabae*, *heliotropa*, and *cepae* shared a common 20-bp deletion at the same nucleotide position. The longest deletion in the IGS sequence was observed within f. sp. *albedinis* (22 bp). The *lactucae* race 2 isolates differed from race 1 isolates by the presence of three deletions, 11, 4, and 3 bp in length, as well as several insertions at various nucleotide positions.

Analysis done specifically with the *lactucae* race 1 IGS sequence revealed the presence of several perfect and imperfect tandem repeats ranging in size between 12 and 36 bp. The consensus sequence GGTGTAGGGTAG was found within most of these repeats. Analysis of the repeat pattern of the IGS sequence without allowing for mismatch of base pairs yielded six repeats, with the longest being 22 bp. These repeat subunits were scattered over more than two-thirds of the IGS region, with the last 700 bases toward the 3′ end having no perfect repeats (Fig. 1B). Allowing for only one mismatch, the repeats extended to the 3′ end of the IGS and their number increased from 6 to 14 (data not shown).

Combined data analysis. The mtSSU, EF-1α, and IGS sequences were combined into a single matrix to conduct both the Mickevich-Farris and Partition-Homogeneity tests for data congruence and combinability for improvement of resolution of the taxa. The Mickevich-Farris test produced an index of 0.000 for the mtSSU and EF-1α sequences, indicating total congruency between the two data sets (Table 4). Parsimony analysis of the combined mtSSU and EF-1α yielded one most parsimonious trees of 112 steps (CI = 0.946, RI = 0.964) with a topology nearly identical to that obtained with the EF-1α data partition (data not shown). The same topology was obtained
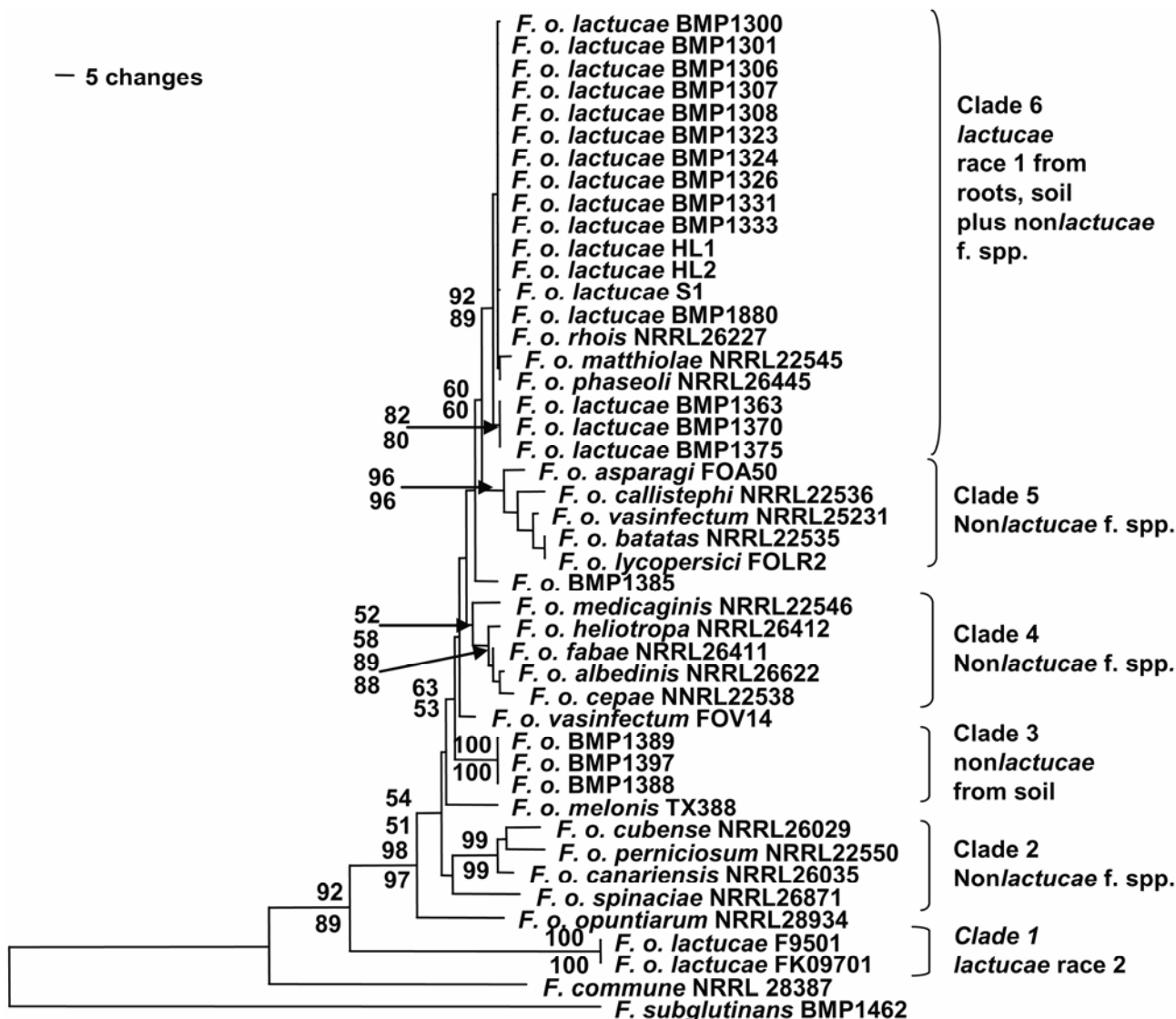

Fig. 4. One of 40 most parsimonious trees generated with the intergenic spacer sequence data. *Fusarium subglutinans* and *F. commune* are included as outgroups. Bootstrap values are indicated as percentages above the nodes for maximum parsimony analysis (1,000 replications) and below the nodes for maximum likelihood analysis (500 replications). The tree length is 657 steps, and the consistency index and retention index are 0.822 and 0.810, respectively.

when gaps were set either as missing data or as a fifth state (data not shown).

The Mickevich-Farris test produced an index of 0.078 for the mtSSU and IGS data sets, which can be interpreted as 7.8% incongruence between data partitions (Table 4). The corresponding P value obtained with the P-H test for these partitions was 0.0500, suggesting incongruence if the threshold for incongruence is set at $P \leq 0.05$. Parsimony analysis of the combined mtSSU and IGS yielded 120 most parsimonious trees of 705 steps (CI = 0.818, RI = 0.807), with a topology nearly identical to that obtained with the IGS data set (data not shown). The primary differences observed in topologies from the combined data set compared with the IGS data set were the clustering of f. sp. *opuntiarum* with the nonpathogenic isolates from soil and the formation of an independent lineage by f. sp. *medicaginis*. Slight differences in topologies were observed when gaps were set either as missing data or as a fifth state (data not shown). On the tree generated with gaps treated as missing data, the f. sp. *vasinfectum* 2 and *melonis* clustered together and f. spp. *spinaciae* and *medicaginis* constituted independent lineages. When gaps were treated as a fifth state, the f. spp. *melonis* and *spinaciae* clustered together and f. sp. *vasinfectum* 2 clustered with non-*lactucae* isolates of clade 3 of the IGS phylogram. In addition, f. sp. *medicaginis* grouped with isolates of clade 4 and f. sp. *opuntiarum* formed an independent lineage. Maximum likelihood analysis resulted in a tree nearly identical (slight differences in relationships within clades 4, 3, and 2) to that obtained in parsimony analysis with similar bootstrap values (data not shown).

The I_{MF} for the combined EF-1α and IGS data sets and the combined mtSSU, EF-1α, and IGS data sets were 0.121 and 0.155, respectively, revealing considerably more character incongruence than that found between the mtSSU and IGS data sets (Table 4). P-H test values for both of these sets were 0.0100, supporting the findings of the I_{MF}. Parsimony analysis of the three combined data sets yielded 800 MPT with topologies very similar to that of the IGS data set (steps = 792, CI = 0.816, RI = 0.813) (Fig. 5). The primary differences observed in topologies from the combined data set compared with the IGS data set were the inclusion of isolate BMP1385 within clade 4, the movement of clade 4 relative to clade 5 of the IGS tree, and the clustering of f. sp. *spinaciae* with the f. spp. *melonis* and *vasinfectum* 2. The f. sp. *medicaginis* still formed an independent lineage in most trees. Similar topologies were obtained when gaps were set either as missing data or as a fifth state (data not shown). Maximum likelihood analysis resulted in a tree nearly identical to the most parsimonious tree presented in Figure 5 (slight differences in branch lengths) with similar bootstrap values (Fig. 5).

Test of hypothesis. Three hypotheses of groupings were tested with the parsimony criterion (Fig. 6). The first hypothesis (A) was drawn from the grouping based on the mtSSU sequence data (Fig. 6A). In this hypothesis, there are four lineages of *F. oxysporum*, with the *lactucae* race 2 isolates forming the first lineage and the

lactucae race 1 and 17 other formae speciales forming the fourth lineage. The second hypothesis was drawn from groupings based on the EF-1α data set (Fig. 6B). In this hypothesis, there are six lineages of *F. oxysporum*, four of which correspond to the fourth lineage in hypothesis A. The third hypothesis (C) was drawn from groupings based upon the IGS data (Fig. 6C). In this hypothesis, there was further resolution of most of the lineages in hypothesis B. In particular, the *melonis–vasinfectum* 2-*spinaciae* group, which formed a monophyletic lineage in hypotheses A and B, was resolved into three different lineages in hypothesis C.

Heuristic search with the mtSSU data under the constraint of Figure 6B and C produced one equally parsimonious tree of 49 steps and two equally parsimonious trees of 61 steps, respectively. The null hypothesis of no difference between the best trees and trees reconstructed under the constraint of Figure 6B was not rejected by the KH, Templeton, and WS tests, implying that hypotheses B is consistent with the mtSSU data (Table 5). However, the null hypothesis was rejected under the constraint of Figure 6C, implying that hypothesis C is inconsistent with the mtSSU data.

Heuristic search with the EF-1α data under the constraints of Figure 6A and C produced one parsimonious tree of 64 steps (CI = 0.953, RI = 0.967) and two equally parsimonious trees of 80 steps (CI = 0.762, RI = 0.791), respectively. The null hypothesis of no difference between the best tree and trees reconstructed under the constraint of Figure 6A was not rejected, implying that hypothesis A was consistent with the EF-1α data (Table 5). However, the null hypothesis was rejected under the constraint of Figure 6C, implying that hypothesis C is inconsistent with the EF-1α data.

Heuristic search with the IGS data under the constraints of Figure 6A and B produced 100 equally parsimonious trees of 689 steps (CI = 0.772, RI = 0.743) and 6 equally parsimonious trees of 727 steps (CI = 0.732, RI = 0.681), respectively. The null hypothesis of no difference between the best tree and trees constructed under the constraints of Figure 6B and 6C was rejected by KH, Templeton, and WS tests (Table 5), which implies that both hypothesis A and B are inconsistent with the IGS data.

DISCUSSION

The primary focus of this study was to examine phylogenetic relationships among f. sp. *lactucae* isolates and between *lactucae*, other formae speciales, and nonpathogenic isolates of *F. oxysporum*, which resulted in several significant conclusions. First, this study revealed that *F. oxysporum* f. sp. *lactucae* is composed of at least two independent clonal lineages. All race 1 isolates from Arizona, California, and Japan had identical mtSSU and EF-1α sequences, and nearly identical IGS sequences, which suggests a common origin. Similarly, both race 2 isolates had identical mtSSU, EF-1α, and IGS sequences. However, all loci showed very significant sequence divergence between race 1 and 2, and phylogenetic analyses revealed that the races were only distantly related. These findings were similar to those obtained by Fujinaga et al. (11) using only partial IGS sequences. Fujinaga et al. also examined race 3, which was not available for this study, and found that it was genetically distinct from both race 1 and 2, suggesting yet another lineage of *F. oxysporum* f. sp. *lactucae*. In addition, Ogiso et al. (29) found a strong correlation between race and vegetative compatibility in *F. oxysporum* f. sp. *lactucae*, and revealed that California race 1 isolates were of the same vegetative compatibility group (VCG) as race 1 from Japan and that all race 2 isolates were of a separate VCG. Similarly, Pasquali et al. (30) found that race 1 isolates from Italy, Arizona, and California and type 1 isolates from Taiwan were all of the same VCG as the race 1 isolates from Japan, which was different from that of race 2 from Japan. These studies further demonstrate that pathogenicity of *F. oxysporum* to lettuce evolved in two independent

TABLE 4. Congruency of data partitions as evaluated by Mickevich-Farris incongruence metrics (I_{MF}) and the Partition-Homogeneity (P-H) test implemented in PAUP[x]

| | Steps | | | |
Data set[y]	Parsimony	Minimum	I_{MF}	P-H test (P)[z]
mtSSU/EF-1α	112	106	0.000	1.0000
mtSSU/IGS	705	577	0.078	0.0500*
EF-1α/IGS	725	593	0.121	0.0100*
mtSSU/EF-1α/IGS	780	638	0.155	0.0100*

[x] Tree lengths (steps) used to determine the I_{MF} are indicated for maximum parsimony analysis and minimum number of synapomorphies.
[y] mtSSU = mitochondrial small subunit, EF-1α = elongation factor 1-α, and IGS = intergenic spacer.
[z] Asterisk (*) indicates $P \leq 0.05$ is considered to be significantly incongruent.

lineages, given the apparent polyphyly. What is most interesting is the fact that all three f. sp. *lactucae* races first were detected and currently are found together only in Japan, revealing considerable diversity in *F. oxysporum* strains from this geographic region. Indeed, O'Donnell (27) reported similar genetic diversity among *F. oxysporum* formae speciales of Asian host genera *Musa*, *Albizia*, and *Phoenix* and suggested that Asia may be the center of origin of the *F. oxysporum* complex.

A second finding was that, of the three loci analyzed (mtSSU, EF-1α, and IGS), the IGS region contained the most sequence variation and provided the best resolution of the *lactucae* isolates as distinct taxa. Based upon the IGS sequences, f. sp. *rhois* was genetically identical to isolates of f. sp. *lactucae* recovered from lettuce tissue (pathogenicity tests conducted on the three lettuce cultivars with f. sp. *rhois* were negative). The next most closely related taxa were the f. spp. *matthiolae* and *phaseoli*, which also grouped with race 1 isolates. These relationships were consistent among the three data sets. Although the EF-1α gene has been used in most studies for phylogenetic resolution within and between some formae speciales of *F. oxysporum*, it did not contain

enough variation to separate the f. sp. *lactucae* race 1 isolates from seven other formae speciales. In previous studies using EF-1α and mtSSU sequences, *F. oxysporum* was shown to comprise three major clades, within which could be found a combination of formae speciales forming distinct lineages (5,27). The trees generated from the mtSSU data set in this study also revealed three primary clades. The EF-1α data generated four clades, two of which were equivalent to clade 3 in the mtSSU analysis. However, the IGS data set generated six clades, with many formae speciales unclustered as separate lineages. In addition, analysis of the IGS region even revealed differences among f. sp. *lactucae* race 1 isolates, with three of the isolates recovered from soil clustering separately. For both mtSSU and EF-1α alignments, race 2 isolates clustered with three other formae speciales in a single clade that was basal to the other formae speciales examined. However, analysis of the IGS data resolved the lactucae race 2 isolates as a monophyletic group basal to all other formae speciales examined. Additional taxa that may group within the race 2 clade can be ascertained only through a more comprehensive review of all formae speciales within *F. oxysporum*. Con-
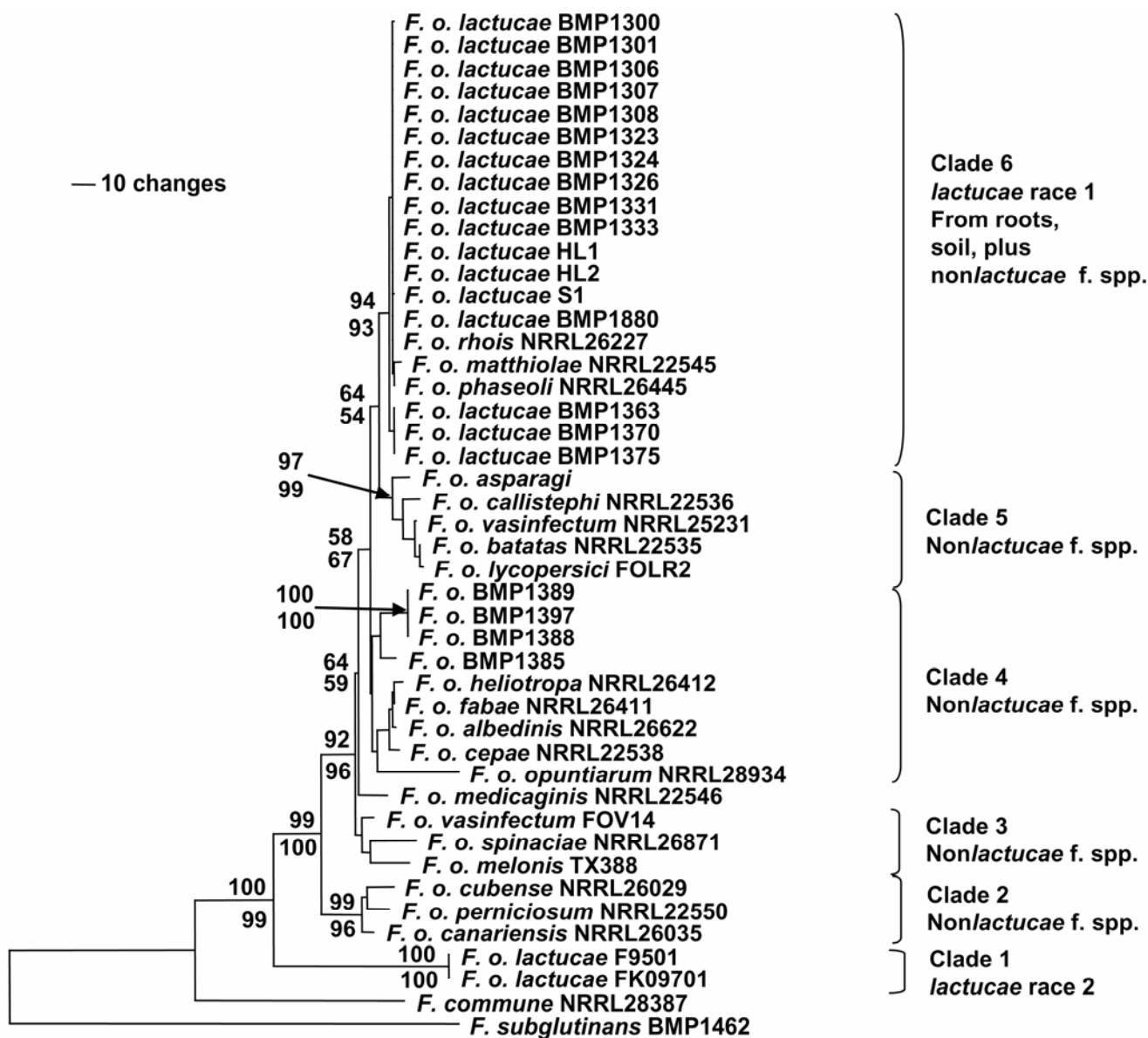

Fig. 5. One of 800 most parsimonious trees generated with combined mitochondrial small subunit, elongation factor 1-α, and intergenic spacer sequence data. *Fusarium subglutinans* and *F. commune* are included as outgroups. Bootstrap values are indicated as percentages above the nodes for maximum parsimony analysis (1,000 replications) and below the nodes for maximum likelihood analysis (500 replications). The tree length is 792 steps, and the consistency index and retention index are 0.816 and 0.813, respectively.

sidering there are over 120 reported formae speciales (4), it is highly likely that future analyses will reveal taxa that are closely related to and cluster with the race 2 isolates of f. sp. *lactucae*.

The IGS and the mtSSU and EF-1α trees presented conflicting topologies, as was demonstrated by differences in the relative positions of the taxa *medicaginis*, *opuntiarum*, and *spinaciae* and the nonpathogens from soil. This conflict was revealed in various tests used to determine the combinability of the data sets as well as examine conflicts between their topologies. The results of the

Mickevich-Farris (I_{MF}) and P-H tests suggested that the IGS and the mtSSU and EF-1α data sets were incongruent. Tests of topological hypotheses supported this conclusion. Whereas hypothesis A, based upon mtSSU sequence data, was consistent with the EF-1α data sets, it was inconsistent with the IGS data set. Hypothesis B, based upon EF-1α sequence data, was consistent with the mtSSU data but inconsistent with IGS data. Likewise, hypothesis C, based on IGS sequence data, was inconsistent with both the mtSSU and EF-1α data. The incongruence between these three
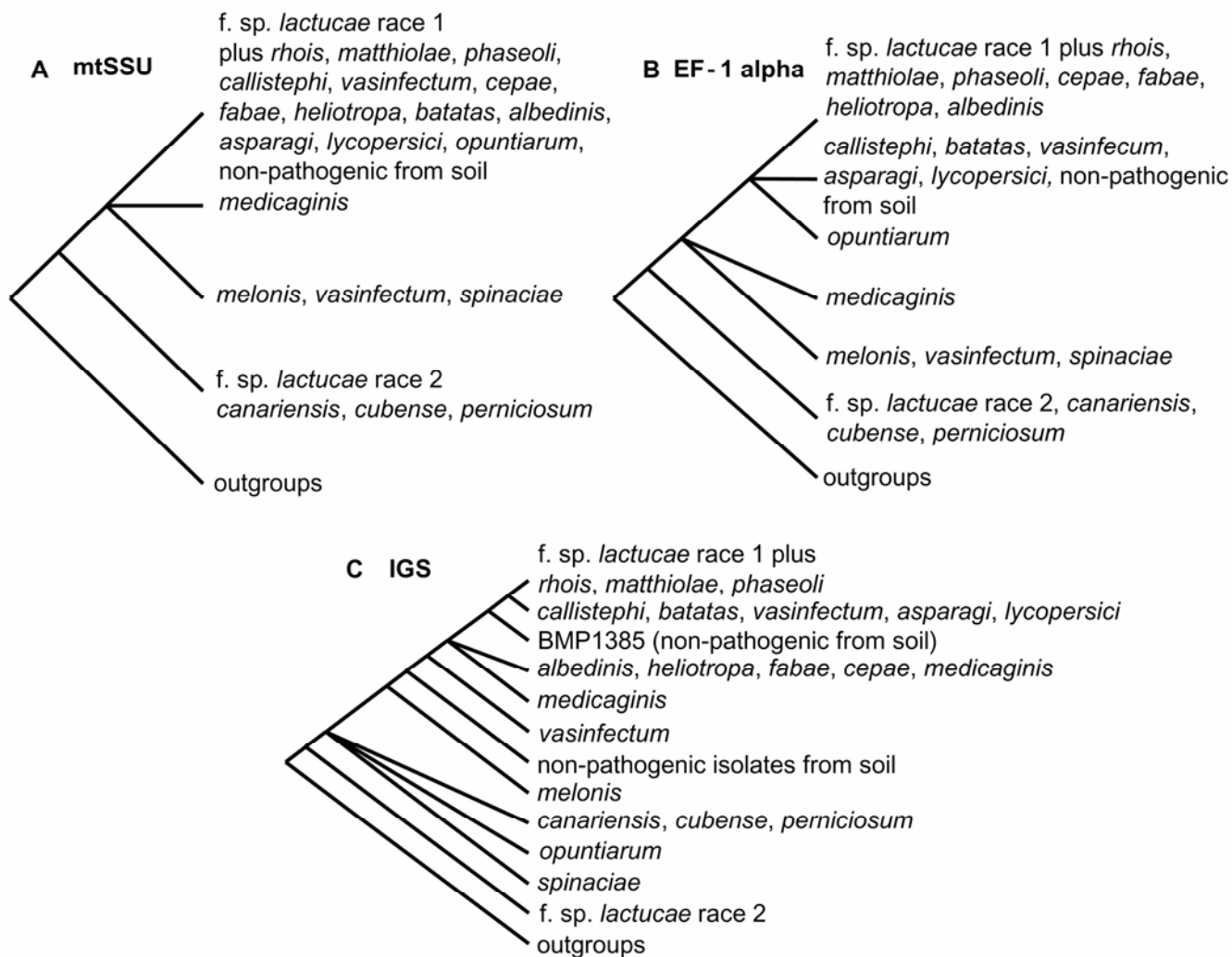

Fig. 6. Three strict consensus trees generated in PAUP from the different data sets used in the hypothesis tests. **A,** Mitochondrial small subunit (mtSSU) data set; **B,** elongation factor 1-α (EF-1α) data set; and **C,** intergenic spacer (IGS) data set.

TABLE 5. Test of topological hypotheses under varying constraints of strict consensus trees

Data set[w]	Constraints[x]	Steps[y]	P values[z]		
			KH	TT	WS
mtSSU	EF-1α	49 (49)	1.0000	…	…
	IGS	61 (49)	0.0104*	0.0139*	0.0156*
EF-1α	mtSSU	64 (63)	0.3177	0.3173	1.0000
	IGS	80 (63)	0.0022*	0.0027*	0.0010*
IGS	mtSSU	689 (646)	<0.0001*	<0.0001*	<0.0001*
	EF-1α	738 (646)	<0.0001*	<0.0001*	<0.0001*

[w] Values in parentheses represent steps of the unconstrained best tree.

[x] Values shown for Kishino-Hasegawa (KH), Templeton (TT), and Winning-sites (WS) tests. The rank sums for both the WS and TT tests were 0.00 for mitochondrial small subunit (mtSSU) data set under hypothesis B constraint; thus, P values were not generated; * indicates that $P \leq 0.05$ is considered to be significantly incongruent.

[y] EF-1α = elongation factor 1-α and IGS = intergenic spacer.

[z] The parsimony criterion also was used to test congruency of data sets against hypotheses for groupings drawn from results from mtSSU, EF-1α, and IGS sequence data. For each data set, a strict consensus tree was generated and used as a topological hypothesis.

data sets suggests that combining them may result in misrepresentation of phylogenetic relationships. Proponents of conditional combination recommend analyzing incongruent data partitions individually until more information is available on the nature of incongruency (7). However, the question as to what constitutes incongruency and when to combine data partitions for phylogenetic analysis remains controversial. Farias et al. (8) considered a significance of $P \leq 0.05$ for the P-H test, which is a frequently used threshold to determine data incongruency, to be too conservative and suggested that a significance of $P \leq 0.01$ is a more appropriate threshold. Cunningham (7) further demonstrated that, at heterogeneity values of $P > 0.01$, combining data still could improve phylogenetic accuracy, whereas combining data at $P < 0.001$ could lead to wrong phylogenetic interpretations. However, for the data sets used in this study, tests of hypotheses supported maintaining a P-H test threshold of $P \leq 0.05$ as a measure of incongruence. When the three data partitions were combined ($P = 0.01$) and analyzed, clade 2 (f. spp. *melonis*, *vasinfectum* FOV14, and *spinaciae*), which was present on the mtSSU and EF-1α trees but not on the IGS tree, was recovered. However, other significant conflicts remained. Resolution of such conflicts may be found in recent studies on topology tests which revealed that single or few genes could have significant probabilities of supporting conflicting topologies, particularly if there is insufficient representation among taxa (32). Because this study included only 40 taxa, including 20 different formae speciales of *F. oxysporum*, there is a possibility of under representation. Indeed, O'Donnell et al. examined a larger number of *F. oxysporum* isolates ($n = 131$) obtained from more diverse host and geographic sources, and subsequent topology tests revealed that EF-1α and IGS data were not significantly incongruent (28). This suggests that the inclusion of additional and more diverse isolates may resolve some of the conflict revealed in this study. The inclusion of more taxa also may improve low bootstrap support evident on several of the intermediate nodes.

Regardless of taxon representation, discordance between rDNA genealogies and those of protein-coding genes has been documented previously. In studies on phylogenetic relationships within the *Gibberella fujikuroi* complex of *Fusarium* and related species, it was discovered that all taxa examined ($n = 23$) contained two nonorthologous nuclear rDNA internal transcribed spacer (ITS)2 types whose genealogies were different from those of the mtSSU rDNA, nuclear 28S rDNA, and β-tubulin gene (26). It was hypothesized that the divergence between ITS2 types was due to an ancient interspecific hybridization event or gene duplication that occurred prior to the evolutionary radiation of the fungal lineages. A nonuniform distribution of the ITS2 types among subsequent species lineages resulted in a homoplastic pattern of evolution which obscured true phylogenetic relationships. Similarly, different IGS sequence types have been reported in an isolate of *F. oxysporum* f. sp. *melonis*, as well as in two putatively nonpathogenic forms of the fungus (3). Therefore, similar systematic problems may be expected with the use of IGS sequences in phylogenetic analyses. Other factors, such as unequal rates of evolution between loci and incomplete concerted evolution within loci, also have been proposed to explain discrepancies between rDNA and other data sets (34). These factors also may have relevance in the apparent discordance between IGS-based genealogy of *F. oxysporum* formae speciales and those based upon mtSSU and EF-1α data that was revealed in this study.

The IGS sequence of *F. oxysporum* f. sp. *lactucae* contains several repeated subunits, most of which contained a common consensus sequence of 12 bp and several imperfect permutations of this same repeat. Analysis of the repeats was done without allowing for any mismatch of base pairs and only six repeated sequences were obtained. However, when one mismatch and two mismatches of base pairs were allowed, the number of repeats increased to 21 and 28, respectively. Similar analysis of IGS

sequences of three *Verticillium* spp. also revealed several repeats located between nucleotides 203 to 1,264 bp from the 5′ end. In that study, one *V. longisporum* contained 14 perfect 39-bp repetitive motifs; whereas, in other isolates of *V. longisporum*, *V. dahliae*, and V. *albo-atrum*, the corresponding regions were made up of imperfect permutations of the 39-bp motif. The rest of the IGS contained no repeated sequences (31). In contrast, the perfect repeats found in f. sp. *lactucae* were distributed along more than two-thirds of the IGS, with only the far 3′ end being free of repeat units. This portion of the IGS was relatively conserved among the *F. oxysporum* isolates examined in this study, and it has been hypothesized that conserved IGS regions may accommodate functions related to rDNA production or processing (31). Additional studies are needed to determine whether the use of this region in the absence of the much larger portion of the IGS that contains repeat units improves phylogenetic utility of IGS data and results in less conflict with other genetic loci.

In summary, potential conflicts between IGS data and those from other genetic loci may make portions of the IGS deficient for robust resolution of *F. oxysporum* formae speciales phylogeny. However, considerable sequence variation among closely related taxa suggests that it can be used effectively as a diagnostic marker to differentiate among pathogenic and nonpathogenic isolates of *F. oxysporum*, including the f. sp. *lactucae*. Based on the heterogeneity of the IGS region, sensitive and specific PCR primers currently are being developed for the diagnosis of *F. oxysporum* f. sp. *lactucae* in field soil, lettuce seed, and in planta as an additional tool for the management of Fusarium wilt of lettuce in Arizona and California.

ACKNOWLEDGMENTS

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