PHYLOGENY, MOLECULAR DETECTION, AND GENETIC VARIATION OF
FUSARIUM OXYSPORUM, VASCULAR WILT PATHOGEN OF LETTUCE

by

Gladys Yekebui Chia Mbofung

A Dissertation submitted to the Faculty of the
DEPARTMENT OF PLANT SCIENCES
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN PLANT PATHOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

2006
As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Gladys Y. Chia Mbofung Entitled Phylogeny, molecular detection, and genetic variation of *Fusarium oxysporum*, vascular wilt pathogen of lettuce
And recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Date: November 27, 2006

Barry M. Pryor

Date: November 27, 2006

C. William Birky, Jr.

Date: November 27, 2006

Hans D. VanEtten

Date: November 27, 2006

Michael E. Matheron

Date: November 27, 2006

Thomas R. Gordon

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.
I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Date: November 27, 2006

Dissertation Director: Barry M. Pryor
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Gladys Y. C. Mbofung
ACKNOWLEDGEMENTS

I wish to sincerely thank my research supervisor, Dr. Barry M. Pryor, distinguished scientist and scholar, yet full of humility, for his ever-ready support and counseling, encouragement during discouragement, pulling me forward when all my desire was to stagnate, and for finally getting me to this next level. My heartfelt thanks also go to Professor Hans D. VanEtten and Martha Hawes for their bountiful help. To both, I say words are inadequate to express my gratitude. Special thanks to Professor C. William Birky of the department of Ecology and Evolutionary Biology, University of Arizona; Professor Thomas R. Gordon of the Department of Plant Pathology, UC-Davis, and Dr. Michael E. Matheron of the Department of Plant Pathology, University of Arizona for their unwavering support.

I also want to thank especially, the Arizona Lettuce Board and the Lettuce Seed Trade Association for Funding this research. And finally, I would like to thank Patricia Figuli, my lab mates Dr. Soon Gyu Hong, Ravi, Myung Soo Park, my helpers Nikolai, Louie, and Trahern, for their kind help and for making the lab a good place to be. To my kids, Rina, Kah, Momoh, and Marc for never complaining, and to say if I did it they are more than able to do same. To my Pastors, Randy, Lori, Ben, Francisco, and Liz for their spiritual support without which it would have been more difficult, I extend special thanks.
# TABLE OF CONTENTS

LIST OF FIGURES ...........................................................................................................10

LIST OF TABLES .............................................................................................................12

ABSTRACT .......................................................................................................................14

I INTRODUCTION ..........................................................................................................16

*Fusarium oxysporum* Schlecht. emend Snyd. & Hans ..................................................24

Taxonomy .......................................................................................................................24

The nomenclature of forma specialis ...........................................................................26

Vegetative compatibility groups ....................................................................................27

Pathogenic races ............................................................................................................29

Phylogeny of *F. oxysporum* species ..............................................................................30

Transposable elements in *Fusarium oxysporum* .......................................................33

Sexual reproduction in *Fusarium oxysporum* ...............................................................34

Host range and the infection process of *Fusarium oxysporum* ..................................36

Molecular diagnostics of fungal plant pathogens .........................................................40

Selection of molecular markers .....................................................................................46

DNA fingerprinting and genotyping of plant fungal pathogens ......................................48

II PRESENT STUDY .......................................................................................................53

REFERENCES ..................................................................................................................61

APPENDIX A: PHYLOGENY OF *Fusarium oxysporum* f. sp. lactucae INFERRED FROM MTSSU, EF-1 A, AND NUCLEAR RIBOSOMAL IGS SEQUENCE DATA.73
TABLE OF CONTENTS - Continued

ABSTRACT...........................................................................................................74
INTRODUCTION .................................................................................................75
MATERIALS AND METHODS.............................................................................77
  Fungal isolates ..........................................................................................77
  Pathogenicity tests ....................................................................................78
  DNA isolation and sequence amplification ............................................79
  Phylogenetic analysis .................................................................................80
  Combined data analysis .............................................................................81
  Tests of hypothesis ...................................................................................81
RESULTS .............................................................................................................82
  Pathogenicity tests ...................................................................................82
  DNA amplification ...................................................................................83
  mtSSU phylogeny .....................................................................................83
  EF-1α phylogeny ......................................................................................84
  IGS phylogeny ..........................................................................................85
  Combined data analysis ...........................................................................87
  Test of hypothesis ....................................................................................89
DISCUSSION .....................................................................................................90
REFERENCES ....................................................................................................97
APPENDIX B: A PCR-BASED ASSAY FOR DETECTION OF *Fusarium oxysporum f. sp. lactucae* IN LETTUCE SEED .................................................................115
TABLE OF CONTENTS - Continued

ABSTRACT .................................................................................................................. 115

INTRODUCTION ........................................................................................................ 116

MATERIALS AND METHODS .................................................................................. 120

  Fungal isolates ...................................................................................................... 120
  DNA isolation ........................................................................................................ 120
  IGS sequencing and primer design ...................................................................... 121
  Sensitivity and specificity of *F. oxysporum* f. sp. *lactucae* primer sets .................. 122
  Seed assay protocol .............................................................................................. 122
  Assay of commercial seed lots ............................................................................. 124
  Pathogenicity of *F. oxysporum* isolates recovered from seed .............................. 125

RESULTS ................................................................................................................... 126

  IGS sequencing and primer design ...................................................................... 126

  Sensitivity and specificity of *F. oxysporum* f. sp. *lactucae* primer sets ............... 127
  Seed assay protocol .............................................................................................. 127
  Assay of commercial seed lots ............................................................................. 128

DISCUSSION .............................................................................................................. 128

REFERENCES ......................................................................................................... 134

APPENDIX C: POTENTIAL FOR DISPERSEL OF *Fusarium oxysporum* f. sp.

*lactucae* BY INFESTED LETTUCE SEED ............................................................... 142
TABLE OF CONTENTS - Continued

ABSTRACT .......................................................................................................................... 142
INTRODUCTION ................................................................................................................ 143
MATERIALS AND METHODS .......................................................................................... 145

- Systemic movement of *F. oxysporum* f. sp. *lactucae* in lettuce ...................... 145
- Seed contamination by infested plant debris ......................................................... 147
- Seed infestation and germination potential ......................................................... 148

RESULTS ............................................................................................................................... 149

- Systemic movement of *F. oxysporum* f. sp. *lactucae* in lettuce ...................... 149
- Seed contamination by infested plant debris ......................................................... 151
- Seed infestation and germination potential ......................................................... 152

DISCUSSION ...................................................................................................................... 152
REFERENCES .................................................................................................................... 157

APPENDIX D: GENETIC VARIATION WITHIN *Fusarium oxysporum* f. *sp. lactucae* .............................................................................................................. 168

ABSTRACT ......................................................................................................................... 168
INTRODUCTION ................................................................................................................ 169
MATERIALS AND METHODS .......................................................................................... 173

- Fungal isolates ........................................................................................................... 173
- DNA extraction and amplification ........................................................................... 174
- ISSR- and minisatellite-primed PCR ..................................................................... 174
- Data analysis .............................................................................................................. 175
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>RESULTS</td>
<td>177</td>
</tr>
<tr>
<td>DNA amplification</td>
<td>177</td>
</tr>
<tr>
<td>Data analysis</td>
<td>177</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>179</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>184</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure I.1. Lettuce field showing dead lettuce heads due to infection with *Fusarium oxysporum* f. sp. *lactucae* .................................................................52

Figure A.1. Diagrammatic representation of the rDNA IGS region within one tandem Repeat .................................................................................................109

Figure A.2. The single most parsimonious tree generated with mtSSU gene sequence Data...........................................................................................................110

Figure A.3. The single most parsimonious tree generated with EF-1α gene sequence Data...........................................................................................................111

Figure A.4. One of 40 most parsimonious trees generated with the IGS sequence Data...........................................................................................................112

Figure A.5. One of 800 most parsimonious trees generated with combined mtSSU, EF-1α, and IGS sequence data.........................................................113

Figure A.6. Three strict consensus trees generated in PAUP from the different datasets used in the hypothesis tests.........................................................114

Figure B.1. The intergenic spacer (IGS) ribosomal DNA showing primary and nested primer sets..........................................................................................138

Figure B.2. A. Specificity of the primer pair GYCF1 and GYCR4C .........................139

Figure B.3. A. Limit of detection of primer pair GYCF1 and GYCR4C .................140

Figure B.4. A. Limit of detection of *F. o. f. sp. lactucae* in lettuce seeds having different levels of infestation with the nPCR-based seed assay ...............141
LIST OF FIGURES - continued

**Figure C.1.** Recovery of *F. o. f. sp. lactucae* along the stem of lettuce plants inoculated at transplant and grown to maturity in the greenhouse ........................................162

**Figure C.2.** Lettuce cultivars Vulcan (purple) and King Henry three weeks after inoculation and transplanting into trays ...............................................................163

**Figure C.3.** Longitudinal sections through the stem of the cultivar Sharpshooter showing vascular discolorations in the basal portion of the stem ........................................164

**Figure C.4.** External structure of the lettuce seed (achene) .............................................................165

**Figure C.5.** The effect of fungal infestation on seed germination .........................................................166

**Figure C.6.** *Fusarium oxysporum* f. sp. *lactucae* race 1 isolate BMP1300 ....................167

**Figure D.1.** Dendrogram derived from fingerprint analysis of 33 *F. oxysporum* f. sp. *lactucae* isolates using ISSR primers (ACA)$_5$, (CAA)$_5$, (GACA)$_4$, (GTG)$_5$, and TGTC ............................................................................................................195

**Figure D.2.** Dendrogram derived from fingerprint analysis of 32 *F. oxysporum* f. sp. *lactucae* isolates using ISSR primers (GACA)$_4$, (GTG)$_5$ ....................................196

**Figure D.3.** Dendrogram of combine data of all the *F. oxysporum* isolates .............................197

**Figure D.4.** Diagrammatic representation of the distribution of the 30 haplotypes of *F. oxysporum* f. sp. *lactucae* .......................................................................................198

**Figure D.5.** Amplification patterns obtained with ISSR primer (GTG)$_5$ ..................................199

**Figure D.6.** Arizona winter lettuce production fields along the Gila valley ..........................200

**Figure D.7.** Patterns of distribution of Fusarium wilt symptoms ..............................................201
LIST OF TABLES

**Table A.1.** Isolates used in this study and associated GenBank accession numbers

noted.....................................................................................................................102

**Table A.2.** Primers pairs for genomic regions amplified and sequenced in this study ...104

**Table A.3.** Disease incidence and disease index of *F. oxysporum* isolates on 3 iceberg

lettuce cultivars commonly used in Arizona........................................................105

**Table A.4.** Congruency of data partitions as evaluated by Mickevich-Farris

incongruence metrics (*I*<sub>MF</sub>) and the Partition-Homogeneity test implemented in

PAUP ...................................................................................................................107

**Table A.5.** Test of topological hypotheses under varying constraints of strict consensus
trees......................................................................................................................108

**Table B.1.** Fungi isolates used in this study..............................................................137

**Table C.1.** Evidence of systemic movement of *F. oxysporum* f. sp. *lactucae* within

lettuce plants calculated as percentage of stem sections from which the pathogen

was recovered at maturity from plants grown in the greenhouse ......................159

**Table C.2.** Percent recovery of *F. oxysporum* f. sp. *lactucae* from seeds harvested from

inoculated lettuce plants in the greenhouse ..........................................................160

**Table C.3.** Percentage contamination of seeds after *F. oxysporum* f. sp. *lactucae*-free

seeds were mixed with different amounts of infested debris obtained from the

greenhouse experiment .......................................................................................161

**Table D.1.** Isolates used in this study and their geographic origin .........................188

**Table D.2.** Minisatellite and ISSR primers used in this study ..................................191
LIST OF TABLES - continued

Table D.3. Haplotypes of *F. oxysporum* f. sp. *lactucae* recovered in Arizona over a
five year period .................................................................192

Table D.4. Genetic diversity analysis of populations of *Fusarium oxysporum* f. sp.
*lactucae* from Arizona, California ........................................193

Table D.5. Genetic identity represented in the upper diagonal and genetic distance
represented in the lower diagonal ........................................194
ABSTRACT

This work encompasses studies on the phylogeny of *F. oxysporum* f. sp. *lactucae*, the development of a PCR-based seed assay for the detection of this fungus in seed, the potential of seed transmission of the fungus that may result in seed dissemination, and the genetic variation existing within pathogen populations. In phylogenetic analysis, the mtSSU and EF-1α sequences provided limited phylogenetic resolution and did not differentiate the *lactucae* isolates from other *F. oxysporum* isolates, while the IGS region resolved *lactucae* race 1 isolates as a monophyletic group with three other f. spp. of *F. oxysporum*. In all analyses, *lactucae* race 2 isolates comprised a separate lineage that was phylogenetically distinct. Based the IGS, PCR primers were designed for detection of the fungus, and a PCR-based seed assay was developed for detection of the fungus in seed. This assay allowed for detection of the pathogen from artificially infested seed lots with infestation rates as low as 0.5%. To investigate seedborne transmission, the moderately resistant cultivars Sharpshooter, Vulcan, and King Henry were inoculated and grown to maturity in the greenhouse. The pathogen was recovered from sections of surface disinfested inflorescence stalks at rates of 14.3 – 62.7% but not from the floral parts. The incidence of recovery from nondisinfested seeds was between 0.02% and 0.08%. The pathogen was not isolated from surface disinfested seeds suggesting that it was externally seedborne. The pathogen was recovered from pathogen-free seeds mixed with infested debris suggesting infested seed may contribute to recently documented dissemination of this pathogen worldwide. Isolates of *Fusarium oxysporum* f. sp. *lactucae* were analyzed for genetic diversity using inter-simple sequence repeat molecular markers. Results
revealed 2 main groups within the Arizona isolates corresponding to eight haplotypes in 2005, which evolved from 2 haplotypes in 2001. Haplotype 1-05 was widespread, occurring in two of the four countries where *F. o. f. sp. lactucae* has been reported. 23 haplotypes were identified among the California isolates that clustered into two subgroups. The clustering of isolates from Arizona suggests that there has been more than one introduction of the pathogen into Arizona.
Lettuce (Lactuca sativa L.) is a common vegetable belonging to the family Asteraceae that is cultivated and consumed worldwide. It is most commonly recognized as seven horticultural types: crisphead, butterhead, romaine (or cos), leaf, stem, latin, and oilseed (Ryder 1998). The United States is second only to China in world production of lettuce and the greater part of the lettuce produced in the U.S. is consumed locally. Among the existing horticultural types, head lettuce accounts for the bulk of production followed by romaine and leaf. The vast majority of US production takes place in the states of California and Arizona which produce more than 96% of the country’s commercial iceberg lettuce and more than 98% of the leaf type. In 2005, lettuce was among the top five fresh-market vegetables in the U.S. with a total farm value of $1.98 billion (USDA-National Agricultural Statistic Service). Production has steadily increased over the years, and total exports valued at $39.3 million in 1989 more than quadrupled to $225.9 million in 2002, revealing the importance of lettuce to the economies of the two primary production states (Agricultural Marketing Resource Center, University of California, 2003).

In Arizona, lettuce production began during the Territorial days, but became a significant commodity in the late 1970s with the increased demand for winter lettuce nationally. Presently, Arizona ranks second among states in lettuce production with head, leaf, and romaine lettuces constituting the State’s leading cash crops totaling $500,749 million in value (USDA-National Agricultural Statistic Service). Over 28,000 ha are devoted to lettuce production in Arizona, 95% of which are located in the lower
Colorado River and Gila River Valleys of Yuma County (Matheron et al. 2005). Other production areas in the State include Cochise County in southeastern Arizona and Maricopa, Pinal, and Pima counties in central Arizona. Lettuce production in Arizona and California has been organized in a precise sequence to ensure year-round supply for national consumption. Production is focused in the Salinas Valley, CA, from April through October, then briefly in Huron, CA, in the fall, followed by the winter growing season in Yuma, AZ, and Imperial Valley, CA, from November through March. Another brief production period in Huron follows in March and April, with production returning to Salinas in the spring (Ag Outlook, April 2001, USDA Publications, 2001).

The most commonly grown type of lettuce in the U.S. is crisphead, which is second only to potatoes as the most popular vegetable in the U.S. However, per capita consumption of this type has declined 13% since a peak in 1989 due to an increased demand for leaf and romaine types. As Americans aspire to improve their diet, the consumption of all lettuce types has increased to the current record of 8.3 pounds annually per capita (Ag Outlook, April 2001, USDA Publications, 2001). This has driven, in part, a corresponding increase in the production of romaine and mixed lettuces grown under intensive production for pre-packaged salad mixes. Both diversification and intensive production has led to the appearance of several new diseases of the crop. One of these diseases is Fusarium wilt.

*Fusarium oxysporum* f. sp. *lactucae* Matuo and Motohashi, causal agent of Fusarium wilt of lettuce, was first described in Japan in 1965. At the time of its description, the disease was only known to affect butter head type cultivars (Tsuchiya et
al. 2004). Twenty eight years later (1995), the disease was common on crisphead type in the major lettuce producing area of Nagano prefecture, Japan. Fusarium wilt is presently recognized as a serious constrain to lettuce production in Japan as the total acreage affected by the disease is rapidly increasing (Tsuchiya et al. 2004). In the United States, the first report of Fusarium wilt was in California in 1990 on crisphead types, and the pathogen responsible was described as *F. oxysporum* f. sp. *lactucum* (Hubbard and Gerik 1993). The disease reappeared in 2001 in both California and Arizona where significant losses in yield were registered (Matheron 2003; Grube et al. 2002). In Arizona, the disease has spread rapidly and has recently been reported in 27 fields (Matheron 2005). In addition to Arizona and California, the disease has also been newly reported in Taiwan in 1998 (Huang and Lo 1998), in Iran in 1999 (Millani *et al.* 1999), and in Italy in 2002 (Garibaldi et al. 2002).

In 2001, isolates of the pathogen from Japan were grouped into two physiological races, race 1 and race 2, based upon pathogenicity on differential lettuce cultivars (Fujinaga *et al.*, 2001). Subsequent studies based on vegetative compatibility determined that *F. o. f. sp. lactucum* from California and *F. o. f. sp. lactucae* race 1 from Japan were identical and the taxa were subsequently synonymized (Ogiso *et al.* 2002). In that study, all strains of race 1 were assigned to VCG 1 while strains of race 2 were assigned to VCG 2 revealing a strong correlation between race and VCG (Ogiso 2002). In Taiwan, *lactucae* isolates have been divided into two morphological types, type I and type II without race designation (Huang *et al.* 1998), but were subsequently determined to be genetically distinct through vegetative compatibility studies (Pasquali *et al.* 2005). Type
I isolates were vegetatively compatible to race 1 isolates from Italy, Japan, and the U.S. and were assigned to VCG 0300, while type II were assigned to VCG 0301. In a parallel study, Fujinaga et al. (2005) identified three VCGs corresponding to the three races 1, 2, and 3, which he named VCG-1, VCG-2, and VCG-3, respectively. All three races (1, 2, and 3) of the pathogen have been identified in Japan, but only race 1 so far has been reported in the other countries of occurrence (McCreight, 2005). Currently, race differentiation is performed using the three lettuce differentials Banchu Redfire, Costa Rica # 4, and Patriot. Banchu Redfire is resistant to race 2 of the pathogen but susceptible to races 1 and 3. Costa Rica # 4 is resistant to race 1 but susceptible to races 2 and 3. Patriot is susceptible to races 1, 2, and 3. The cultivar River Green is a fourth lettuce differential identified and its physiological reaction to the three races is similar to that of Costa Rica # 4.

*F. oxysporum* is a well characterized soilborne pathogen and many phytopathogenic strains have been reported to be seedborne. *Fusarium o. f. sp. lactucae* is no exception to this general characteristic as the fungus has also been reported soilborne and seedborne (Matheron 2005; Pasquali et al. 2005). The disease cycle begins with planting of either *F. oxysporum*-infested seed or planting in infested soil. As the lettuce seeds germinate, the pathogen grows from the seeds and colonizes the seedlings, or grows from the soil and colonizes the roots as they emerge from the seed. The fungus is capable of causing wilt in both seedlings and mature plants. The first symptoms of Fusarium wilt typically occur as early as 3-4 weeks when young plants wilt and die. In more mature plants, a characteristic red-brown streak extends from the upper taproot into
the cortex of the crown. Older affected heads often exhibit a tip burn (Fig. 1), yellow leaves, and a brown to black streaking of the foliar vascular tissue while younger plants may be stunted and fail to form heads (Hubbard and Gerik 1993).

The number of fields infested with the pathogen in Arizona has been increasing and is predicted to continue to increase via contaminated soil inadvertently introduced into pathogen-free fields. The main means of dispersal of the pathogen locally is hypothesized to be through infested farm equipment and through movement of mud-encrusted irrigation pipes used to germinate seeds following planting (Matheron 2005). However, the occurrence of this disease in countries far from the original discovery area reveals the potential of the pathogen to spread long distance and suggests the possibility of seedborne dispersal. Although long-distance seedborne dispersal has not been proven, the occurrence of the fungus on lettuce seed was recently documented in Italy (Garibaldi et al. 2004). Moreover, this is consistent with the mode of dispersal of many other *Fusarium* species.

The original fields infested in Arizona were all planted to crisphead lettuce from mid-October through early January suggesting a link between planting date and lettuce type to the disease (Matheron et al. 2005). Recent variety trials revealed no resistance present in crisphead types, but romaine lettuce offered some form of tolerance to the pathogen (Matheron 2005). Presently, the only effective control measure is to avoid planting crisphead lettuce in infested fields for at least several years. In recent studies, planting during the coolest winter months and the use of romaine type lettuce resulted in the lowest incidence of disease (Matheron 2005). Thus, factors such as lettuce cultivar,
planting date, and soil temperature were shown to affect the incidence and severity of the disease. Alternative control methods with potential for the future include soil solarization, biological control with antagonistic microorganisms, and the use of resistant cultivars (Di Pietro et al. 2003; Matheron et al. 2005).

Tsuchiya et al. (2004) evaluated a total of 155 existing lettuce accessions for resistance to *F. o. f. sp. lactucae*. Compared to the genetic resources from Japan, they reported a larger number from outside Japan showing resistance, with the cultivars Kikugawa 102 and Blizzard possessing the most resistance to race 2 of the pathogen. Cultivars Batavia Tezier and Batavia Blonde de Paris were resistant to both race 1 and 2. The same Japanese group has developed a hybrid lettuce from two crisphead lines B155 and B42, which is resistant to race 1 of *F. o. lactucae* and is slow bolting and can be grown in summer when the soil temperatures are conducive for the development of the disease. These features of the hybrid are required for growth in the race 1-infested region of Nagano in Japan (Tsuchiya et al. 2004). In a study to evaluate resistance to Fusarium wilt in lettuce adapted to Arizona and California growing conditions, the cultivars Salinas and Salinas 88 were determined to be resistant to Arizona and California isolates of *F. o. lactucae*. Furthermore, limited F1 and F2 data showed that resistance to race 1 in Costa Rica No. 4 and Salinas was recessive and that the cultivar Calmar which is the ancestor of Salinas and Salinas 88 was the source of resistance in these two cultivars (McCreight et al. 2005). These cultivars may likely provide a base of genetic resource on which resistance against all races of the pathogen might be built.
Although VCG analysis provides a measure of similarity between the different races, it does not unravel the origin of the inoculum responsible for the different epidemics. Intraspecific and phylogenetic relationships among the races of *F. o. f. sp. lactucae* using multigene genealogies are of great importance. Recently, phylogenetic analysis using only the partial sequence of the intergenic spacer region of the rDNA has revealed that the three races are genetically distinct (Fujinaga et al. 2005). More comprehensive genotyping of pathogen populations will be very important in monitoring the dynamics of pathogen populations within a certain area and also for predicting resistance breakdown in resistant varieties of lettuce that are being developed. Since the pathogen continues to spread in the prime lettuce production area in Arizona, it is critical that spread of the pathogen be contained until the necessary research in disease management can be conducted. As a necessary component for successful containment program, methods need to be available for the detection of the pathogen in potential sources of contamination such as commercial lettuce seed.

This dissertation focuses on several aspects of the etiology and epidemiology of Fusarium wilt of lettuce, namely, (i) the phylogeny of *F. oxysporum f. sp. lactucae* inferred from sequences of the translation elongation 1 alpha factor, the mitochondrial small subunit, and the intergenic spacer region of the rDNA, (ii) the development of a PCR-based seed assay for the detection of *F. oxysporum f. sp. lactucae* in seed, (iii) the potential of seed transmission of the *F. oxysporum f. sp. lactucae* resulting in seed dissemination, and (iv) the genetic variation that exist within pathogen population sampled over a period of four years. An applied objective of this work was to determine
the relationships between the forma specialis *lactucae* and other formae speciales in order to identify genetic markers for seed health testing and other diagnostic activities. The following Introduction chapter provides a review of *Fusarium oxysporum* taxonomy, its host range as defined by the different formae speciales, and the vegetative compatibility groupings that exist within the different form species. Included also is the state of knowledge of the different transposable elements found within the fungus and how these affect the genetic variability in *Fusarium oxysporum*. Of particular interest is how the fungus invades its host (infection process) and how this relates to its seedborne and soilborne nature. A discussion on the available molecular diagnostic methods and the ease of their integration into the pest management strategies is presented as well.
**Fusarium oxysporum** Schlecht. emend. Snyd. & Hans.

**Taxonomy**

*Fusarium* is a form genus (asexually reproducing genus) of filamentous fungi in the class Sordariomycetes (Subclass: Hypocreomycetidae) and order Hypocreales. The taxonomy of *Fusarium* was initiated in 1809 when the genus was first described by Link. The morphology of microscopic characteristics, particularly the shape and dimensions of the macroconidia, the production of microconidia, chlamydospores, sclerotia, sexual stages and pigmentation, are the primary means used to identify and define *Fusarium* species. However, these characters must be described under very specific and environmental conditions because of inherent cultural variability of morphological traits.

Wollenweber and Reinking (1935) organized the species of *Fusarium* into 16 sections containing 65 species, 55 varieties and 22 forms, using characteristics such as shape and shape of both the macroconidia and microconidia, and presence and location of chlamydospores. The authors used six different media for growth and morphological characterization. The 16 sections comprised *Eupionnotes*, *Macroconia*, *Spicarioides*, *Submicrocera*, *Pseudomiccroceria*, *Arachnites*, *Sporotrichella*, *Roseum*, *Arthrosporiella*, *Gibbosum*, *Discolor*, *Lateritum*, *Liseola*, *Elegans*, *Martiella*, and *Ventricosum*. The characters used to separate sections were the presence or absence of microconidia, the shape of the microconidia, the presence or absence of chlamydospores, the location of the chlamydospores (intercalary or terminal), the number of septations in the macroconidia, and the length and width of the macroconidia. It is worth noting that the characters used by Wollenweber and Reinking for grouping the species, varieties, and forms were not
stable and varied considerably on the six different culture media they used. In addition, morphological mutations in plate cultures and variation that exists within the genus *Fusarium* were likely not recognized (Nelson et al. 1994). Since then, the taxonomic system used for *Fusarium* has evolved over the years with significant changes to the number of recognized species each time a revision was made.

Snyder and Hansen (1940, 1941, 1945) reduced Wollenweber and Reinking’s 65 species to 9 based on the morphology of the macroconidia and an extensive study of the general nature and variability of the *Fusarium* species. Their specific work on the species *Fusarium oxysporum* and *Fusarium solani* was accepted by most mycologists, but the remainder of their proposed system generated a great deal of confusion and controversy (Nelson et al. 1994). In 1971, Booth recognized 44 species and 7 varieties within the genus, adding information on perfect states, conidiophores, and conidiogenous cells. Gerlach and Nirenberg (1982) reorganized the system into 73 species and 26 varieties, while Nelson, Toussoun, and Marasas (1983) expanded Snyder and Hansen’s 9 species to 30. In their comprehensive work, Nelson, Toussoun, and Marasas selected what they considered to be the best parts from the existing systems and combined them with their research results to develop a practical identification scheme. Thus over the years as few as 9 species and as many as 73 species have been recognized within *Fusarium* based on the morphological taxonomic system.

Despite its initial controversy, the nine species system of Snyder and Hansen has continued in use throughout the twentieth century and its influence has been maintained to this day. Although the genus is mainly made up of asexual species, species with known
teleomorphs are placed in the genera *Gibberella* and *Nectria* (Gams and Nirenberg 1989). The morphology of macroconidia, the presence or absence of microconidia, the morphology of microconidiophores, and the presence and absence of chlamydospores are now accepted as the most stable taxonomic characters and is presently being used in identification system of the different *Fusarium* species (Nelson et al. 1994).

Isolates of *Fusarium oxysporum* were grouped under the section *Elegans* by Wollenweber and Renking (1935) together with nine other species that were further divided into three subsections. Due to the fact that the morphological divisions within this section were small and the features were highly variable and changed with environmental influence, Snyder and Hansen (1940) collapsed the section into a single species, *F. oxysporum* Schlecht. emend. Snyd. & Hansen. Later, they proposed the adoption of the botanical term, cultivar, for certain infraspecies populations differing in conidial morphology which has developed into the forma specialis nomenclature today (Nelson et al. 1994).

**The nomenclature of forma specialis**

Within the species of *F. oxysporum*, the concept of formae speciales was applied to recognize physiological strains that are indistinguishable from saprophytic strains of the same species but have the ability to cause disease on different host plants (Booth 1971). Isolates within *F. oxysporum* with the same or similar host ranges are assigned to the same forma specialis, which usually reflects the name of the common host(s). Previously, pathogenicity of each forma specialis was thought to be highly host specific. But it was later discovered that although host specificity occurs for most formae
speciales, it could not be strictly applied to all strains (Armstrong and Armstrong 1975). To date, more than 150 host-specific formae speciales have been described for the species *F. oxysporum* (O’Donnell et al. 1999).

The categorization of strains by host range does not lead to a natural subdivision within the species as some isolates belonging to different formae speciales may be more closely related than isolates within the same forma specialis. Moreover, considerable genetic diversity exists between pathogenic and nonpathogenic strains of *F. oxysporum* isolated from the same host (Kistler 1997). Genetic diversity studies have been conducted for a large number of formae speciales. However, most of these studies focused on single forma specialis because they were undertaken by phytopathologists interested in disease on a single crop (Kistler 1997). The relationship among isolates across different formae speciales has not been fully addressed.

**Vegetative compatibility groups**

Vegetative compatibility (VC) in Ascomycetes refers to the ability of two related strains of fungi to fuse hyphae and form a heterokaryon. Heterokaryon formation is considered important in the fungal cycle because it serves as the first step in the parasexual cycle and also for the transmission of hypovirulent factors such as dsRNAs. It is a means by which normally haploid fungi become functional diploids and acquire the benefits of complementation or heterosis (Leslie 1993). Most Ascomycetes possess genetic systems governing vegetative compatibility based upon a set of *het* loci whose product mediates either vegetative compatibility or incompatibility leading to cell lysis after hyphal fusion.
There are presently more than 125 vegetative compatibility groups (VCGs) identified among plant pathogenic isolates of *F. oxysporum* (Kistler et al. 1998). One of the techniques for identification of VCGs involves creating spontaneous auxotrophic mutants on chlorate medium (nit mutants) incapable of utilizing nitrate as a sole nitrogen source. This results in the generation of a series of nit mutants affected at different steps in the pathway involved in nitrogen reduction. The mutants are then paired on minimal medium and pairs that fuse to produce a heterokaryon complement each other and result in wild type growth. For two strains to be vegetatively compatible and be able to fuse, alleles at all loci must be identical. Only strains very similar genetically and isoallelic at all VC loci will form heterokaryons and are considered to belong to the same VCG. Although, membership in a VCG reflects genetic similarity, differences in VCGs give no indication of the degree of genetic difference (Kistler 1997). Broadly, VCGs may give an indication of the reproductive strategy of a species and population structure of the pathogen.

In an asexual fungus such as *Fusarium oxysporum*, strains related by clonal descent should be within the same VCG except for vegetatively self-incompatible strains (Kistler 1997). Heterokaryon self-incompatible strains are considered as those that carry mutations that prevent them from fusing to form hetrokaryons and such strains have been identified in field populations of *F. oxysporum* (Bosland and Williams 1987; Jacobson and Gordon 1991; Jacobson and Gordon 1988). Clonal reproduction greatly limits the reassortment of genes for heterokaryon incompatibility. VCGs were thought to be quite distinct from one another in that inter-isolate heterokaryosis was confined to isolates
within a VCG. There is now emerging evidence that some cross-VCG compatibility may occur (Katan and Katan 1999). Pullaha (1985) was the first to attempt utilizing vegetative compatibility to classify strains of *F. oxysporum*. According to a summary of 37 formae speciales of *F. oxysporum*, the number of VCGs within a forma specialis ranges from 1 to 24, averaging four VCGs per forma specialis (Katan 1999).

The discovery of multiple VCGs within the forma specialis of *F. oxysporum* suggests that VCGs could become a useful indicator of genetic diversity for the study of these important pathogens. It is presumed that pathogenic strains that belong to the same VCG originate from the same clone even if they are geographically isolated, and that isolates that are similarly pathogenic and belong to different VCGs are assumed not to be clones (Leslie 1993). Thus, vegetative compatibility groupings within a pathogen population may enable the investigator to distinguish between pathogenic strains that have arisen locally and those that have been imported from another location. VCG analysis can also be applied to test for genetic homogeneity to determine if isolates are clones of a common progenitor or not.

**Pathogenic races**

Pathogenic races refer to cultivar-level specificity of the host genotype which is often determined by single genes in the host. So far, the highest number of races identified within a single formae speciales is eight (Aloi and Baayen 1993). Race or disease specificity to particular host species genotypes, may or may not correlate with clonal lineage. For example, a single clonal lineage of *F. oxysporum* f. sp. *melonis* contained all known races of the pathogen, yet races 1 and 2 of *F. o. f. sp. lycopersici*
were distributed randomly among lineages belonging to four VCGs (Elias et al. 1993; Gordon 1993; Jacobson and Gordon 1988). A forma specialis may either consist of one or more vegetative compatibility groups (VCG) or pathogenic races. However, race in some instances has been defined by selectivity of isolates to distinct plant species (Armstrong and Armstrong 1966).

**Phylogeny of *F. oxysporum* species**

*F. oxysporum* is a large, diverse taxonomic unit to which several analytical techniques have been employed to differentiate between formae speciales, races, and clonal lineages (VCGs) (Kistler 1999). These studies have used VCGs, isozymes, DNA fingerprinting, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), electrophoretic karyotype (EK), and DNA sequence analysis as basis for comparison between isolates. Many of these genetic diversity studies have sought to determine whether *F. oxysporum* can be considered a single species. However, the answer to this question is not straightforward as many definitions have been applied to the species concept (Avise and Ball 1990). Cracraft (1983) defined a phylogenetic species as the smallest diagnosable cluster of individual organisms sharing a common ancestry. Nixon and Wheeler (1990) define species as “... the smallest aggregation of populations (sexual) or lineages (asexual) within which there is a pattern of ancestry and descent, and which is diagnosable by a unique combination of character states in comparable individuals (semaphoronts).” Slightly modifying Nixon and Wheeler’s definition, Harrington and Rizzo (1999) defined a species simply as “... the smallest aggregation of populations with a common lineage that share unique,
diagnosable phenotypic characters.” This definition corresponds to the phylogenetics species concept that incorporates the important features of population, lineage, and phenotype into a workable species definition.

The resolution of ancestry may be based upon a single gene, however, the concordance of more than one gene genealogy is generally required to determine species limit in fungi. This genealogical concordance concept was used by Taylor et al. to identify species limit in fungi (Taylor et al. 2000). In fungi, a single morphological species generally corresponds to many biological or phylogenetic species (Taylor et al. 2000). Biological species or mating populations have been defined as groups of cross-fertile isolates. In nature, different mating populations could arise due to separation of life cycles in time and/or space. A biological species classification has been developed for species within the Gibberella fujikuroi species complex (Leslie 1995) and the F. solani species complex (Matuo and Snyder 1973). In a study of the F. solani species complex, mating populations corresponded to phylogenetic species (O'Donnell 2000). However, there are several reasons why the application of a biological species concept to Fusarium oxysporum is a problem, top among which is the purely asexual reproductive mode. Regarding the phylogenetic species concept, questions on how an asexual species should be defined and on how many nucleotides constitute a species divergence in Fusarium and other fungi are yet to be answered (Steenkamp et al. 2002).

Unlike the use of morphological traits, molecular traits provide large numbers of characters for comparing organisms. Genetic diversity studies have been conducted for a large number of formae speciales. Some studies have indicated that isolates within
certain formae speciales are genetically very similar and are monophyletic. Using the nuclear EF-1α and the mitochondrial small subunit rDNA O’Donnell et al. (1998) showed that *F. oxysporum* comprises dozens of phylogenetically distinguishable species spread among 3 well-supported clades. In a related study, isolates within the f. sp. *cubense* fell into five phylogenetically distinct lineages distributed within two of the three major clades described by O’Donnell (Baayen et al. 1999). Using an extended dataset of 332 strains of *F. oxysporum*, O’Donnell and Cigelnik (1999) obtained four clades within the *F. oxysporum* complex in which approximately 80% of the formae speciales with more than two VCG groups appeared to be either para- or polyphyletic in origin. In another study using AFLP fingerprints, it was observed that the forma specialis *opuntiarum* consisted of a monophyletic group of isolates while formae speciales *dianthi* and *gladioli* had paraphyletic origins (Baayen et al. 1999). Thus, the the phylogenetic basis of species identification in *Fusarium oxysporum* is yet to be defined.

Results show that VCGs could be a good predictor of genetic similarity based on RFLP analysis, but difficulties may arise when isolates cannot be assigned to any specific VCG. Furthermore, when pathogenic and nonpathogenic strains of *F. oxysporum* are considered, the consistency of VCGs with genetic similarity is not absolute (Kistler 1997). A good example of a monophyletic lineage is *F. o. f. sp. albedinis*, the causal agent of Bayoud disease of date palm. A collection of 120 isolates from several geographic regions was found to belong to a single mtDNA haplotype and a single VCG (Fernandez et al. 1995). The forma specialis *lycopersici*, however, consisted of isolates that fall into more than two VCGs. Within the forma specialis *cubense*, 16 VCGs were
identified within a world wide collection of more than 600 isolates (Kistler 1997). Similar observations within forma specialis melonis showed that VCGs were aligned with distinctive mtDNA haplotypes but with a few exceptions (Appel and Gordon 1995). Thus, it is apparent that although the species F. oxysporum is a monophyletic group, the designation of forma speciales has little phylogenetic utility.

Race or host specificity to a particular genotype, may or may not always correlate with clonal lineage. Races of forma specialis dianthi correlated with VCGs and DNA fingerprint patterns (Manicom and Baayen 1993). In forma specialis lycopersici, all races belonged to a single clonal lineage. Thus, race determinants are generally considered to be simple and highly mutable (Gordon 1993).

**Transposable elements in Fusarium oxysporum**

The size of the genome of F. oxysporum is estimated to be 18.1 to 51.5 Mb with chromosome numbers varying between 7 and 14, including linear plasmids (Kistler and Leong 1986). It has been estimated that approximately 5% of the Fusarium genome is made up of transposable elements (TEs). These elements are thought to constitute the primary means by which diversity is introduced and maintained in the genome (Daboussi et al. 2002). To date, seventeen TEs have been characterized in F. oxysporum grouped into twelve families. These include the whole spectrum of eukaryotic elements from class I (those that transpose by reverse transcription of an RNA copy of the element) to class II (those that transpose directly through the DNA copies).

Identification of TEs is performed by several methods including trapping into a target gene, cloning dispersed repetitive DNA, or using a heterologous probe (Daboussi
The LTR-retrotransposons *Foret1, Skippy*, and the LINE-like element *Palm* belong to class I, while the class-II elements include Fot1, Fot2, *impala, Hop, Tfo1, Ac*-like (hornet), *mimp, han, marsu*, and *Folyt* elements (Hua-Van et al. 2000). Some of the elements are intact and active (*impD* and *Fot3*) while others seem to be relics of retroelements such as the *han* elements. The number and distribution of the elements vary in the different *F. oxysporum* strains and this has contributed to local rearrangements of the regions containing them.

Since *F. oxysporum* has no known sexual stage, the considerable amount of genetic variation observed in this fungus has been attributed in part, to the presence of the active TEs and perhaps a parasexual cycle following heterokaryosis (Daboussi 1996; Daboussi and Capy 2003; Teunissen et al. 2002). TEs induce mutations in the host by promoting changes in gene expression, gene sequence or chromosome structure. Clustering of TEs on chromosomes bring about chromosome rearrangements. Thus, TEs have been suggested to be agents of rapid genomic restructuring in natural populations of *F. oxysporum* influencing genetic diversity. Depending on where they insert, *Fot 1* elements leave footprints of different sizes. When they insert in an intron, excision leaves a footprint of 3 or 4 bp, and when they insert into an exon, excision result in three additional nucleotides encoding for an extra amino acid or no foot prints (Daboussi and Capy 2003).

**Sexual reproduction in *Fusarium oxysporum***

Sexual reproduction in the genus *Fusarium* is governed by bipolar heterothallism in which mating is controlled by one pair of mating type loci (MAT): MAT1-1 and
Compatible strains must have complementary alleles for successful sexual reproduction to occur. Field isolates of *F. oxysporum* contain either *MAT1*-1 or the *MAT1*-2 idiomorph that are very similar to those of the heterothallic species *Gibberella fujikuroi* suggesting that *F. oxysporum* is capable of sexual reproduction. However, there is no known sexual stage in the life cycle of *F. oxysporum*, although RT-PCR analysis has revealed that the *MAT* genes in *F. oxysporum* are expressed and processed correctly (Yun et al. 2000). In a study in which 106 isolates of nonpathogenic *Fusarium oxysporum* were characterized for mating type genes by PCR, 101 were of type 2 and 5 of type 1. RFLP analysis of these strains revealed all possible recombinant types in the sample. These results were interpreted as support for the hypothesis of the existence of a cryptic form of genetic recombination in *Fusarium oxysporum*. Furthermore, the presence of the two mating types suggests that sexual recombination may be present but infrequent (Yun et al. 2000).

The parasexual cycle in filamentous fungi was first discovered in *Aspergillus niger* and has been reported as occurring in other species of the genus (Tuveson and Garber 1959). It is believed that *F. oxysporum* may possess a cryptic parasexual cycle but this has not proven beyond the laboratory (Pontecorvo 1956). The parasexual cycle is defined as the production of a heterokaryon through the fusion of unlike haploid nuclei to form a diploid. Occasionally during this process, there is mitotic-crossing over during the multiplication of the diploid nuclei and occasional breakdown of the diploid nuclei to produce haploids through haploidization. Even though the parasexual cycle was demonstrated to exist in *F. o. f. sp. pisi*, the authors concluded that it not useful for
genetic analysis due to the fact that the fungus did not undergo conjugate nuclear division during the process (Tuveson and Garber 1959). It could be concluded that in the absence of an identified sexual reproduction, genetic exchange therefore in *F. oxysporum* is limited to mutations or parasexual cycle through heterokaryosis.

**Host range and the infection process of *Fusarium oxysporum***

*Fusarium oxysporum* is considered the most common of the species of *Fusarium* (Roncero et al. 2003). With the exception of some grasses and most tree crops, few widely cultivated plants are non-hosts to a pathogenic form of *F. oxysporum*. The fungus causes vascular wilts in such a wide range of economically important crops that it is believed that pathogenic forms must have evolved from the ubiquitous nonpathogenic forms. Both formae speciales and races within forma specialis differ in symptomatology, epidemiology, and cultivar susceptibility and can be differentiated by pathogenicity tests with appropriate host cultivars. Monogenic, dominant resistant traits have been described for known races of some notable *Fusarium* formae speciales (Diener and Ausubel 2005). Thus, the interactions between *Fusarium oxysporum* races and host cultivars are considered to be a gene-for-gene relationship (Ori et al. 1997).

As a soil inhabitant, *F. oxysporum* survives for extended periods in the soil as chlamydospores. The proximity of roots to these chlamydospores induces the dormant propagules to germinate. After germination, the infection hyphae adhere to the roots and penetrate directly (Di Pietro et al. 2001; Bishop and Cooper 1983). The mycelium advances intercellularly through the root cortex until it reaches the xylem vessels and then enters through the xylem pits (Bishop and Cooper 1983). The fungus grows within
the xylem vessels using them as avenues to rapidly colonize the host by producing microconidia that detach and are carried upward in the sap stream. The microconidia germinate and the hyphae penetrate the upper walls of the vessels and produce more microconidia. A combination of pathogen activities such as the accumulation of fungal mycelia, spores, and toxin production, and the host response by production of gels, gum, tyloses, and proliferation of adjacent parenchyma cells that crush xylem vessels results in the occlusion of the vascular system causing the characteristic wilt symptoms (Kovachich 1948; Beckman 1987).

The fungus remains limited to the xylem vessels and a few surrounding cells as long as the plant is alive, but invades the parenchymatous tissue and sporulates profusely on the plant surface upon death of the plant (Di Pietro et al. 2003). The spores may then serve as secondary inoculum and initiate secondary infections on other nearby plants. Successful infection of a host requires several stages that include the recognition of roots through unknown host signals, root surface attachment and differentiation of penetration hyphae, and penetration of the root cortex. The pathogen then has to degrade the physical host barriers such as the endodermis in order to reach the vascular tissues where it lodges. Once in the vascular tissues, it has to adapt to the hostile plant environment and tolerate plant antifungal compounds. It is only after hyphal proliferation that production of microconidia within the xylem vessels and secretion of virulence determinants such as small peptides or phytotoxins occurs (Di Pietro et al. 2003).

*Fusarium oxysporum* is believed to perceive environmental cues via cell receptors and signal transduction cascades which regulate expression of effector genes that are
directly involved in pathogenesis. The resting chlamydospores immediately germinate in the presence of a potential host stimulant such as soluble or volatile compounds from root exudates. The exact nature of the stimulating compounds is yet to be identified but nutrients and certain flavonoids have been implicated (Osherev and May 2001; Ruan et al. 1995). Ambient pH is thought to act as a key signal modulating cell growth, development, and possibly pathogenicity in *F. oxysporum*. A pH signaling cascade with a terminal zinc finger transcription factor PacC, which acts as a negative regulator of acid-expressed genes was shown to be important in pathogenicity (Caracuel et al. 2003). An orthologous mitogen-activated protein kinase (MAPK) signaling pathway has also been identified in *F. oxysporum* and is thought to be required for direct root attachment and penetration (Schoffelmeer et al. 2001).

Cell wall-degrading enzymes are of key importance in two phases of the development of vascular wilt: during penetration of the different layers of the root cortex to enter the xylem vessels and during spread within the host upward through the xylem vessels. When *F. oxysporum* contacts the host cell wall, it secretes a set of extracellular cell wall-degrading enzymes that are regulated by substrate induction and catabolite repression. Among these enzymes are pectate lyase I (PLI) and pectate lyase II (PLII), both of which were isolated from *F. oxysporum* f. sp. *ciceri* (Artes et al. 1990). Pectate lyase has also been detected inside tomato stem and root tissues infected with *F. oxysporum* f. sp. *lycopersici* and the transcription of the *plI* was observed during different stages of the disease cycle (Di Pedro and Roncero 1996). Three similar pectate lyases, *pelA, pelB*, and *pelD*, which are involved in the depolymerization of pectin by trans-
elimination of pectate, were also identified during colonization of pea roots by *F. solani* (Rogers et al. 2000). Also polygalacturonases (PGs) have been shown to be important in cell wall degradation, and the major ones identified to date are three endoPGs, (*PG1*, *PG4*, and *PG5*) and two exoPGs, *PG2* and *PG3*. The endoPGs are involved in plant tissue maceration by depolymerising the homogalacturans, which are major components of the plant cell wall (Garcia-Maceira et al. 2000; Garcia-Maceira et al. 1997; DePietro and Roncero 1996). This fungus is also capable of targeting the major component of hemicellulose, xylan, by secreting xylanolytic enzymes (Alconada and Martinez 1994). Targeted disruption of most of these genes involved in cell wall degradation did not show any reduction in virulence in most cases due to their redundancy within the fungus or lack of involvement.

*Fusarium oxysporum* penetrates roots directly without the need for fully differentiated infection structures. Upon penetration, the fungus is assailed by numerous defensive compounds from within the plant such as phytoanticipins and phytoalexins. The best studied example is the tomato saponin alpha-tomatine in tomato and acetophenone in carnation. Different formae speciales of *F. oxysporum* are known to produce inducible extracellular alpha-tomatinase that cleaves the alpha-tomatine into tetrasaccharide lycotetrose and an aglycone tomatidine which is less toxic to the fungus (Ford et al. 1977; Lairini et al. 1996; Lairini et al. 1997). *F. oxysporum* f. sp. *dianthi* detoxifies the carnation phytoanticipin acetophenone to phenyethanol which has lower fungitoxic activity (Curir et al., 2000). Other nondegradative tolerance mechanisms employed to overcome the host antimicrobial compounds have been identified. The class
V chitin synthase responsible for the synthesis of chitin is required for pathogenicity and has been shown to play an important role in tolerance to plant antifungal compounds (Madrid et al. 2003). Since pathogenic strains of *F. oxysporum* are thought to have derived from nonpathogenic ones, and considering the great number of specific forms recognized, it is possible that different combinations of genes are responsible for pathogenicity on the different host plants.

**Molecular diagnostics of fungal plant pathogens**

Pathogens of plants most often are found associated with other microorganisms and the identification of the causal organism of a particular disease is often complicated by this association. For a given crop, the ability to distinguish between different diseases usually dictates the appropriate control measures to be applied. All aspects of plant pathology including disease epidemiology, implementation of plant disease regulations through quarantine, and seed phytosanitation require the ability to accurately diagnose the presence of pathogens and identification of virulent pathotypes within pathogen population (Atkins and Clark 2004; McCartney et al. 2003).

Historically, conventional methods of identifying fungal plant pathogens have relied on interpreting the visual symptoms, isolation, culturing, and laboratory identification by microscopy. The accuracy and reliability of the methods have depended on most part on the experience and skill of the person making the diagnosis. Usually these conventional methods are time consuming and impractical when rapid results are required. Thus, the development of rapid DNA-based methods is desired. The development of DNA-based diagnostics requires three basic steps: the selection of the
genomic region to be used to recognize the pathogen; extraction of fungal DNA from the sample; and a method to identify the presence of the target DNA in the sample (MacCartney et al. 2003). Current diagnostic methods ranged from immunoassays to nucleic acid-based techniques.

Although, serological methods have been available for several years, their application to plant pathology has seen a modification towards an advanced technological system (Martin et al. 2000). Immunoassays are being used routinely for the detection of plant pathogens in vegetatively propagated material and seeds in conjunction with quarantine seed-testing, seed-certification, and pathogen-indexing programs. Briefly, immunoassay techniques involve the binding of a specific antigen that is made visible through the use of an enzyme which acts upon its substrate and generates a colored product. Presently, the enzyme linked immunosorbent assay (ELISA) kits for the detection of some plant pathogenic viruses, bacteria, and fungi are commercially available (Martin et al. 2000).

Other variants of immunoassays exist, such as the use of colloidal gold which is conjugated to an antibody that binds to the antigen and the ensemble is viewed with a microscope (Martelli and Walter 1998). Immunofluorescent assays use fluorescent dyes such as fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC) to quantify, detect, and localize fungi in plant material and soil. However, use of these methods has been restricted by the autofluorescence of plant material and fungicides in soil samples and the adsorption of tagged antibodies to soil particles (Schots et al. 1994; Kuske et al. 1998). Another variant of the immunological method is radioimmunoassay
that is used routinely in clinical microbiology laboratories but infrequently for plant
pathogen detection due to the risk associated with radioisotopes (Dobrowolski and
O’Brien 1993, Palfreyman 1998). Taken together, imunoassay application has been
complicated by the issue of selection of the type of immunogen to use (whole cells, cell
walls, cell membranes etc), and the difficulty in producing highly specific monoclonal
antibodies (MABs). Nevertheless, MABs do exist that have been developed for a few
plant pathogens including Phytophthora cinnamomi (Goodwin et al. 1990; Gundersen
etal. 1994).

The first DNA-based method developed was nucleic acid hybridization which
depends on the high degree of specificity inherent in the pairing of nucleotide base
sequences. This method used labeled probes or restriction fragment length polymorphic
markers to detect the target organism. The technique involves labeling one of the
nucleotide sequences to provide the necessary signal to follow the hybridization. The
most commonly used label is $^{32}$P which is incorporated into the “detecting” strand of
nucleic acid. However, nonradioactive compounds are presently available and are
replacing $^{32}$P (Maritin et al. 2000). Nucleic acid probes offer an advantage over
serological methods in that a test can be made for a whole set of genes within a given
pathogen. One method that offers considerable potential for plant-pathogen detection is
the dot-blot assay (Ling et al. 1998). The target nucleic acid from a plant- or insect-
vector sample is spotted onto a solid matrix (nitrocellulose), and bound by baking. Free
binding sites on the nitrocellulose are then blocked with nonhomologous DNA usually
salmon sperm or calf-thymus DNA and protein source (usually bovine serum albumin
(BSA) or nonfat dried milk). Thereafter, hybridization with a labeled probe is carried out. The label is then detected by autoradiography, for $^{32}$P labels, or a colorimetric reaction if an enzyme label is used.

DNA probes are usually designed from plasmid libraries of random fragments of fungal DNA and then tested for their specificity as probes. This technique was successfully used for the identification of *Phytophthora citrophthora*, *P. parasitica* (Goodwin et al. 1989; Goodwin et al. 1990). Instead of using random probes, a sequence specific to a species can be selected and used as an oligonucleotide hybridization probe. The use of restriction fragment length polymorphisms (RFLPs) has been increasing in fungal taxonomy in recent years (Martin et al. 2000). The technique involves restriction enzyme digested genomic DNA that is hybridized to a probe which could be random or specific. Most RFLP work with fungi has focused on mtDNA which is smaller than genomic DNA in order to get distinct banding patterns (Di Bonito et al. 1995).

Diagnostic methods based on the polymerase chain reaction (PCR) amplification of specific nucleic acid sequences constituted the major breakthrough in the field of molecular taxonomy and detection (Henson et al. 1993). The specificity of PCR ultimately depends on the design of proper PCR primers that are unique to the target organism. Several genomic targets for designing primers have been used ranging from single proteins unique to the microorganism to the multicopy genes of the rDNA (Schaad and Frederick 2002). Primers may also be developed using randomly amplified polymorphic DNA (RAPDs) and amplified fragment length polymorphic DNA (AFLPs) markers (Pooler et al. 1997; Pryor and Gilbertson 2001). The region of the genome
coding for ribosomal DNA has been the most extensively used in phylogenetic analysis. Within this region is the internal transcribed spacer region which has been used preferentially to design primers for species differentiation. Primer pairs that are specific for *Phytophthora citricola* was designed from within the ITS1 and ITS2 and was used to detect the fungus in inoculated plants (Schubert et al. 1999). Other genomic regions have been utilized and sometimes two different but linked loci have been used. An example of a primer pair used in the detection of *Phytophthora fragariae* was designed from part of the intergenic spacer region (IGS) and the 5' end of the large subunit rDNA. Sequences from the elicitin gene *ParA1* was also used in the detection of *Phytophthora nicotianae* directly from inoculated tomato and tobacco plants (Lacourt and Duncan 1997). In situations where the primers lacked sensitivity in a single round of PCR and there is cross-reaction, improvement was brought about by using nested PCR (Bonants et al. 1997).

A less time consuming technique is the use of real-time PCR. During real-time PCR, the accumulation of the PCR products is measured automatically during each cycle in a closed tube format using an integrated cycler/fluorimeter. The initial amount of target DNA in the reaction is related to a cycle threshold (*ct*) defined as the cycle number at which there is a statistically significant increase in fluorescence. Target DNA is quantified by construction of a calibration curve that relates *ct* to known amounts of template DNA. PCR products can be monitored using either fluorescent DNA intercalating dyes such as SYBR Green 1, or sequence specific probe based assays using TaqMan probes or molecular beacons (Wittwer et al. 1997; Tyagi et al. 1998). The use
of intercalating dye is less specific as it binds to all double stranded DNA present including any primer dimers and can lead to false positives. The specificity of the sequence probe is such that it can allow for single nucleotide polymorphisms to be targeted, and therefore, can be isolate specific. Probes with different reporter dyes can be used to allow several isolates to be monitored together.

Three methods are available for detecting the production of PCR amplicons: these are TaqMan® probes, fluorescent resonance energy transfer (FRET), and molecular beacons (Mackay et al. 2002). The TaqMan® system uses an oligonucleotide that is labeled at both the 5’ and 3’ ends with fluorochromes. During PCR amplification, the probe is degraded by the 5’-3’ exonuclease activity of the Taq polymerase and the fluorophore is released. The amount of fluorescence is proportional to the amount of PCR product generated (Lee et al. 1993). Presently, less expensive fluorogenic primers are replacing the TaqMan probes in real-time PCR technology to make the technique more available to users (Nazarenko et al. 2002). The FRET probes require labeling two oligonucleotide probe sequences that anneal head to tail on the target sequence and fluorescence is measured during the annealing step of each of the amplification cycles when both probes hybridize to the PCR amplicon (Cockerill and Smith 2002). Molecular beacons are fluorescent oligonucleotide probes that are designed as stem loops with a fluorescent chromophore attached at the 5’ end and a quencher molecule attached to the 3’ end. They are complementary to the nucleotide sequence of the target amplicon. During amplification the fluorophore and quencher are separated from each other and fluorescence can be detected (Didenko 2001; Cockerill and Smith 2002).
Real-time PCR has many advantages over classical PCR. It is a more contained system and less prone to cross contamination, optimization of PCR protocol is easier, and it can be used for multiplex PCR (Schaad and Frederick 2002). Even though real-time PCR assays have been developed to detect and identify several fungal species, several challenges remain to developing successful real-time PCR assays for fungal pathogens. The most important being the availability of efficient protocols for extraction of DNA from plant materials (Schaad and Frederick 2002) and the availability of highly specific probes.

**Selection of molecular markers**

Two approaches are in use for the selection of target DNA: 1) identify known conserved genes with sufficient sequence variation within and between them, and 2) screen random parts of the fungal genome to find regions that show the required specificity. The most common conserved region targeted for fungal diagnostics is the ribosomal DNA (White et al. 1990). This region presents a number of useful features including presence in all organisms at high copy number that allows for very sensitive detection. rDNA is encoded in both the nucleus and the mitochondrion. The nuclear rDNA consists of a transcriptional repeat unit made up of three genes, the small subunit (18S), a 5.8 rDNA gene, and the large subunit (28S). The three genes are separated by two internal transcribed spacers, ITS1 and ITS2. Each repeated unit is separated from the next by a non-transcribed spacer, the intergenic spacer region (IGS), which lies between the large subunit and the small subunit. The large subunit and the small subunits are well conserved between species but the spacer regions and the mitochondrial rDNAs
are more variable within species (MacCartney et al. 2003). Sequence polymorphisms are often identified by using restriction enzyme analysis of PCR amplified products to differentiate between the strains. Probes can also be generated from amplified products and used in identification (Ward and Gray 1992). More commonly, taxon-specific PCR primers are designed from amplified products to target the DNA sequences that are unique to the organism in question. When rDNA sequence variation is not suitable for production of a taxon-specific diagnostic, other markers such as β-tubulin and mating type genes have been employed (Hirsch et al. 2000; Fraaije et al. 2001). These markers have the disadvantages of being single copy in the genome, thus, sensitivity of detection is reduced.

Diagnostic genetic markers may also be obtained by screening random regions of the genome to find sequences that are unique to a particular taxon. Traditionally, fragments of the pathogen genome are cloned and each clone is used as a probe against related genomes to find ones that are specific to the taxon in question. The specific clones are then sequenced and used to design specific primers for detection (Henson, 1989). Repetitive sequences of the genome can be used in designing specific primers. PCR-based markers commonly used to generate randomly amplified fragments of the genome are random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs), and intersimple sequence repeats (ISSRs). The amplified products are then separated by gel electrophoresis and the patterns of bands from the group of fungal isolates are compared. Potentially diagnostic bands may then be
sequenced and used to design specific sequence characterized amplified regions (SCARs) primers (Nicholson et al. 1998).

One of the main limitations with PCR is quantification. Although the technique can detect minute amounts of target signal, without a means of quantification, assessment of the incidence of the disease or decisions on whether to treat the disease, is usually delayed until methods of quantification such as culturing on agar plates can be performed. Techniques have been developed and are now available that can be used to quantify the PCR target signal. Fluorescent in situ PCR provides a direct method of quantification of a fungus from a fixed sample. Competitive PCR involves an additional target sequence that is added to the product of a different size to the fungal DNA to be amplified. The added target sequence is serially diluted over a range of PCR reactions and the level is calculated before addition to the PCR reaction. The additional target sequence is recognized by the same primers and therefore competes for them during the reaction (Mauchline et al. 2002). Presently, it is possible to label the specific primers for use in real-time PCR, thus making quantification possible (Schaad and Frederick 2002).

**DNA fingerprinting and genotyping of plant fungal pathogens**

DNA fingerprint methods directly measure DNA differences among strains within a species. Over the years, the increase in molecular genetic approaches has resulted in a concomitant increase in DNA fingerprinting methods for a variety of fungal plant pathogens. DNA fingerprint techniques provide reliable tools for tracking strains, identifying the sources of particular infections, assessing the genetic relatedness of isolates of the same species within a pathogen population, understanding the dynamics of
a pathogen in a host population, deciphering the complex relationship between a saprobe and pathogen, and monitoring the emergence of host resistant strains. An isolate has been defined as a clone collected independently of other isolates, in contrast to a strain which is a collection of isolates of the same species that are highly related or genetically indistinguishable (Soll 2000). Many fingerprint methods are presently in use but in order to be effective, a fingerprint method must have sufficient resolving power to discriminate one particular strain from a majority of other strains in the same population or other populations. Furthermore, the method must be amenable to computer-assisted analysis and be reproducible and quantitative (Soll 2000).

One of the first DNA fingerprint methods used to assess strain relatedness in the fungi is restriction fragment length polymorphism analysis (RFLP). In this method, DNA is extracted from the target pathogen, digested with one or more endonucleases and the resolved in an agarose gel. In this case, the resolution is poor due to the fact that all restriction fragments are stained. To visualize particular fragments in the pattern, a southern blot of the RFLP gel is performed with a radio-labeled or biotinylated DNA sequence that recognizes one or more fragments as a result of sequence homology. Thus rDNA probes have been developed for the use in DNA fingerprinting of fungi (Magee and Magee 1987; Mercure et al. 1993). However, the level of resolution using total genomic DNA is low and the patterns generated were not complex enough for effective fingerprinting. Consequently, more complex probes have been cloned from fragments containing repetitive genomic sequences. Probes containing repeat sequences have been used to discriminate among strains of Cryptococcus neoformans and Aspergillus flavus.
Among the variety of PCR-based methods developed for DNA fingerprinting purposes (Caetano-Anollés 1993), randomly amplified polymorphic DNA has emerged as the most popular method. In this method, random primers of approximately 10 mers are used in a genome-wide amplification. Several RAPD primers have been developed but for each particular species, a number of oligonucleotides have to be tested and those that provide the best variability among independent isolates selected (Soll 2000). This method has been successfully applied to *Candida albicans*, *Aspergillus flavus*, *Histoplasma capsulatum* and *Blastomyces dermatidis* (Bart-Delabesse et al. 1995; Buffington et al. 1994; Kersulyte et al. 1992; Yates et al. 1995). Despite the testing, several probes have emerged with the use of this method but it is difficult to reproduce results not only among laboratories but also within the same laboratory due to the methodological aspect of PCR associate with it (Ellsworth et al. 1993).

Amplified fragment length polymorphism is a modification of the RAPD method that selectively amplifies restriction fragments in a genomic DNA digest. Selective amplification is achieved by addition of selective bases to the 3’ end of primers (Vos et al. 1995). This method is gaining popularity in genotyping plant pathogenic fungi. Another fingerprinting method is the use of microsatellites. The primers for the repeat regions can be designed outside of the region or within the repeats. Microsatellites and minisatellite primers are reported to be more effective since the sequences are usually dispersed throughout the genome. Just as with repeat sequence probes, variability due to high frequency of change in the sequences may reduce the effectiveness of the method in
clustering moderately related isolates. The choice of DNA fingerprinting method therefore, will depend on the epidemiological questions to be answered and on its efficacy in accessing strain relatedness at four levels of resolution namely: identify the same strain in independent isolates, identify microevolutionary changes in a strain (recognize highly related but nonidentical isolates), cluster moderately related isolates, and identify completely unrelated isolates (Soll 2000).
Figure I.1. Lettuce field showing dead lettuce heads due to infection with *Fusarium oxysporum* f. sp. *lactucae*. (Photo courtesy of Dr. Barry Pryor, Department of plant Sciences, 2003).
II PRESENT STUDY

This work involved a number of studies on the fungus *Fusarium oxysporum* f. sp. *lactucae*, causal agent of Fusarium wilt of lettuce. The main objectives were to study its evolutionary history compared to other formae speciales of *Fusarium oxysporum* inferred from three genomic regions, and to use this knowledge to develop a PCR-based detection method in seed. In the course of this study several additional studies on the genetic variation and nature of transmission were conducted. It was the aim of these investigations to further develop basic knowledge of *Fusarium oxysporum* f. sp. *lactucae*, in order to provide a strong foundation for more applied aspects of Fusarium wilt management. The methods, results, and conclusions of these studies are presented in the sections appended to this dissertation. Following is a summary of the methodology employed, the most important findings, and the conclusions drawn from such findings.

All *F. oxysporum* f. sp. *lactucae* strains collected in this study were recovered from infected lettuce tissue and from field soil using standard phytopathological isolation techniques and standard soil dilution plate methods, respectively. For recovery from lettuce, lettuce roots were 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 (Komada 1975). For both isolations from roots and soil, putative *F. oxysporum* colonies were subcultured on identification media (Komada’s, potatoes dextrose agar [(PDA) Difco brand; Becton, Dickinson and Co., Franklin Lakes, NJ] and 0.5% KCl medium (Burgess et al 1994) and their identity was confirmed by morphological characterization.
All other isolates were obtained from collaborators as specifically acknowledged in the
different sections of the dissertation.

Pathogenicity of putative *F. oxysporum* f. sp. *lactucae* isolated from diseased
lettuce tissue and field soil, and isolates obtained from collaborators was assessed on
three major commercial lettuce cultivars (Beacon, Lighthouse and Winterhaven). These
tests were set up in the greenhouse using ten-day-old seedlings and 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 to control, 2 = plant severely stunted, and 3 =
plant dead. Disease incidence was calculated for each isolate/cultivar combination.

DNA extraction was performed from lyophilized mycelia cultured on PDA using
the BIO 101 Fast DNA kit (Qbiogene, Irvine, CA) according to the manufacturer’s
instructions. For each study genomic DNA concentrations were adjusted to 10 ng/µl.
Polymerase chain reaction (PCR) was performed to amplify the mtSSU, EF-1α gene, and
IGS rDNA using specific primers. Each PCR reaction contained 0.2 mM
deoxynucleotide triphosphates, 2.5 mM Amplitaq MgCl₂, 0.8 µM of each primer, 1X
Amplitaq reaction buffer, and 2 units 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 standardized steps for each polymerization. The
amplified products were purified with the aid of a QIAquick PCR purification kit (Qiagen
Inc., Stanford, CA) and all sequencing was done at the DNA Sequencing Facility,
University of Arizona. Phylogenetic analyses were performed on the aligned sequences
using the Phylogenetic Analysis Using Parsimony package (PAUP, ver. 4.0b10; Sinauer Associates).

Sequence analysis of the IGS rDNA revealed polymorphisms that were used to design primer sets for PCR-based detection of *F. oxysporum* f. sp. *lactucae*. Sensitivity of the primers were assessed with 10-fold serial dilutions of *F. o.* f. sp. *lactucae* total genomic DNA and the capacity of the primers to direct the amplification of the target DNA fragment from decreasing concentrations of DNA was determined. Specificity was determined by testing the primers pairs on total genomic DNA (10 ng/μl) from 13 fungal species. Based on this nested-PCT (nPCR) method a seed assay was developed for the detection of *F. oxysporum* f. sp. *lactucae* in lettuce seed. An incubation period was incorporated in the seed assay during which fungal biomass on the seed was increased. PCR with DNA recovered from seed was carried out with the addition of 1 ul of 10% skim milk. Eighty-eight seed lots obtained from commercial seed producers in Arizona and California were assayed for the presence of *F. oxysporum* f. sp. *lactucae* using both the agar plate method and nPCR-based assay.

Lettuce cultivars Sharpshooter, Vulcan, and King Henry each belonging to head, leaf, and romaine types, respectively, were selected for studies on the potential and mode of seedborne transmission of *F. oxysporum* f. sp, *lactucae*. These cultivars were chosen based on their moderate resistance to *F. o.* f. sp. *lactucae* because susceptible cultivars would not survive infection until seed production. Ten-day-old seedlings of these cultivars were either root-dip inoculated or transplanted into soil inoculated with the pathogen. The third method of inoculation that consisted of spraying the flowers with a
spore suspension of the pathogen was included as a positive control. The experiment was set up in a completely randomized design and was repeated twice in the greenhouse. The lettuce was grown to maturity and whole plants were harvested for systematic plating to recover the pathogen. Seeds harvested from each treatment were divided into two parts; one part was plated out on Komada’s medium without surface disinfestations and the other part was surface disinfested in 10% NaOCl for 3 minutes, and plated out on Komada’s medium for reisolation of the pathogen. Plant debris from the root and flower sprayed treatments were used to infest pathogen-free seed and the level of contamination determined to assess the possibility of infested plant debris contaminating seed under field conditions. Two seed lots artificially infested with \textit{F. oxysporum} f. sp. \textit{lactucae} stored at room temperature for over a year and four months, respectively, were evaluated for germination potential in the greenhouse.

Isolates from Arizona and California were collected over a period of four years from disease lettuce roots and infested field soil and were used in the population study of isolates of \textit{F. oxysporum} f. sp. \textit{lactucae} currently present in both Arizona and California. A total of nine isolates including one race 1 and two race 2 isolates from Japan, three race 1 isolates from Italy, and three race 1 isolates from Taiwan were also included in the study. Inter-simple sequence repeat and minisatellite primers were used to generate fingerprints for these isolates and cluster analysis were performed using similarity coefficient as estimated by Jaccard’s method. The dendrograms were generated using the NTSYSpc2.0 program (Exeter Software, Setauket, NY). Bootstrap values (1000 replicates) for each branch were calculated using Phylogenetic Analysis Using Parsimony
package (PAUP, ver. 4.0b10; Sinauer Associates). Population structure and differentiation were estimated using total genetic variation partitioned among geographic origin and gene diversity within population were calculated using POPGENE 1.31 (Yeh et al., 1999).

Several significant findings emerged from these studies that have direct bearing on the management of Fusarium wilt. In particular, the fact that the two races have acquired pathogenicity independently was reflected in the sequences of the genomic regions used in the phylogenetic analysis. Work by others suggests that the 3rd race also evolved independently. The phylogenetic studies conducted also included various f. spp. of *Fusarium oxysporum* and should be of widespread interest due to the economic importance of this fungus. Analysis of sequence divergence within the Ef-1α, the mtSSU and the intergenic spacer rDNA has provided a new insight into the phylogenetic relationships within the forme species and between the forme species and other formae speciales. The phylogeny revealed a close relationship between *F. o. f. sp. lactucae* to f. spp. *matthioli* and *phaseoli*. Even though these two strains are not pathogenic on lettuce, it nevertheless shows that divergent pathogenicity can evolve independently in *Fusarium oxysporum* strains with similar genetic backgrounds. Additional work is necessary to more fully resolve the taxonomy of the formae speciales, and this should include at least three isolates of each forma specialis.

Of most significance is the development of the PCR-based method for the detection of the pathogen in seed. Primers for the detection of *F. o. f. sp. lactucae* were designed, and their use in this PCR-based assay allowed for the detection of the fungus in
seed lots with infestation as low as 0.1%. The sensitivity of the method was greatly improved by the nested primer pair used in the second PCR. The PCR-based detection method can also be applied to a limited extent to detect the pathogen in disease tissue. This method can be adapted for use in detecting the fungus in soil if DNA extraction kits that will limit the amount of PCR inhibitors in the DNA extract from soil are used and the reaction conditions optimized. By providing a method to detect *Fusarium oxysporum* f. sp. *lactucae* in seed, we were able to screen commercial seed lots for the presence of the pathogen and our results suggest that these particular seed lots may not be involved in the spread of the pathogen in Arizona.

The results of the greenhouse experiment indicates that *F. o. f. sp. lactucae* is not internally seedborne and that seed transmission from one location to the another is probably through inoculum on the surface of the seed. In this study, it was difficult to reisolate the pathogen from the mature lettuce plants and the method of direct systematic plating was time consuming. In the future, an easier method may be to label the pathogen with green fluorescent protein before inoculating the plants so that identification could be done by confocal laser scanning microscopic analysis, a technique which has been used successfully with *F. o. radicis-lycopersici* (Lagopodi et al., 2002). This technique will positively detect the movement of the pathogen up the stem and will also easily distinguish between any nonpathogen found on/in the seed from the inoculum used.

The variability within the species was also examined in this study. The results indicate that there are 8 haplotypes in Arizona and 23 haplotypes in California. The greater genetic variation within the California isolates was to be expected since the
disease appeared there first. *Fusarium oxysporum* is endemic and cosmopolitan in soils worldwide where they aggressively colonize the root cortex of native hosts without causing disease. The emergence of Fusarium wilts in agricultural systems has been attributed to the selective pressure of monocultures on nonpathogens of this fungus. Movement of the pathogen to other locations has been basically through genotype flow. Different monocultures select for different *F. oxysporum* formae speciales from the nonpathogenic ancestral populations (Gordon and Martyn, 1997). The same forma specialis can originate independently in different agroecosystems, thus generating wilt strains with different genetic backgrounds. This has been documented for the formae speciales that are polyphyletic in origin and this hypothesis is consistent with our findings (Koenig et al., 1997). For those formae speciales that are monophyletic and widespread, the spread is thought to be potentiated by genotype flow to other locations (Baayen et al., 1998, Jimenez-Gasco et al., 2002). As the forma specialis *lactucae* is polyphyletic, a determination of its center of origin will need a more representative sampling of the different races and isolates from around the world.

Most genetic diversity studies on *F. oxysporum* have focused on preexisting subspecific categories such as forma specialis, VCG, and race. Most often a new form is tested only on the host from which it was isolated. The effect of plant cultivar on the profile of isolates recovered from the field should be assessed. Sampling the pathogen populations may establish host preference or selectivity of genotypes and the potential for cultivars to have long-term effects on the composition of the pathogen population. Future work therefore, should concentrate on how populations of *F. oxysporum* change over
time and the factors that influence that change. The mechanisms available for genetic change in *F. oxysporum* are still largely unknown, but numerous possibilities exist beyond simple sexual or clonal reproduction. Recently, simple sequence repeat markers for species in the *Fusarium oxysporum* complex have been developed (Bogale et al., 2005). These markers may be the most appropriate for studying the genetic variation within populations of this important plant pathogen. Furthermore, the results obtained with one set of markers must be compared to other appropriate markers such as AFLPs or nuclear repetitive DNA sequences to further support data derived from these studies.
REFERENCES


APPENDIX A

PHYLOGENY OF *Fusarium oxysporum* f. sp. *lactucae* INFERRED FROM MTSSU, EF-1A, AND NUCLEAR RIBOSOMAL IGS SEQUENCE DATA

This manuscript, which is in press, will be published January 2007 in the journal *Phytopathology*. Research involving sequencing, phylogenetic analysis and characterization of the EF-1α, mtSSU and IGS rDNA was performed exclusively by Gladys Mbofung in the Pryor Laboratory with the exception of sequence alignments and editing which were performed by Dr. Soon Gyu Hong and Gladys Mbofung. This manuscript was written by Gladys Mbofung, based on her research data and was revised and corrected by Drs. Soon Gyu Hong and Barry M. Pryor.
Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* inferred from mtSSU, EF-1α, and nuclear ribosomal IGS sequence data.

Gladys Y. Mbofung¹, Soon Gyu Hong¹,², and Barry M. Pryor¹.

¹Division of Plant Pathology and Microbiology, Department of Plant Sciences, College of Agriculture and Life Sciences, University of Arizona, Tucson, Arizona 85721, USA
²Polar Biocenter, Korea Polar Research Institute, KORDI, Songdo Technopark, Songdo-dong 7-50, Yeonsu-gu, Incheon, 406-840, Korea

ABSTRACT

*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 alpha (EF-1α) gene, and the nuclear rDNA 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 *forme speciales* with the *lactucae* isolates. Analysis of the IGS region revealed numerous sequence polymorphisms among *F. oxysporum forme 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 f. spp. of *F. oxysporum*. In all analyses, *lactucae* race 2 isolates comprised a separate lineage that was phylogenetically distinct and distantly related to the *lactucae* race 1 isolates thus indicating a polyphyletic origin for the two races.

**INTRODUCTION**

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* Schlechtend: Fr. f. sp. *lactucae* Matuo and Motohashi (22) and pathogenicity on lettuce was confirmed. In the US, the disease was first reported in the San Joaquin valley of California in 1993, where it was responsible for lettuce losses in 3 fields near the town of Huron (18). The disease was subsequently reported in Arizona in 2001 in five fields near the town of Yuma and in 11 additional fields during the 2002-2003 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 is commonly 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 *forma specialis* (f. spp.) 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 *Fusarium oxysporum* species 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* has often 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 alpha (EF-1α) gene, and nuclear rDNA intergenic spacer (IGS) rDNA 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, 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 A.1. *Fusarium oxysporum* isolates from Arizona were recovered 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, potatoes 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, ten grams of each soil sample (approx. 200-300 g total for each field soil sample) were suspended in 200 ml of 1% sodium hexametaphosphate and stirred for 8 minutes. Twenty milliliters of the suspension were added to 80 ml of 0.1% water agar and stirred for 5 minutes. Five plates of Komada’s selective medium were inoculated each with 1 ml of the suspension per soil sample. Following 7 d incubation at 25°C, putative *F. oxysporum* colonies were then 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 ten-day-old seedlings were washed in running tap water, and approximately 1 cm of root tip was removed. The wounded roots were dipped in a spore suspension containing $1 \times 10^6$ spores/ml for 10 minutes. For each fungal isolate/lettuce cultivar combination, eight inoculated seedlings were transplanted to 20 cm wide plastic pots containing a sterile mix of 1 part peat, 1 part vermiculite, and 2 parts sand (1:1:2). The experiment was set up in the greenhouse (24-29 C, RH=50-60%) in a completely randomized design (CRD). 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 to control, 2 = plant severely stunted, and 3 = plant dead. Disease incidence was calculated for each isolate/cultivar combination. In addition, a disease index (DI) was calculated for each isolate/cultivar combination using the formula: $\frac{(n_0) + (n_2 \times 2) + (n_3 \times 3)}{(n_0 + n_1 + n_2 + n_3)}$, 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 ANOVA (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 potato dextrose agar 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/mycelia suspension (2 ml) was used to inoculate 100 ml of liquid growth medium (1.2 g of DL-asparagine, 0.2 g NaCl, 1.2 g K₂HPO₄·3H₂O, 0.25 g MgSO₄, 0.5 g yeast extract, and 20.7 g D-glucose in one 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 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 A.2). Each PCR reaction contained 0.2 mM deoxynucleotide triphosphates, 2.5 mM AmpliTaq MgCl₂, 0.8 μM of each primer, 1X AmpliTaq reaction buffer, and 2 units 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 (approximately 2600 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 A.2). The map of the amplification and sequencing primers for the IGS region are presented in Fig. A.1A. All IGS as well as the mtSSU and EF-1α sequences have been submitted to GenBank (Table A.1) and their alignments have been submitted to TreeBASE (SN2653-10318).

**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 5th 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 (AIC) using Modeltest (ver. 3.07; http://darwin.uvigo.es/software/modeltest.html). Clade stability was assessed using 500 bootstrap replicates with rearrangements limited to 10,000 per
replicate to reduce computational time. Two *Fusarium* species, *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, N.S., 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) was also 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 to the best trees generated without any constraint by Kishino-Hasegawa, Templeton, and Winning-sites tests implemented in PAUP. The Kishino-Hasegawa 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 datasets. 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
Winning-sites 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 A.1).
Among the *F. oxysporum* isolates recovered from soil in Arizona (n = 38), approximately
65% were pathogenic on all the 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 A.3). Thus, the determination of pathogenicity was
unambiguous and the utilility 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 A.3). There was no significant difference (*P = 0.071*)
in disease index among isolates on the cv. Winterhaven (Table A.3). However, there
were significant differences ($P < 0.001$) in disease index among isolates on the cvs. Beacon and Lighthouse (Table A.3). When the experiment was repeated, results were very similar with the exception of BMP1324 on the 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 A.1).

**DNA amplification**

The mtSSU primers NMS1 and NMS2 amplified fragments ranging in size between 730 and 739 base pairs (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 2547 and 2600 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 dataset 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. A.2]. The major clade 3 included all *lactucae* race 1 isolates, 13 other non f. sp. *lactucae* isolates, and nonpathogenic isolates with strong bootstrap support (96%). Clade 2, grouping together 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. sp. perniciosum, cubense, and canariensis with a bootstrap support of 86%. When gaps were coded as a 5th character state, the same phylogeny was obtained with similar bootstrap support values (data not shown). Maximum likelihood analysis resulted in a near identical tree (slight differences in branch lengths) as that obtained in parsimony analysis with similar bootstrap values (Fig. A.2).

**EF-1α phylogeny**

Alignment of the EF-1α sequences generated a dataset 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. A.3). The lactucae race 1 isolates grouped together with seven other f. spp, 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 5th character
state revealed the same phylogeny with similar bootstrap support values (data not shown). Maximum likelihood analysis resulted in a near identical tree as that obtained in parsimony analysis with similar bootstrap values (Fig. A.3).

IGS phylogeny

Assembly of the IGS contigs and their alignment resulted in a dataset of 2226 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. A.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, 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 addition, f. sp. *medicaginis* oscillated between clade 3 and forming an
independent lineage. When gaps were coded as a 5th character state, the same phylogeny was obtained with similar bootstrap support values (data not shown). Maximum likelihood analysis resulted in a near identical tree as that presented in Fig. 4 obtained in parsimony analysis (slight differences in relationships within clades 6 and 2) with similar bootstrap values (Fig. A.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 *cephae* 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 GGTGTAGGGGTAG 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 towards the 3’ end having no perfect repeats (Fig. A.1B). Allowing for only 1 mismatch, the repeats extended to the 3’ end of the IGS and their number increased from six to fourteen (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 datasets (Table A.4). Parsimony analysis of the combined mtSSU and EF-1α yielded 1 most parsimonious trees of 112 steps (CI = 0.946, RI = 0.964) with a near identical topology to that obtained with the EF-1α data partition (data not shown). The same topology was obtained when gaps were set either as missing data or as a 5th 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 A.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 near identical topology to that obtained with the IGS data set (data not shown). The primary differences observed in topologies from the combined data set compared to 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 5th 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 5th 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 near identical tree (slight differences in relationships within clades 4, 3, and 2) as 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 datasets (Table A.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 dataset (steps = 792, CI = 0.816, RI = 0.813; Fig 5). The primary differences observed in topologies from the combined data set compared to 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 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 5th state (data not shown). Maximum likelihood analysis resulted in
a near identical tree to the most parsimonious tree presented in Fig. A.5 (slight
differences in branch lengths) with similar bootstrap values (Fig. A.5).

**Test of hypothesis**

Three hypotheses of groupings were tested with the parsimony criterion (Fig. A.6). The first hypothesis (A) was drawn from the grouping based on the mtSSU
sequence data (Fig. A.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
seventeen other f. spp. forming the fourth lineage. The second hypothesis was drawn
from groupings based on the EF-1α dataset (Fig. A.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. A.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 the hypotheses A and B, was resolved into three different lineages in
hypothesis C.

Heuristic search with the mtSSU data under constraint of Fig. A.6B and C
produced 1 equally parsimonious tree of 49 steps and 2 equally parsimonious trees of 61
steps, respectively. The null hypothesis of no difference between the best trees and trees
reconstructed under the constraint of Fig. A.6B was not rejected by the KH, Templeton
and WS tests, implying that hypotheses B is consistent with the mtSSU data (Table A.5).
However, the null hypothesis was rejected under the constraint of Fig. A.6C, implying that hypothesis C is inconsistent with the mtSSU data.

Heuristic search with the EF-1α data under constraints of Fig. A.6A and C produced one parsimonious tree of 64 steps (CI = 0.953, RI = 0.967) and 2 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 Fig. 6A was not rejected, implying that hypothesis A was consistent with the EF-1α data (Table A.5). However, the null hypothesis was rejected under the constraint of Fig. A.6C, implying that hypothesis C is inconsistent with the EF-1α data.

Heuristic search with the IGS data under the constraints of Fig. A.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 Fig. A.6B and 6C was rejected by KH, Templeton and Wining-sites tests (Table A.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 polyphyletic. All race 1 isolates from Arizona, California, and Japan had identical mtSSU and EF-1α sequences, and near
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 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 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 VCG as race 1 from Japan and that all race 2 isolates were of a separate VCG. Similarly, Pasquali et al (30) found 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 lineages given the apparent polyphyly. What is most interesting is the fact that all three f. sp. *lactucae* races were first detected and are currently 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* f. spp. of Asian host genera *Musa*, *Albizia* and *Phoenix* and suggested that Asia may be the center of origin of the *Fusarium 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. *rhouis* was genetically identical to isolates of f.sp. *lactucae* recovered from lettuce tissue (pathogenicity tests conducted on the three lettuce cultivars with f. sp. *rhouis* were negative). The next most closely related taxa were the f. sp. *matthiolae* and *phaseoli* which also grouped with race 1 isolates. These relationships were consistent among the three datasets. 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 *forme 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 dataset 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 dataset 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 only be ascertained through a more comprehensive review of all *formae speciales* within *F. oxysporum*. Considering 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*, *spinaciae*, and the nonpathogens from soil. This conflict was revealed in various tests used to determine the combinability of the datasets as well as to examine conflicts between their topologies. The results of the Mickevich-Farris (*IMF*) and Partition-Homogeneity tests suggested that the IGS and the mtSSU and EF-1α datasets were incongruent. Tests of topological hypotheses supported this conclusion. Whereas hypothesis A, based upon mtSSU sequence data, was consistent with the EF-1α datasets, it was inconsistent with the IGS data set. Hypothesis B, based upon EF-1α sequence data, was consistent with the mtSSU data, but was 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 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 could still improve phylogenetic accuracy while 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). As this study included only 40 taxa including 20 different formae speciales of F. oxysporum, there is a possibility of underrepresentation. 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 may also improve low bootstrap support evident on several of the intermediate nodes.

Irrespective of taxon representation, discordance between rDNA genealogies and those of protein-coding genes has been previously documented. 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 ITS2 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 non-uniform 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 have also been proposed to explain discrepancies between rDNA and other data sets (34). These factors may also 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 3 *Verticillium* species also revealed several repeats located between nt 203-1264 bp from the 5’ end. In that study, one *V. longisporum* contained fourteen perfect 39 bp
repetitive motifs, while in other isolates of V. longisporum, V. dahliae, and V. albo-
atrium, 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 if 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 effectively used 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 are
currently 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.
REFERENCES


Table A.1. Isolates used in this study and associated GenBank accession numbers noted.

All mtSSU, EF-1, and IGS sequences were determined in this study with the exception of those with accession numbers in bold.

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

All strains preceded by: BMP are from B. M. Pryor, Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721; 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, AZ 85721; all other strains are from T. Gordon, Department of Plant Pathology, University of California, Davis.

*(p) = pathogenic on lettuce, (np) = not pathogenic on lettuce, (nd) = pathogenicity on lettuce not determined. ‘N/a = geographic origin of the strain is not available. The lactucae isolates recovered from soil are noted (soil). ²ARS = IGS sequences of these isolates were obtained through courtesy of Kerry O’Donnell.*
Table A.2. Primers pairs for genomic regions amplified and sequenced in this study.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial small subunit: mtSSU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMS1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’-CAGCAGTGAGGAATATTGGTCAA TG- 3’</td>
<td>Li et al 1994</td>
</tr>
<tr>
<td>NMS2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-GCAGGATCATCGAATTAAATAACAT- 3’</td>
<td>Li et al 1994</td>
</tr>
<tr>
<td>Translation elongation factor: EF1-α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF-1H&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’-ATGGGTAAGGAAGCAAGAC- 3’</td>
<td>O’Donnell et al 1998</td>
</tr>
<tr>
<td>EF-2T&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-GAAGGTACCAGTGATCATGTT- 3’</td>
<td>O’Donnell et al 1998</td>
</tr>
<tr>
<td>Intergenic spacer Region: IGS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNL12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’-CTGAACGCTCTTAAGTGCG- 3’</td>
<td>Anderson &amp; Stasovski 1992</td>
</tr>
<tr>
<td>CNS1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-GAGACAAGCATATGACTACTG- 3’</td>
<td>White et al., 1990</td>
</tr>
<tr>
<td>U46.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-AATAGACGACGCGCGACAC- 3’</td>
<td>Appel &amp; Gordon 1996</td>
</tr>
<tr>
<td>RU46.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-GTGTCGCGGTGCTTGATT- 3’</td>
<td>This study</td>
</tr>
<tr>
<td>CN61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’-GGTTCAATTTGATGTCGGCT- 3’</td>
<td>This study</td>
</tr>
<tr>
<td>RCN61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-AGCGACATCAAATTGACC- 3’</td>
<td>This study</td>
</tr>
<tr>
<td>RU3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’-GTGTGAATTTGGAAGTCCG- 3’</td>
<td>This study</td>
</tr>
<tr>
<td>RRU3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-CCGACTTTCCATAATTTCACAC- 3’</td>
<td>This study</td>
</tr>
<tr>
<td>CN34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-CCAACACATGGGTGTTACCG- 3’</td>
<td>This study</td>
</tr>
<tr>
<td>IGSF4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5’-CCAGACTTCCACTGGCGTTC- 3’</td>
<td>This study</td>
</tr>
<tr>
<td>CNS12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-GCACGCCAGGACTGCCTCGT- 3”</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> Forward primers  <sup>b</sup> Reverse primers
Table A.3. Disease incidence and disease index of *F. oxysporum* isolates on 3 iceberg lettuce cultivars commonly used in Arizona: Beacon, Lighthouse, and Winterhaven.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Beacon</th>
<th>Lighthouse</th>
<th>Winterhaven</th>
<th>path.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL2</td>
<td>100/2.6ab</td>
<td>100/3.0a</td>
<td>100/2.8a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>HL1</td>
<td>100/2.6ab</td>
<td>100/2.6ab</td>
<td>100/2.6a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>JCP007</td>
<td>100/3.0a</td>
<td>100/2.6ab</td>
<td>100/2.6a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1323</td>
<td>100/2.8ab</td>
<td>100/2.9a</td>
<td>100/2.6a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1324</td>
<td>100/3.0a</td>
<td>87.5/1.8b</td>
<td>100/2.5a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1326</td>
<td>100/3.0a</td>
<td>100/2.5ab</td>
<td>100/2.6a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1331</td>
<td>100/2.9a</td>
<td>100/2.8a</td>
<td>100/2.9a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1300</td>
<td>100/2.2b</td>
<td>100/2.2ab</td>
<td>100/2.6a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1301</td>
<td>100/2.6ab</td>
<td>100/2.6ab</td>
<td>100/2.6a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1306</td>
<td>100/2.5ab</td>
<td>100/2.9a</td>
<td>100/2.8a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1308</td>
<td>100/2.5ab</td>
<td>100/2.6ab</td>
<td>100/3.0a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1307</td>
<td>100/3.0a</td>
<td>100/3.0a</td>
<td>100/2.5a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1333</td>
<td>100/3.0a</td>
<td>100/2.1ab</td>
<td>100/3.0a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1880</td>
<td>100/3.0a</td>
<td>100/3.0a</td>
<td>100/3.0a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1375</td>
<td>100/3.0a</td>
<td>100/3.0a</td>
<td>100/3.0a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1363</td>
<td>100/2.8ab</td>
<td>100/3.0a</td>
<td>100/3.0a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>BMP1370</td>
<td>100/2.9a</td>
<td>100/3.0a</td>
<td>100/3.0a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>F9501</td>
<td>100/3.0a</td>
<td>100/2.9a</td>
<td>100/3.0a</td>
<td>pathogenic</td>
</tr>
<tr>
<td>Sample</td>
<td>Score 0</td>
<td>Score 1</td>
<td>Score 2</td>
<td>Score 3</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>FK9701</td>
<td>100/2.9a</td>
<td>100/2.9a</td>
<td>100/3.0a</td>
<td></td>
</tr>
<tr>
<td>S-1</td>
<td>100/3.0a</td>
<td>100/2.9a</td>
<td>100/2.6a</td>
<td></td>
</tr>
<tr>
<td>BMP1389</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>BMP1397</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>BMP1388</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>BMP1385</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>NRRL26227</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>0.5% KCl</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td></td>
</tr>
</tbody>
</table>

Each treatment consisted of eight inoculated plants per cultivar. The disease incidence/severity was assessed with a 0-3 scale where 0 = no disease, 1 = plants stunted, 2 = plants severely stunted, 3 = plants dead. The disease index was calculated using the formula 
\[
\text{Index} = \frac{n_0 + n_1 \times 2 + n_2 \times 3}{n_0 + n_1 + n_2 + n_3},
\]
where \(n_0\) is the number of plants with a score of 0, \(n_1\) is the number of plants with a score of 1, \(n_2\) = number of plants with a score of 2, and \(n_3\) represents the number of plants with a score of 3. Disease index values followed by a different letter were significantly different \(P \leq 0.05\) based upon Tukey’s test. *Path. = determination of pathogenicity: P = pathogenic and NP = non-pathogenic.*
Table A.4. Congruency of data partitions as evaluated by Mickevich-Farris incongruence metrics ($I_{MF}$) and the Partition-Homogeneity test implemented in PAUP. Tree lengths (steps) used to determine the $I_{MF}$ are indicated for maximum parsimony analysis and minimum number of synapomorphies.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Steps (parsimony)</th>
<th>Steps (minimum)</th>
<th>$I_{MF}$</th>
<th>Partition-Homogeneity test ($P$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtSSU/EF-1α</td>
<td>112</td>
<td>106</td>
<td>0.000</td>
<td>1.0000</td>
</tr>
<tr>
<td>mtSSU/IGS</td>
<td>705</td>
<td>577</td>
<td>0.078</td>
<td>0.0500*</td>
</tr>
<tr>
<td>EF-1α/IGS</td>
<td>725</td>
<td>593</td>
<td>0.121</td>
<td>0.0100*</td>
</tr>
<tr>
<td>mtSSU/EF-1α/IGS</td>
<td>780</td>
<td>638</td>
<td>0.155</td>
<td>0.0100*</td>
</tr>
</tbody>
</table>

* $P \leq 0.05$ is considered to be significantly incongruent.
Table A.5. Test of topological hypotheses under varying constraints of strict consensus trees.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Constraints</th>
<th>Steps</th>
<th>$P$ (KH) $P$</th>
<th>$P$ (Wining sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mtSSU</td>
<td>EF-1 $\alpha$</td>
<td>49 (49)</td>
<td>1.0000</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IGS</td>
<td>61 (49)</td>
<td>0.0104*</td>
<td>0.0139*</td>
</tr>
<tr>
<td>EF-1 $\alpha$</td>
<td>mtSSU</td>
<td>64 (63)</td>
<td>0.3177</td>
<td>0.3173</td>
</tr>
<tr>
<td></td>
<td>IGS</td>
<td>80 (63)</td>
<td>0.0022*</td>
<td>0.0027*</td>
</tr>
<tr>
<td>IGS</td>
<td>mtSSU</td>
<td>689 (646)</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td>EF-1 $\alpha$</td>
<td>738 (646)</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

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

*$P \leq 0.05$ is considered to be significantly incongruent.

bValues in parentheses represent steps of the unconstrained best tree.

cThe rank sums for both the Wining sites and Templeton tests were 0.00 for mtSSU data set under hypothesis B constraint, thus, $P$ values were not generated.
Figure A.1. A. Diagrammatic representation of the rDNA 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 *F. oxysporum* f. sp. *lactucae* using the program DNA strider allowing for no mismatch of base pairs and no overlap of repeat sequence.
Figure A.2. The single most parsimonious tree generated with mtSSU 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 (CI) and retention index (RI) are 0.918 and 0.946, respectively.
Figure A.3. The single most parsimonious tree generated with EF-1α 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 CI and RI are 0.968 and 0.978, respectively.
Figure A.4. One of 40 most parsimonious trees generated with the IGS 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 CI and RI are 0.822 and 0.810, respectively.
Figure A.5. One of 800 most parsimonious trees generated with combined mtSSU, EF-1α, and IGS 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 CI and RI are 0.816 and 0.813, respectively.
Figure A.6. Three strict consensus trees generated in PAUP from the different datasets used in the hypothesis tests; A. mtSSU dataset, B. EF-1α dataset, C. IGS dataset.
APPENDIX B

A PCR-BASED ASSAY FOR DETECTION OF *Fusarium oxysporum* f. sp. *lactucae* IN LETTUCE SEED

ABSTRACT

A nested polymerase chain reaction (nPCR)-based assay was developed and evaluated for the rapid detection of *Fusarium oxysporum* f. sp. *lactucae* in seed of lettuce. Four primers were designed from pathogen intergenic spacer rDNA and were used in the PCR amplifications. The first amplification employed the pair of outer primers GYCF1 and GYCR4C and produced a 2200-bp primary product. The second amplification employed the nested primer P1 and R943 and produced a 1000-bp PCR product. The nPCR protocol developed successfully detected the target sequence in 20 fg genomic DNA. A nPCR-based seed assay was developed that included a 4-day incubation step in which seed was maintained under high humidity conditions in order to increase fungal biomass. The seed was then incubated with lysis buffer, extracted with phenol-chloroform, and the fungal DNA recovered using a silica matrix. In seed lots prepared by mixing known amounts of *F. oxysporum* f. sp. *lactucae*-infested seed with noninfested seed, this assay allowed for the detection of the pathogen from lots with infestation rates as low as 0.1%. Samples of lettuce seeds obtained from 88 commercial seed lots were assayed for the pathogen by direct plating on agar medium and by using the nPCR assay. *Fusarium oxysporum* f. sp. *lactucae* was not detected by either diagnostic method, suggesting the seed lots were pathogen-free or the level was below detection limits. However, the samples tested were non-representative, and future tests will employ
statistically robust seed sampling protocol to more accurately access the occurrence of *F. oxysporum* f. sp. *lactucae* in commercial lettuce seed.

**INTRODUCTION**

*Fusarium oxysporum* Schlechtend: Fr. f. sp. *lactucae* Matuo and Motohashi, causal agent of Fusarium wilt in lettuce (*Lactuca sativa*), has recently become a serious fungal pathogen of lettuce in several lettuce production areas of the world. The fungus was first described in 1967 when it was responsible for significant losses in lettuce in Japan and pathogenicity on lettuce was confirmed (Matuo and Motohashi 1967). In the U.S., the disease was first reported in the San Joaquin valley of California in 1993, where it was responsible for lettuce losses in 3 fields near the town of Huron (Hubbard and Gerik 1993). The disease was subsequently reported in Arizona in 2001 in five fields near the town of Yuma and in 11 additional fields during the 2002-2003 cropping season (Matheron and Koike 2003). 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) (Garibaldi et al. 2002; Huang and Lo 1998; Millani et al. 1999).

The fungus is primarily a root and crown pathogen and causes severe stunting of lettuce with affected plants showing dark streaking of the vascular tissue extending from the tap root into the stem (Hubbard and Gerik 1993). The nature of dissemination of the pathogen in the U.S. is still unknown but seed transmission and infested farm equipment has been suspected (Matheron 2005). In Italy, however, the pathogen was isolated from
commercial seed lots used for sowing in farms where the disease was first reported (Garibaldi et al. 2004). In Taiwan, Japan, and Iran the mode of transmission of the pathogen has not been documented.

Testing for seedborne pathogens is important for quarantine purposes to avoid the spread of disease to new regions, to make accurate decisions regarding the appropriate use of seed treatment, and for seed certification programs. The conventional methods for seed health testing include the freezer blotter technique or plating on selective media (International Seed Testing Association 1966, Neergaard 1977). Identification of the pathogens present is by microscopy. These methods are slow, labor-intensive, require skilled personnel, and are not suited for rapid and high-throughput testing often required for screening commercial seeds (Ward et al. 2004). These important factors have prompted the development of more sophisticated diagnostic methods based on antibody- or DNA-based techniques. Antibody-based techniques include generating polyclonal or monoclonal antibodies that recognize specific antigens associated with a specific plant pathogen. The main objective of such assays is to detect or quantify the binding of the diagnostic antibody to the target antigen. These assays have been used mostly for the detection of bacteria and viral pathogens (Griep et al. 1999). However, the development of immuno-assays for fungal plant pathogens has been restricted by the identification of antibodies that will give the required specificity when challenged with a complex antigen mixture (Ward et al. 2004).

Recent advances in molecular biology and biotechnology has seen a corresponding increase in the development of rapid, specific and sensitive tools for the
detection of plant pathogens. Nucleic acid-based methods using probes and/or polymerase chain reaction (PCR) are increasingly being used in diagnostic assays for plant pathogens (Martin et al. 2000). These methods more commonly target DNA, which is more stable than RNA, and are highly specific. The first molecular-based diagnostic techniques were nucleic acid hybridization tests which use labeled probes to detect specific pathogens. The probes employed are generally single stranded DNA or RNA molecules that have been labeled with a reporter molecule and bind to complementary DNA or RNA sequences (Ward et al. 2004).

More recently, PCR-based methods have become the methods of choice because of their specificity and the requirement of only small amounts of DNA for successful detection (Lee and Taylor 1990). Different versions of the technique have been developed depending the system under study. Immunocapture PCR involves immobilizing an antibody on a microtitre plate surface that captures the target pathogen DNA (Shamloul and Hadidi 1999). Multiplex PCR is used when there is the need to detect several pathogens simultaneously on a host plant. Nested PCR is used when there is a need to improve the sensitivity and/or specificity of the PCR assay (Henegariu et al. 1997; Schesser et al. 1991). The problem with standard PCR, however, is that it is not inherently quantitative, which makes it difficult to relate the amount of product obtained to the concentration of the original target DNA used. This problem has been solved with the advent of real-time PCR.

The principle underlying real-time PCR is that the larger the amount of target DNA present in the sample being tested, the quicker the reaction progresses and enters an
exponential phase of amplification (Ward et al. 2004). The advantages of real-time PCR over classical PCR include fewer problems with contamination, the ease of optimization of the PCR protocol, and the possibility of multiplexing (Schaad and Frederick 2002). Even though real-time PCR assays have been developed to detect and identify several fungal species, there still exist several challenges to developing successful real-time PCR assays for fungal pathogens. The most important of these are the availability of efficient protocols for extraction of DNA from plant materials (Schaad and Frederick 2002) and the availability of highly specific probes.

The rapid spread of Fusarium wilt in Arizona within the previous five years is particularly troubling to the lettuce industry. An important aspect of an integrated pest management program is the availability of methods of pathogen detection in propagation material (i.e., seed) to curb the spread of the causal fungus. This may be possible with the use of rapid and sensitive detection techniques like PCR in place of conventional methods. Diagnostic methods based on the polymerase chain reaction amplification of specific nucleic acids have high analytical sensitivity to discriminate between different strains of fungi and have been used to detect a number of f. spp. within \textit{F. oxysporum} such as f. spp. \textit{phaseoli}, \textit{lycopersici}, \textit{radicis-lycopersici}, \textit{basilici}, \textit{niveum}, and \textit{canariensis} (Alves 2001; Hirano and Arie 2006; Chiocchetti et al. 2001; Zhang et al. 2005; Plyler et al. 1999). In addition to high sensitivity and specificity, PCR-based methods have the advantage in that a large number of samples can be processed within a short period of time and can be conveniently applied to seed testing and certification. However, a major drawback is the presence of amplification inhibitors in plant materials that often require
optimization of the DNA isolation procedure to ensure subsequent detection. This point is even more critical when performing isolations from seed.

The objective of this study was to design a PCR-based method to detect *Fusarium oxysporum f. sp. lactucae* in seed and to adapt it to a seed assay that will limit the inhibitory effects of seed DNA extracts to PCR. A second objective was to use this method to assay commercial lettuce seed in order to develop preliminary data as to the presence of the pathogen in lettuce seed used in Arizona.

**MATERIALS AND METHODS**

**Fungal isolates.**

Test isolates of *F. oxysporum f. sp. lactucae* used in this study were recovered from diseased lettuce roots and soil. Other fungal isolates used and their sources are listed in Table B.1. Cultures were grown on potato dextrose agar (PDA; Difco Laboratories, Plymouth, MN) at 23°C and stored on silica gel at 4°C. Each isolate was identified based upon standard morphological criteria, comparisons with type or representative isolates, and published descriptions (Hubbard and Gerik 1993).

**DNA isolation.**

Liquid cultures of all isolates were prepared by flooding agar plates (PDA) containing 5-day-old cultures of test fungi with 10 ml of sterile H_2O and dislodging mycelia and conidia with a pipette tip. A suspension (2 ml) was added to 100 ml of sterile liquid growth medium (20 g of D-glucose, 1.2 g of DL-asparagine, 1.2 g of K_2HPO_4, 0.5 g of yeast extract, 0.5 g of MgSO_4.7H_2O, and 0.1 g of NaCl per liter) in a 250 ml Erlenmeyer flask. The flasks were incubated on a rotary shaker at 120 rpm for 5
days at 22°C. Mycelia were harvested by filtration through Miracloth (Calbiochem, EMD Biosciences Inc., San Diego, CA), lyophilized, and stored under desiccation at 4°C. Extraction of fungal total genomic DNA was accomplished using the BIO 101 Fast DNA kit (Qbiogene, Irvine, CA) according to the manufacturer protocols.

IGS sequencing and primer design

Polymerase chain reaction (PCR) was used to amplify the full length intergenic spacer (IGS) rDNA using primers developed in a previous study (Appendix A; Mbofung et al., 2006 in press). Each PCR reaction contained 0.2 mM deoxynucleotide triphosphates, 2.5 mM Amplitaq MgCl2, 0.8 µM of each primer, 1X Amplitaq reaction buffer, and 2 units 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 35 cycles. 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 (approximately 2600 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. 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). The regions of the IGS rDNA sequence that were specific to select *Fusarium* spp. and to the *lactucae* isolates were used to design outer primers and inner primers, respectively (Fig. B.1).

**Sensitivity and specificity of *F. oxysporum* f. sp. *lactucae* primer sets**

To determine the sensitivity of the primers, 10-fold serial dilutions of *F. o. f. sp. lactucae* total genomic DNA were prepared in TE buffer (concentrations ranged from 10 ng/µl to 1 fg/µl), and the capacity of the primers to direct the amplification of the target DNA fragment from decreasing concentrations of DNA was determined. To determine specificity, the primers pairs were tested on total genomic DNA (10 ng/µl) from 13 fungal species (Table B.1). In both experiments, the first PCR was carried out in a volume of 50 µl containing 2 µl of total genomic DNA, 0.06 mM of each primer, 2 mM of each dNTP, 2 mM of MgCl₂, and 0.03 U of Amplitaq DNA polymerase in 1X Amplitaq buffer II. PCR reactions were carried out in a PTC-100 thermal cycler programmed for the following parameters: for the initial PCR, the parameters were 94° C for 1 min, 35 cycles at 94° C for 30 secs 65° C for 2 mins 24 secs, and 72° C for 1 min. For the nested PCR, the parameters were 94° C for 1 min, 35 cycles at 94° C for 30 secs, 60° C for 30 secs, 72° C for 1 min, and a final extension period of 10 min at 72°. PCR-amplified DNA fragments were fractionated in 1% agarose gels in TBE buffer and visualized in ethidium bromide.

**Seed assay protocol.**

For each seed assay, 1g of lettuce seed (approximately 1200 to 1300 seeds) per seed lot was evenly distributed over the bottom of a 15 mm plastic Petri dish. The dish
was placed on a rigid mesh support in a plastic container (31 cm by 22 cm by 10 cm) and water was added beneath the mesh support to a depth of 1.5 cm. A fine mist of sterile water was initially applied to the seeds in the dish with a spray bottle (approximate duration of misting was 1 to 2 seconds). After misting, the Petri dish cover was placed slightly ajar and the top was replaced over the plastic container to generate conditions of high humidity. The set-up was incubated in the dark for 4 days at 28°C.

After incubation, 5 ml of lysis buffer (1% sodium deodecyl sulfate, 10 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0, and 0.5 M NaCl) was added to the dish and the dish was rocked to fully moisten all seeds and fungal hyphae growing on the seed. The dish was then incubated on a rotary shaker at 60 rpm for 15 min at 23°C. The contents of the dish (seeds, mycelia, and lysis buffer) were transferred into a 15 ml centrifuge tube and 3 ml of phenol-chloroform (1:1, vol/vol) was added. The tube was vigorously shaken for 1 min and centrifuged at 9,000 x g for 20 min at 4°C. The aqueous phase was transferred into a new centrifuge tube and a second extraction performed with 3 ml of chloroform. Approximately 3 ml of aqueous phase was recovered, and 500 ul of 10% polyvinyl-polyprroloidone was added. The tube was gently rocked on a rotary shaker for 5 min and then centrifuged at 9,000 x g for 10 min at 4°C. Supernatant (2 ml) was transferred to a new tube and 0.9 ml of Bio101 binding matrix was added. The solution was incubated for 20 min at 23°C with periodic agitation, and then centrifuged at 12,000 x g for 1 min. The matrix pellet was washed two times with 0.5 ml of 70% EtOH. The matrix pellet was dried at 37°C overnight. DNA was eluted from the dried matrix by adding 100 ul of TE buffer and incubating for 10 min at 55°C. After incubation, matrix and buffer were
transferred to a 1.5 ml microfuge tube, centrifuged at 12,000 x g for 1 min, and the TE buffer solution containing DNA was recovered.

PCR with DNA recovered was carried out as previously described for total genomic DNA, except that (i) 2 ul of DNA extract was used and (ii) 1 ul of skim milk (Difco Laboratories; 10% solution in sterile H2O) was added to the PCR reaction mixture to a final concentration of 0.2%. To determine whether the skim milk altered primer sensitivity or specificity, both primer sets were tested as previously described, except in the presence of 0.2% skim milk. To determine the limit of detection of PCR-based seed assay, samples of noninfested seed were mixed with artificially infested seed (wt/wt) to generate lots with 100, 20, 10, 2, 1, 0.5, 0.2, and 0.1% levels of contamination. Each seed lot was assayed in duplicate and the experiment was conducted twice.

**Assay of commercial seed lots**

Samples of lettuce seed (n=88) obtained from commercial seed lots were assayed for the presence of *F. oxysporum* f. sp. *lactucae* by both the agar plate method and nPCR-based assay. The samples were provided by various commercial seed producers in Arizona and California and were representative of lots used for planting production fields in Arizona. For agar plate method, isolations were carried out on subsamples of nondisinfecting seeds (720-1200 seeds per lot). Twelve agar plates (Komada’s medium) per lot were incubated for 4 days under white fluorescent light (12 hours light / 12 hours dark) at 25°C. Putative *F. oxysporum* colonies were subcultured on three media (Komada’s, PDA, and 0.5% KCl) and their identity was confirmed by morphological
characterization (Burgess et al. 1994). For the nPCR-based assay, DNA extraction was performed as described previously and each seed lot was assayed four times.

**Pathogenicity of *F. oxysporum* isolates recovered from lettuce seed**

Cultures of *F. oxysporum* isolates obtained from assayed seed were grown on PDA for ten days under 12 hours of fluorescent light. Each plate was then flooded with 20 ml of 0.5 % KCl and the spores dislodged using a rubber spatula to generate a spore suspension. The spore suspensions were filtered through four layers of cheese cloth to remove fungal hyphae and the spore concentration was adjusted to 1 X 10^6 spores/ml. Putative *F. oxysporum* isolates were tested for pathogenicity on the commercial lettuce cultivar Winterhaven. Roots of ten-day-old seedlings were washed in running tap water, and approximately 1 cm of root tip was removed. The wounded roots were dipped in a spore suspension for 10 minutes. For each fungal isolate, eight inoculated seedlings were transplanted to 20 cm wide plastic pots containing a sterile mix of 1 part peat, 1 part vermiculite, and 2 parts sand. The experiment was set up in the greenhouse (24-29 C, RH=50-60%) in a completely randomized design. Negative control plants were prepared similarly but the wounded roots were dipped in 0.5 % KCl. Plants inoculated with the *F. o. f. sp. lactucae* isolate HL1 constituted the positive control. Plants were examined for wilt symptoms 7 days after inoculation and were scored for disease 20 days after inoculation.
RESULTS

IGS sequencing and primer design.

The IGS primers CNL12 and CNS1 generated a product between 2547 and 2600 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. Analysis of the complete IGS sequence revealed numerous polymorphisms among the isolates, some of which were unique to the *lactucae* 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. 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.

Part of the nucleotide sequences of 28S and 18S rDNA including the 5’ and 3’ end of the IGS with polymorphisms unique to the *lactucae* isolates and several *Fusarium* spp. were determined and from these sequences, the first PCR primer pair was designed:

Forward primer: GYCF1 5’-CTCCGGATTCTGGAGACTTG-3”; and
Reverse primer: GYCR4C, 5’-ACTATCGTGTGCCGGGGTTG-3”

The nested primer set was designed based upon a Hind III restriction site and sequence polymorphisms unique to *lactucae* isolates within the IGS region (Appendix A; Mbofung et al., 2006 in press) (Fig. B.1).

Forward primer: P1, 5’-ATCCTTTTCAGACGACTTAGC-3”; and
Reverse primer: R943, 5’-CCCATACTATATACCAGACG-3’
Sensitivity and specificity of the *F. oxysporum* f. sp. *lactucae* primer sets

In specificity tests with total genomic DNA from 13 other fungal species, the outer primer pair directed the amplification of approximately 2200 bp target fragment from total genomic DNA from the *lactucae* isolates, two nonpathogenic *F. oxysporum* isolates, and *F. subglutinans* (Table B.1). No DNA fragments were amplified from total genomic DNA of the other 11 fungal species (Fig. B.2). The nested primers directed the amplification of an approximately 1000 bp target fragment from the primary PCR product only from the *lactucae* isolates. No DNA fragments were amplified from all the other 13 fungal species. In sensitivity tests with serial dilutions of *F. oxysporum* f. sp. *lactucae* total genomic DNA, the limit of detection (i.e., the lowest concentration of total genomic DNA from which the fragment was amplified) for the outer primer set was 200 pg, and that for the nested primer pair was 20 fg (Fig. B.3).

Seed assay protocol

In experiments to determine the limit of detection (sensitivity) of the *F. oxysporum* f. sp. *Lactucae* - specific primers in seed assay (i.e., the lowest level of seed contamination that could be detected), the target DNA sequence was detected from duplicate samples of undiluted DNA extracts from seed lots at infestation levels of 10% in the first PCR, while the subsequent nPCR detected the target fragment in samples at infestation levels as low as 0.5% (results not shown). However, without skim milk the amplification of the target sequence from undiluted extracts from seeds was inconsistent for the first PCR reaction. Therefore, skim milk was included in all subsequent amplifications with the first primer set. The second set of seed lots were artificially
infested at levels of 100, 20, 10, 2, 1, 0.5, 0.2, and 0.1%. The first primer set directed the amplification of the target sequence from undiluted extracts with infestation levels as low as 1% (data not shown). The nested primer consistently amplified the target sequence from seed lots with infestation levels as low as 0.1% (Fig. B.4). No DNA fragments were amplified from equivalent extracts prepared from noninfested seed lots (Fig. B.4).

**Assay of commercial seed lots**

Direct plating assay revealed several of the commercial seed lots were infested with other saprobic fungi including *Alternaria* spp., *Penicillium* spp., and *Aspergillus* spp. In only three seed lots were *F. oxysporum* colonies isolated. Nine putative *F. oxysporum* f.sp. *lactucae* isolates were recovered from the seed lots. In pathogenicity tests on the lettuce cultivar Winterhaven in the greenhouse, none of the isolates obtained were pathogenic (results not shown). In addition, no target sequence was amplified from any of the commercial seed lots using the nPCR-based seed assay (results not shown).

**DISCUSSION**

The PCR-based assay developed for detection of *F. o. f. sp. lactucae* on lettuce seed represents the results of numerous experiments aimed at optimizing parameters to allow for reliable PCR-based detection of the fungus from infested seed. Two major obstacles had to be overcome in the development of the assay: (i) low levels of the fungus (i.e., target DNA) associated with seed and (ii) PCR inhibitors associated with lettuce seed. Fungi associated with seed often occur at very low infestation levels, and DNA extracts prepared from this seed will contain low levels of the target DNA sequence. To overcome this problem, a preassay incubation step was used to increase fungal biomass
on seeds. This step has also been used in assays for detection of other fungi in seeds (Taylor et al. 2001; Pryor and Gilbertson 2001). In the development of PCR-based assays for other fungi, it may be necessary to use similar preassay incubation steps in order to increase the amount of target fungus on seed.

A variety of incubation methods have been previously tested to facilitate rapid and consistent growth of fungi on seed, including incubating seed in liquid culture, in culture tubes, or on moistened filter paper (Pryor and Gilbertson 2001). Some of these methods did allow for increased biomass of the fungal pathogen on seed, but PCR amplification of the target DNA sequence from DNA extracts prepared from seed lots following such incubation methods were reported to be inconsistent even for highly infested seed lots. Also, in some cases bacteria overgrew or suppressed fungal growth. When seeds are simply placed in a plastic Petri dish in the absence of nutrients but under conditions of high humidity, abundant fungal growth develops on the seed surface with little or no bacterial growth. This pre-assay incubation method produced sufficient fungal growth for the consistent recovery of fungal genomic DNA from which the target sequence could be amplified. In addition, 1 g of lettuce seed (approximately 1200-1300 seeds) could be assayed in a single 100-by-15-mm Petri dish. Larger amounts of seed may be assayed by increasing the number of Petri dishes or by using a larger-sized dish.

PCR inhibitors associated with seeds can constitute obstacles in the development of assays of this kind. Such inhibitors have been commonly encountered in PCR assays for the detection of organisms or DNA from complex environmental samples (De Boer et al. 1995; Jobes et al. 1995). In this study, inhibitors appeared to come from the lettuce
seeds because (i) inhibitors were not associated with fungal DNA extracted from mycelia; (ii) inhibitors were consistently encountered in DNA extracts from lettuce seed, irrespective of whether they were spiked with *F. oxysporum* species or other fungi; and (iii) PCR amplification with the first primer set was often inconsistent. Thus it was necessary to develop a method for recovery of fungal DNA from seed while minimizing the release of inhibitors from the seeds. A variety of methods were tested, but these either failed to generate sufficient amounts of fungal DNA or disrupted seed tissue, resulting in the release of more PCR inhibitors. By adding lysis buffer directly to seeds after incubation, followed by a phenol-chloroform extraction of the total sample (i.e. buffer, mycelia, and seeds) without grinding seeds, a sufficient amount of fungal DNA was recovered with a minimal amount of PCR inhibitors released from seed.

DNA recovered from seed after phenol-chloroform extraction and ethanol precipitation contained brown pigments (e.g. phenolics) and PCR inhibitors that could not be removed by washing with ethanol or repeated precipitation. To reduce these contaminants, silica matrix (Qbiogene, Irvine, CA) purification was used instead of ethanol precipitation to recover the DNA. DNA recovered with this matrix did not contain brown pigments and the target DNA sequence was consistently amplified from seed DNA extracts, although the dilution of extracts was often required. The addition of skim milk to the PCR reaction allowed consistent amplification of the target fragment from undiluted DNA extracts from seed thereby increasing consistency and sensitivity.

The outer primer set was not species specific because it also directed the amplification of an approximately 2200-bp DNA fragment from two nonpathogenic *F.*
oxysporum isolates and from F. subglutinans. However, this should not result in false positives in lettuce seed assays because F. subglutinans fungi are not associated with lettuce seed (Garibaldi et al. 2004). Moreover, the nonpathogenic F. oxysporum isolates did not possess the Hind III restriction site that formed the basis of the improved selectivity of the nested primers. Thus, this initial cross-reaction was eliminated in the subsequent PCR with the nested primers, which greatly improved both the sensitivity and specificity of the assay. New primers can easily be incorporated into this PCR-based assay, thereby providing a means for continually updating and improving the specificity and sensitivity of the assay.

The negative results obtained from the PCR assay on commercial seed lots supports one proposed hypothesis that the introduction and rapid spread of Fusarium wilt of lettuce in Arizona may have been due to contaminated farm equipment moving from California into Arizona and between fields in Arizona (Matheron 2005). However, the original lots of lettuce seed from 2001 used to seed the fields in which the disease was first reported in Arizona were not included in the samples assayed. It is therefore, not known if these original seed lots were infested or not. Moreover, the seed samples that were tested were not robust representatives of their original seed lots as statistically sound seed sampling protocols were not employed when samples were taken. Thus, the negative results from the commercial seed assays may not be a true representation of the infestation level of these lots. These commercial seed lots that were tested were all produced in the Huron area of California where Fusarium wilt of lettuce was first reported in the U.S. and where 80% of all lettuce seed used in Arizona is produced (Ron
Berens, Arizona Seed Trade Association, pers. comm.). Considering that Fusarium wilt is still reported in this area, there is reason to believe that at least a percentage of seed produced in this area has the potential of becoming contaminated and spreading the pathogen.

In Italy, it was established that the spread of the disease was due to the use of contaminated seed as propagation material (Garibaldi et al. 2004). Nine out of 27 samples of lettuce seeds obtained from seed lots used for sowing farms affected by the disease in Italy were contaminated by *F. oxysporum*. Out of sixteen isolates *F. oxysporum* isolated from the seed lots, three were pathogenic on lettuce exhibiting a level of pathogenicity similar to that of race1 isolates of both California and Italy and confirming that *F. oxysporum* f. sp. *lactucae* can become seedborne (Garibaldi et al. 2004).

The PCR-based assay developed for the detection of *F. oxysporum* f. sp. *lactucae* on infested lettuce seed in this study is accurate, sensitive, and reliable, and could easily incorporate other primers for *F. oxysporum* f. sp. *lactucae* race1, or those specific for other races. Recently, Shimazu et al. (2005) developed two sequence tagged sites using RAPD markers for the identification of the three races of *F. oxysporum* f. sp. *lactucae* but, the authors did not incorporate these primers into a comprehensive seed assay protocol. These sequence tagged sites could be incorporated to the seed assay presented in this work to identify all races of *F. oxysporum* f. sp. *lactucae* in a single seed assay. Because it has the 5-day incubation period, this test requires approximately 6 days to complete, compared to 10 days for the freezer blotter test or the direct plating method.
Another advantage of the assay is that it does not rely on visual identification of \textit{F. oxysporum} f. sp. \textit{lactucae} on lettuce seed, which is complicated by the frequent occurrence of morphologically similar saprobes on lettuce seed. Thus, this PCR-based assay has the potential to be used for routine testing of lettuce seed lots for \textit{F. oxysporum} f. sp. \textit{lactucae}. Furthermore, this type of test may have applicability for the detection of other fungal pathogens on seed of other crops, once appropriate primer pairs are developed.
REFERENCES


Table B.1. Fungi isolates used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>sourcea</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>lactucae</em> 1</td>
<td>HL2</td>
<td>TRG</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>lactucae</em> 2</td>
<td>FOL. 10</td>
<td>TRG</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>lactucae</em> 3</td>
<td>2002-06</td>
<td>MM</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>lactucae</em> 4</td>
<td>BMP1382</td>
<td>BMP</td>
</tr>
<tr>
<td><em>F. oxysporum</em> 1</td>
<td>BMP 1388</td>
<td>BMP</td>
</tr>
<tr>
<td><em>F. oxysporum</em> 2</td>
<td>BMP1397</td>
<td>BMP</td>
</tr>
<tr>
<td><em>F. subglutinans</em></td>
<td>BMP1462</td>
<td>BMP</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>31.X4</td>
<td>EN</td>
</tr>
<tr>
<td><em>F. graminearum</em></td>
<td>BMP1461</td>
<td>BMP</td>
</tr>
<tr>
<td><em>F. acuminatum</em></td>
<td>BMP1460</td>
<td>BMP</td>
</tr>
<tr>
<td><em>F. concolor</em></td>
<td>BMP1454</td>
<td>BMP</td>
</tr>
<tr>
<td><em>F. culmorum</em></td>
<td>BMP1453</td>
<td>BMP</td>
</tr>
<tr>
<td><em>Bipolaris tetramera</em></td>
<td>51-31-001</td>
<td>BMP</td>
</tr>
<tr>
<td><em>Exoserothilium pedicillatum</em></td>
<td>61-21-001</td>
<td>BMP</td>
</tr>
<tr>
<td><em>Alternaria alternata</em> 1</td>
<td>0196</td>
<td>BMP</td>
</tr>
<tr>
<td><em>A. alternata</em> 2</td>
<td>0208</td>
<td>BMP</td>
</tr>
<tr>
<td><em>A. alternata</em> 3</td>
<td>EC6</td>
<td>EGS</td>
</tr>
</tbody>
</table>

aAbbreviations for sources of fungal isolates are as follows: BMP = B. M. Pryor, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721; EGS = E. G. Simmons; EN = Edward Nigh, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721; TRG = T. R. Gordon, University of California, Davis
Figure B.1. The intergenic spacer (IGS) ribosomal DNA showing primary and nested primer sets. Specificity of the nested primers was attained by designing the reverse of the nested primers at a unique Hind III site.
Figure B.2. A. Specificity of the primer pair GYCF1 and GYCR4C, The primers were used in the PCR with total genomic DNA (20ng) of various fungal species. The primers cross-amplified a fragment of the same size as the target sequence from two nonpathogenic *F. oxysporum* (lanes 5 and 6) and from *F. subglutinans* (lane 7). B. Improved specificity with nested primer pair P1 and R943. Lanes 1 to 4 represent *F. o. f. sp. lactucae*, lanes 5 to 18 represent *F. oxysporum, F. oxysporum, F. subglutinans, F. proliferatum, F. graminearum, F. acuminatum, F. concolor, F. culmorum, Bipolaris tetramera, Exserohilium pedicillatum, Alternaria alternata 1, A. alternata 2, A. alternata 3*, and negative control. Lane M contains the 1-kb plus DNA ladder.
Figure B.3. A. Limit of detection of primer pair GYCF1 and GYCR4C. The first PCR was performed with serial dilutions of *F. oxysporum* f. sp. *lactucae* total genomic DNA. Lanes 1 to 9 contain 20ng, 2ng, 200pg, 20pg, 2pg, 200fg, 20fg, and 2fg of total genomic DNA and no DNA, respectively. Lane M contains the 1kb plus DNA ladder. B. Limit of detection of nested primer pair P1 and R943. The second PCR was performed with the product of the first PCR. Lane 9 is a positive control showing the amplification of the approximately 1000-bp target DNA fragment amplified from *F. o. f. sp. lactucae* total genomic DNA.
Figure B.4. A limit of detection *F. oxysporum* f. sp. *lactucae* in lettuce seeds having different levels of artificial infestation with the nPCR-based assay. Lanes 1 to 16 show results from two independent DNA extractions for each seed lot having the indicated rates of infestation: Lanes 1 and 2, 100%; lanes 3 and 4, 10%; lanes 5 and 6, 5%; lanes 7 and 8, 1%; lanes 9 and 10, 0.5%; lanes 11 and 12, 0.2%; lanes 13 and 14, 0.1%; and lanes 15 and 16, (for gel 1 are positive controls, for gel 2 are 0% infestation). Lanes 17 and 18 for gel 1 represent 0% infested seed, while for gel 2 represent positive
APPENDIX C

POTENTIAL FOR DISPERSAL OF *Fusarium oxysporum* f. sp. *lactucae* BY INFECTED LETTUCE SEED.

ABSTRACT

In 2001, Fusarium wilt of lettuce was reported in six fields in Yuma, Arizona and significant loss in yield was recorded. To date, the disease has spread to 27 fields in this prime lettuce production area of the state. The origin and dispersal of the pathogen, *Fusarium oxysporum* f. sp. *lactucae*, has yet to be determined but dispersal via infested or infected seed has been suggested. To investigate seedborne transmission, lettuce cultivars Sharpshooter, Vulcan, and King Henry which are moderately resistant to the pathogen, were inoculated and grown to maturity in the greenhouse. Whole plants were harvested and tested for systemic movement of the pathogen from roots to aerial parts. The pathogen was recovered from sections of surface disinfested stem and branches of the panicle at rates of 57.0 – 100% and 14.3 – 62.7%, respectively. However, the pathogen was not recovered from flowers. The rate of recovery decreased as the distance from soil surface to tip of the plant increased. The incidence of recovery from nonsurface disinfested seeds was very low (0-0.3%) and the pathogen was not recovered from surface disinfested seeds suggesting that it was externally seedborne. Furthermore, the pathogen was recovered from pathogen-free seeds that were mixed with infested debris from the greenhouse at seed: debris ratios as low as 400:1. These data indicate that infested seed could result from infected mother plant or contact with infested debris and
may have contributed to the recently documented dissemination of this pathogen worldwide.

INTRODUCTION

*Fusarium oxysporum* f. sp. *lactucae* the causal agent of Fusarium wilt of lettuce has become a serious pathogen of lettuce in several important production areas in the world (Matuo and Motohashi, 1967, Huang and Lo, 1998, Millani et al., 1999, Garibaldi et al., 2002). In Italy, research has shown that dispersal may be the result of planting infested lettuce seeds (Garibaldi et al. 2004). However, in the United States, several hypotheses for the spread of Fusarium wilt into disease-free lettuce-production areas have been proposed (Matheron 2005). Among these hypotheses are the movement of infested farm equipment and the possible use of infested seed material. It has been suggested that contaminated soil is unintentionally introduced into pathogen-free fields through farming activities such as laser-leveling, disking, and other seed bed preparation methods. In addition to these, contamination could also result from the movement of mud-encrusted irrigation pipe used to germinate seed (Matheron 2005). However, these modes of dissemination (movement of infested farm equipment from one field to the next and seed dispersal) are yet to be proven.

Dissemination of *F. oxysporum* by seed has been documented for as many as 20 different formae speciales (Kuniyasu and Takeuchi 1981). In most cases, such transmission has been due to contamination on the seed surface by conidia. These conidia may be transmitted to seed by wind, dislodged from infected plants during threshing, or from contact with infested soil (Besri 1978; Gamliel et al. 1996; Kuniyasu
and Takeuchi 1981; Elmer 1994,). However, some strains of *Fusarium oxysporum* (e.g., f. spp. *largenariae* and *vasinfectum*) are known to infect seeds internally (Bersri 1978; Kuniyasu and Nakamura 1978). Most infection of internal seed tissues has been revealed through histological and surface disinfestations studies (Baker 1948; Bakry and Rizk 1967; Gardner 1980; Haware et al. 1978). These studies generally involve dissecting the fruit or seed into its separate entities and surface disinfesting each part, followed by plating on agar. Results obtained have often been considered controversial because in most cases the seed in question is often of small size, which result in difficulty successfully separating outermost layers from internal layers (Inglis 1980; Gracia-Garza et al. 1999). Regarding lettuce, these types of studies may also be difficult because the lettuce seed is a small achene with the seed coat tightly adhering to the cotyledons. Therefore, physical separation would not be easy. The most robust studies documenting seedborne inoculum were performed on fruit and seed of coca which are relatively large (0.5 to 1.5 cm in diameter). However, the results from these studies clearly indicated that *F. o*. f. sp. *erythroxyli* was externally seedborne (Gracia-Garza et al. 1999).

The occurrence of Fusarium wilt in countries far from the original discovery reveals the potential for the disease to spread by some common means. The fact that the five countries documenting the disease are non-adjacent and separated by great distances suggests that the pathogen may have moved via contaminated seed. Seed dispersal has consistently been linked to the spread of most *Fusarium* spp. diseases (Gracia-Garza et al. 1999). In Arizona, the disease has been reported only in commercial lettuce production fields and isolates of the pathogen have been obtained only from diseased
lettuce plants or from soil collected in fields that harbor such plants. However, the rapid spread of the disease to neighboring fields within four years of its first report has raised concerns as to whether the fungus is primarily seedborne or soilborne. In Italy, *F. o. f.* sp. *lactucae* has been isolated from nondisinfested lettuce seeds and thus was demonstrated to be externally seedborne (Garibaldi et al. 2004). While the presence of external seedborne inoculum has been documented, it is not known if the fungus can move from the roots to the seed through the vascular system of the plant and thus become internally seedborne.

The objective of this study was to investigate the nature of seed infestation, and to examine differences in seedborne potential among lettuce types and between inoculation methods. In addition, the potential of seed to become infested via contact with infested crop debris was examined, as well as the impact of infestation on seed health.

**MATERIALS AND METHODS**

**Systemic movement of *F. o. f.* sp. *lactucae* in lettuce**

Lettuce cultivars Sharpshooter, Vulcan, and King Henry each belonging to head, leaf, and romaine types, respectively, were selected for studies on the potential and mode of seedborne transmission of *F. oxysporum* f. sp. *lactucae*. These cultivars were chosen based on their moderate resistance to *F. o. f.* sp. *lactucae* because highly susceptible cultivars would not survive infection until seed production. Ten-day-old cultures of the *F. oxysporum* f. sp. *lactuca isolate* (isolate HL2) were used to inoculate ten-day-old lettuce seedlings of each cultivar using three different methods. In the first method, the seedlings were lifted from soil and their roots were washed. Approximately, 2 cm of the
root tips were cut and the wounded roots were immersed in a spore suspension of $5 \times 10^6$ spores/ml for 8 minutes. Seedlings were then transplanted into plastic trays filled with sterile soil mix (1 part peat: 1 part vermiculite: 2 parts sand). The second inoculation method consisted of inoculating the sterile soil mix with 10 ml of the spore suspension of the same concentration, and directly transplanting seedlings whose roots have been washed as described above. The third inoculation method consisted of seedlings whose roots were washed and then transplanted directly into the sterile mix. At flowering, the open flowers were sprayed to saturation (8ml) with the same spore concentration as used in method 1. Eight seedlings were inoculated per cultivar per treatment (Fig. C.2). For each trial, the trays were incubated in the greenhouse at day and night temperatures of 25 ± 3°C and 19.0 ± 3 °C, respectively. Seedlings that were root inoculated with 0.5% KCl, soil treated with 10 ml of 0.5% KCl or flowers sprayed with 0.5% KCl, were added as negative control plants for each of the three inoculation methods respectively. The experiment was set up in a completely randomized design and was repeated twice. In the second experiment two isolates of *F. oxysporum* f. sp. *lactucae* were used (HL2 and 2002-06).

The lettuce was grown to maturity and whole plants were harvested for analysis. The elongated stems including inflorescences (5-10 cm in length) were sectioned into basal nodes (BN, 0-2.5 cm), middle nodes (MN, 2.5-5 cm), upper nodes (UN, 5-7.5cm), and flower panicles (FP) for the recovery of the pathogen. The stems sections of each plant were washed and surface disinfested in 10% NaOCl for 10 minutes, rinsed copiously with sterile distilled water, and blotted dry on sterile paper towels. Three
subsections (about 5 mm³) were cut from each main section and plated on Komada’s medium. The plates were incubated under 12 hr fluorescent light and 12 hr darkness for five days, after which they were examined microscopically for the presence of the pathogen. Putative *F. oxysporum* colonies were subcultured on three media (Komada’s, potatoes dextrose agar, and 0.5% KCl medium and their identity was confirmed by morphological characterization (Burgess et al. 1994). Seeds harvested from each treatment were divided into two parts: one part was plated out on Komada’s medium without surface disinfestation and the other part was surface disinfested in 10% NaOCl for 3 minutes, rinsed three times with sterile distilled water and dried in the fume hood on filter paper for about 3 hours. Approximately 100 seeds of both disinfested and nondisinfested seeds were plated per agar plate for the recovery of *F. oxysporum* f. sp. *lactucae* from seed. The rate of recovery was calculated as the number of plants from which the pathogen was recovered to the total number of plants per treatment. The recovery rate for the seed was calculated as the percentage of seeds infested per plant.

**Seed contamination by infested plant debris**

Ten grams of infested debris was suspended in 200 ml of 1% sodium hexametaphosphate and stirred for 8 minutes. Twenty milliliters of the suspension were added to 80 ml of 0.1% water agar and stirred for 5 minutes. The water agar suspension (1 ml) was used to inoculate 10 plates of Komada’s medium. The plates were incubated under 12 hours of white fluorescent light and the number of putative *F. oxysporum* colonies counted after five days to estimate the level of infestation per gram of debris. Representative colonies were subcultured on Komada’s medium and the identity was
confirmed by the development of a grainy texture characteristic (Fig. C.6) of \textit{F. oxysporum} f. sp. \textit{lactucae}.

Subsamples of seeds from commercial seed lots were plated on Komada’s medium to assess the level of contamination and for identification of \textit{F. oxysporum} f. sp. \textit{lactucae}-free seed. Infested debris was then mixed with \textit{F. o. f. sp. lactucae}-free seeds (wt by wt) in the following ratios: 1:5, 1:1, 5:1, 20:1, 100:1, 300:1, and 400:1. The final weights were respectively, 6g, 2g, 6g, 10.5g, 20.2g, 30.1g, and 40.1g. The seed:debris combinations were mixed thoroughly and then were winnowed to remove all the debris. The seeds were then plated out on Komada’s selective medium and incubated under 12 hr fluorescent light for 7 days to determine the level of infestation. Ten plates were plated per seed:debris mix and identification of the fungi growing on the seeds was performed by microscopy and confirmation of colony morphology as previously described.

\textbf{Seed infestation and germination potential}

Five grams each of \textit{F. oxysporum} f. sp. \textit{lactucae}-free seed (as determined in the previous section) were soaked in (15 ml) different spore concentrations (100,000 spores/ml, 10,000 spores/ml, 1000 spores/ml, 100 spores/ml, 10 spore/ml, and 1 spore/ml) of \textit{F. oxysporum} f. sp. \textit{lactucae} isolate HL2 for 5 minutes with occasional agitation. The seeds were drained and dried on sterile filter paper in the laminar flow hood and were plated on Komada’s medium. The plates were incubated under 12 hours of fluorescent light for 3-4 days to determine the level of infestation. The seed lot in which 100% infestation was achieved using the lowest spore concentration possible was chosen to mix with noninfested seed in the creation of lots with different infestation
levels. Seed lots with artificially infested levels of 10, 5, 1, 0.5, 0.2 and 0.1 % infestation were obtained by mixing *F. oxysporum* f. sp. *lactucae* free-seed and the 100% infested seed stock wt/wt. The first set was stored at room temperature for one year and the second set was stored at room temperature for four months. The two seed lots were then tested for the effect of contamination on germination potential in the greenhouse. Three levels of infestation were chosen (100%, 10%, and 5%) and about 216-250 seeds were sown in sterile soil mix (1 part Sunshine planting soil mix: 2 parts sand) in germination trays of 76 cm x 38 cm. Three lettuce seeds were seeded in each seed compartment. The experiment was repeated twice.

**RESULTS**

**Systemic movement of *F. o. f. sp. lactucae* in lettuce**

The first cultivar to reach maturity was Vulcan followed by King Henry, and finally Sharpshooter. By plating different plant parts on *Fusarium* selective medium, the pathogen was recovered from the basal part of the stem to the inflorescence panicles in both root inoculated and soil inoculated plants for all cultivars (Table C.1). The recovery rate decreased as the distance from the root to the tip of the plant increased (Fig. C.1). The highest recovery rate for the soil inoculated plants was at the basal nodes from which 100% of the plants from all the three cultivars contained the fungus. The lowest recovery rate was from the panicles with just 14.3% of Sharpshooter plants harboring the fungus, while the rate of recovery for Vulcan and King Henry was 62.5% each. For the soil inoculation method, the highest recovery rate was also obtained at the basal nodes with 100% of plants of all the cultivars scoring positive for *F. oxysporum* f. sp. *lactucae*. 
The lowest recovery rate was obtained at the panicles, which was 25% for Vulcan and King Henry and 0% for Sharpshooter. In addition, the method of inoculation did not seem to affect the rate of recovery as the pathogen was recovered from most plants, except flowers, inoculated either by the root-dip or by the soil inoculation method. The results were similar for the both experiments.

Symptom development was only visible at the basal nodes of some plants of the cultivars Sharpshooter and Vulcan and never on the upper parts of the stems (Fig. C.3). There were no visible vascular discolorations on the cultivar King Henry in any plant part. The reddish vascular discolorations that were observed were only visible on one side of the stem and there was no sign of wilt on any of the plants at harvest. Thus, outwardly, the plants were asymptomatic.

Using isolate HL2 in the first experiment, the pathogen was not recovered from the seeds obtained from the root-dip or soil inoculated plants of all the three cultivars (Table C.2). For contrast, the infestation rate of seed produced from flowers that were directly sprayed with the pathogen was 0.0% for disinfested seeds and 3.9 % 4.0% for Sharpshooter and King Henry for nondisinfested seed, respectively. In the second experiment using isolate HL2, none of the Sharpshooter seeds harvested from the plants inoculated either by root-dip or by soil inoculation were infested with the pathogen regardless of whether they were disinfested. However, nondisinfested seed from the flower spray plants had a 2.2% infestation rate. Seeds obtained from both root-dip and soil inoculated Vulcan plants were contaminated with the pathogen, with the higher contamination recorded for seed obtained from plants that were soil inoculated (0.3%).
Seed from flower sprayed plants of the cultivar King Henry recorded infestation rates of 8.30% and the root inoculation treatment had an infestation level of 0.03%. However, infestation was only detected on nondisinfested seed and the pathogen was not recovered from the disinfested seed.

In the second experiment using isolate 2002-06, the infestation rate for the cultivar Sharpshooter was 0.08% for nondisinfested seed obtained from soil inoculation treatments while Vulcan had an infestation rate of 0.02% for the same treatment. The pathogen was not recovered from the cultivar King Henry in both root and soil inoculated treatments. Overall, in this experiment, the level of infestation for the flower sprayed plants for the three cultivars were 3.6%, 5.5%, and 8.2% for Sharpshooter, Vulcan, and King Henry, respectively, while disinfested seeds from the same treatment were pathogen-free.

Compared to the infestation levels of the nondisinfested seeds harvested from plants inoculated by flower spray (ranged between 2.2% and 8.3%), the levels of infestation recorded for the various nondisinfested seeds were relatively low (0.01% – 0.3%). Seed from the negative control plants were not infested by the pathogen.

**Seed contamination by infested plant debris**

The number of colony forming units (CFUs) of *F. oxysporum* f. sp. *lactucae* recovered by dilution plating of debris from the greenhouse experiments ranged between $1.3 \times 10^4$ and $7.4 \times 10^4$ CFUs per gram of debris. When the infested debris was mixed in decreasing amounts with noninfested lettuce seeds, the pathogen-free seeds became infested with *F. o. f. sp. lactucae* (Table C.3). The infestation rate ranged between 100%
and 0.08% for infestation ratios of 1:5 to 400:1 respectively. The infestation levels decreased with decreasing amounts of debris.

**Seed infestation and germination potential**

Only the highest spore concentration, 100,000 spores/ml, resulted in 100% infestation of seed (data not shown). The second highest infestation rate (78.1%) was obtained with a spore concentration of 10,000 spores/ml. The seed lot treated with this concentration was used to generate seed lots of decreasing infestation levels. The seed lot that was stored for over a year had a significantly lower germination rate than the newly infested lot. Compared to the noninfested seeds, the germination rate of the infested seeds was reduced by more than 90% (Fig. C.5). Seed lot 6AO (10%) had more germinated seeds than seed lot 5AO which had an infestation level of 5%.

**DISCUSSION**

The purpose of this work was to examine the potential of lettuce to produce infected seed following inoculations with the vascular wilt pathogen *F. oxysporum* f. sp. *lactucae*. Three different lettuce types were used in these experiments and three methods of inoculations were employed. Although there was some variability among treatments, overall results were similar. For all lettuce types, the infected lettuce plants survived, the fungus was recovered at all nodes and the panicles, and infested lettuce seed resulted from infection.

The percent recovery of *F. oxysporum* f. sp. *lactucae* from the mature lettuce plants was the same for the basal and middle nodes for both root inoculated and soil inoculated treatments. However the percent recovery for the upper nodes and panicles in
the root inoculated plants was generally higher in the root-dip inoculated plants than in the soil inoculated plants. This may be due to an increased ability of the fungus to penetrate wounded roots compared to unwounded roots. It has been shown that *Fusarium oxysporum* penetrates the host primarily through roots most likely because root hairs are single cells and are easier to penetrate single cells than multiple cell layers (Lagopodi et al. 2002). Wounding roots likely facilitates access to vascular tissue by eliminating the necessity for additional cell penetration.

The rate of recovery dropped significantly towards the inflorescence stalks for both soil inoculated and root inoculation treatments. Apparently, movement of the fungus along the stalks was restricted by some mechanism. It has been documented that in resistant plants, production of tyloses and gels within the xylem vessels is very rapid and is accompanied by the accumulation of antifungal compounds. Occlusion of the vessels restricts further fungal colonization and allows the accumulation of antifungal compounds to inhibitory levels (Flood 2006). This restriction may have contributed to the moderate resistance to the pathogen exhibited by these cultivars for even though the pathogen was recovered from all plants inoculated, these plants were, for the most part, asymptomatic (Fig. C.3). Similarly, *Fusarium verticilloides* has also been known to infect maize plants without causing noticeable symptoms (Munvold et al. 1997).

A second finding was that the recovery of the pathogen from nondisinfested seed was very low (0.02-10%). Moreover, no fungus was isolated from the disinfested seeds suggesting that the fungus is externally borne. Similarly, work by Garibladi et al. (2004) on seedborne *F. oxysporum* f. sp. *lactucae* in Italy revealed seed infestation levels of 0.06
to 0.6%, and no recovery from surface disinfected seed. Most reports of seed transmission of *F. oxysporum* indicate low seed infestation levels that may result from dispersal of inoculum by mechanical means and not from internal movement of the fungus (Inglis 1980; Gracia-Garza et al. 1999). However, pathogen movement through the vascular system of the pedicel and into the embryo has been reported for other formae speciales such as *matthioli*, *largenariae*, *vasinfectum*, and *pisi* (Baker 1948; Kuniyasu and Kishi 1977; Rudolf and Harrison 1945; Snyder 1932).

Additionally, in fruit-bearing host plants, *F. oxysporum* can penetrate the fleshy fruit easily and then continue on to enter the seed coat resulting in seed infestation. For example, in watermelon that was grafted to bottle gourd root stocks, *F. oxysporum* f. sp. *lagenariae* was commonly detected in mature fruits borne on diseased stems, and could be found in the principal vascular bundles and tissue sections obtained from the mesocarp and seeds (Kuniyasu and Kishi 1977). However, in the young fruits, the fungus was detected in the base of the young peduncles but was not detected from the other parts of the unripe fruit. Regarding lettuce, data from this study suggest the fungus is suppressed from moving up into the flower pedicel to the ovaries where it can become internally seedborne, although this suppression may not be absolute.

A third finding was that seeds of lettuce that had come into contact with infested debris became infested with the fungus as revealed by selective plating on Komada’s medium. This result suggests that field contaminated seed could be a potential source of inoculum for the introduction of Fusarium wilt in new lettuce production areas. In such a situation, fungus spores borne in infested seed likely contact seed during harvest and
threshing and then lodge within the groves of the seed coat. The fungus could then re-
infects the seedling during germination or the spores could germinate and penetrate the
seed coat directly before seed germination. The possibility of contaminated seed giving
rise to infected plants was not investigated in this study but, has been documented for *F.
oxysporum* f. sp. *elaeidis* and other formae speciales (Flood et al. 2006; Bersri 1978;
Kuniyasu and Nakamura 1978)).

Only the highest spore concentration, 100,000 spores/ml, resulted in 100%
infestation of seed. Although in 10,000 spores/ml treatment, each seed had the chance of
coming into contact with approximately 30 spores during incubation, the infestation rate
at this concentration was only 78.1%. This result clearly showed that the low infestation
rate is not due to lack of enough spores to infect seed but may be due to the experimental
protocol. The seeds were infested with occasional agitations and the time allowed for
spores to settle may not have been sufficient and so most of the floating spores may have
drained with the solution.

Germination rate of infested seed stored for over a year was reduced by more than
90% compared to the noninfested seed. Interestingly, this substantial reduction was
apparent regardless of the infestation rate, and was not apparent when seed was stored for
only 4 months. Some species of *Fusarium* (*F. moniliforme*) has been shown to have
variable effects on seed germination in which germination did not always reflect the level
of infestation of the seed. Some seed lots that had high incidence of *F. moniliforme*
infection experienced little or no reduction in germination or seedling growth, while
others were seriously affected by the presence of the pathogen at low rates (Munkvold et
High mortality observed in the seed germination test suggests that the presence of the fungus on some seed in a lot may impact the entire lot and thus have serious consequences on seed quality and subsequent crop yield.

It is concluded from this study that dissemination of the Fusarium wilt of lettuce via seed is possible but that seed transmission may be due to seed contaminated only on the surface by conidia. These data are particularly important for those involved in seed production and seed health. A significant amount of lettuce seed production in California occurs in the vicinity of Huron, CA, where Fusarium wilt was first reported in the U. S. This study suggests there needs to be increased vigilance in the placement of lettuce seed production fields to minimize any potential of seed to contamination by contact with asymptomatic infected plants in production fields or contact with infested soil.
REFERENCES


Table C.1. Evidence of systemic movement of *F. oxysporum f. sp. lactucae* within lettuce plants calculated as percentage of stem sections from which the pathogen was recovered at maturity from plants grown in the greenhouse. Plants from each treatment were cut into main sections and stem pieces of each section were plated out on Komada’s medium for the recovery of the pathogen within.

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Cultivar</th>
<th>Plant parts used for recovery of pathogen*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BN</td>
</tr>
<tr>
<td>Root inoculation</td>
<td>Sharpshooter</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Vulcan</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>King Henry</td>
<td>100</td>
</tr>
<tr>
<td>Soil inoculation</td>
<td>Sharpshooter</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Vulcan</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>King Henry</td>
<td>100</td>
</tr>
</tbody>
</table>

* BN represents the bottom nodes of the stem, MN represents the middle nodes, UN represents the upper nodes, and FP represents the inflorescence panicles. The percentage recovery of the pathogen is the mean recovery for two experiments.
Table C.2. Percent recovery of *F. o. f. sp. latucae* from seeds harvested from inoculated lettuce plants in the greenhouse. The percent infestation was determined on nondisinfested seeds harvested from each of eight plants in each treatment.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Isolate</th>
<th>Inoc. method</th>
<th>No. of seeds infested/ No. plated</th>
<th>Mean (% seed infested)</th>
<th>Max.</th>
<th>Min.</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharpshooter</td>
<td>HL2</td>
<td>root</td>
<td>0/2379</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
<td>0/3123</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vulcan</td>
<td>HL2</td>
<td>root</td>
<td>0/2278</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
<td>0/2935</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>King Henry</td>
<td>HL2</td>
<td>root</td>
<td>0/2487</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
<td>0/2935</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharpshooter</td>
<td>2002-06</td>
<td>root</td>
<td>0/9900</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
<td>1/5560</td>
<td>0.083</td>
<td>0.50</td>
<td>0</td>
<td>0.0722</td>
</tr>
<tr>
<td>Vulcan</td>
<td>2002-06</td>
<td>root</td>
<td>0/7430</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
<td>0/7770</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HL2</td>
<td>2002-06</td>
<td>root</td>
<td>2/10877</td>
<td>0.025</td>
<td>0.10</td>
<td>0</td>
<td>0.0164</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
<td>22/9680</td>
<td>0.275</td>
<td>0.60</td>
<td>0</td>
<td>0.0800</td>
</tr>
<tr>
<td>King Henry</td>
<td>2002-06</td>
<td>root</td>
<td>2/5080</td>
<td>0.025</td>
<td>0.10</td>
<td>0</td>
<td>0.0164</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soil</td>
<td>0/7850</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The mean percent infested seed was calculated from the percent infestation per plant for 8 plants in each treatment. The Max. and Min. were the highest infestation rate and the lowest infestation rate per plant, respectively, for the 8 plants in each treatment.
Table C.3. Percentage contamination of seeds after *F. oxysporum* f. sp *lactucae*-free seeds were mixed with different amounts of infested debris obtained from the greenhouse experiment.

<table>
<thead>
<tr>
<th>Ratio of mixture (wt/wt)</th>
<th>No. of seeds plated</th>
<th>No. of seeds infested</th>
<th>Percent infestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: 5</td>
<td>1640</td>
<td>1640</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1: 1</td>
<td>2988</td>
<td>2976</td>
<td>95.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5:1</td>
<td>820</td>
<td>819</td>
<td>99.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20: 1</td>
<td>1584</td>
<td>7</td>
<td>0.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100: 1</td>
<td>2016</td>
<td>3</td>
<td>0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>300: 1</td>
<td>1800</td>
<td>4</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>400:1</td>
<td>2520</td>
<td>2</td>
<td>0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values followed by the same letter are not significantly different according to Tukey’s test (*P* < 0.05).
Figure C.1. Recovery of *F. o. f. lactucae* along the stem lettuce plants inoculated at transplant and grown to maturity in the greenhouse. During transplanting, the plants were either inoculated by root dip or by soil inoculation. Each stem was partitioned into four sections: BN = basal nodes, MN = middle nodes, UN = upper nodes, and FP = floral panicle.
Figure C.2. Lettuce cultivars Vulcan (purple) and King Henry three weeks after inoculation and transplanting into trays
Figure C.3. Longitudinal sections through the stem of the cultivar Sharpshooter showing vascular discolorations in the basal portion of the stem. The upper portions of the stems most often were symptomless.
Figure C.4. External structure of the lettuce seed (achene). A. Cultivar Sharpshooter showing the seed parachute that aids in dispersal. B. Cultivar Vulcan artificially infested with *F. o. f. sp. lactucae*. C. Cultivar Vulcan uninfested with parachute still attached. D. Microscopic view of the grooves on the seed coat. E a close-up view showing serrations at groove lines.
Figure C.5. The effect of fungal infestation on seed germination. Head type lettuce seeds were artificially infested with a spore suspension of an isolate of *F. o. f. sp. lactucae*. Both trays were sown to seeds with an infestation rate of 100%. The seeds in the tray on the left were sown 1 year after infestation and the seeds in the tray on the right were sown one month after infestation.
Figure C.6. *Fusarium oxysporum* f. sp. *lactucae* race 1 isolate BMP1300 on Komada’s medium showing the characteristic grainy texture 5 days post-inoculation.
APPENDIX D

GENETIC VARIATION WITHIN *Fusarium oxysporum* f. sp. *lactucae*

ABSTRACT

Isolates of *Fusarium oxysporum* f. sp. *lactucae* collected from Arizona, California, Japan, Italy, and Taiwan were analyzed for genetic diversity using four inter-simple sequence repeat (ISSR) primers and two minisatellite molecular markers. Cluster analyses of fingerprint patterns revealed 2 groups within the Arizona isolates. Eight haplotypes were identified in these clusters based on the polymorphic bands generated. Analysis over several years, revealed an increase from two haplotypes in 2001 to eight haplotypes in 2005. Haplotype 1-05 was widespread occurring in two of the four countries where *F. oxysporum* f. sp. *lactucae* has been reported. Three isolates from lettuce and three isolates from soil within the Arizona population constituted the second haplotype group distinct from all the other haplotypes. It is suggested that these may represent the introduction of a new form of the f. sp. *lactucae* based on its marked genetic discrimination from the rest of the haplotype clusters. The number of haplotypes within the California isolates was 23 that clustered into a single group (Group I), reflecting a greater diversity in California compared to Arizona. Even though, the group was subdivided into two subgroups, the genetic similarity among the haplotypes was significantly high (88.5%). Three nonpathogenic field isolates were more closely related to some of the pathogenic isolates than other members of the forma specialis. The presence of two clusters of isolates from Arizona suggests there has been more than one
introduction of the pathogen into Arizona, and this introduction was likely from California.

INTRODUCTION

_Fusarium oxysporum_ f. sp. _lactucae_, causal agent of Fusarium wilt of lettuce has recently emerged as a major pathogen of lettuce causing vascular wilt in a variety of commonly grown lettuce cultivars. The fungus currently can be found in Arizona, California, Japan, Italy, Iran, and Taiwan (Matheron and Koike 2003, Hubbard and Gerik 1993, Matuo and Motohashi 1967, Garibaldi et al. 2002, Millani 1999, Huang and Lo 1998). Three pathogenic races have been distinguished within the pathogen population worldwide based on the susceptibility of the three lettuce differentials, Banchu Red Fire, Costa Rica # 4, and Patriot. Race 1 is widespread and has been reported in California, Arizona, Italy, Taiwan, and Japan, while race 2 and 3 are found only in Japan. Vegetative compatibility tests have revealed three vegetative compatibility groups within race 1 of the pathogen (Pasquali et al. 2005, Fujinaga et al. 2003). In a another related study of vegetative compatibility groupings within the 3 races of _F. oxysporum_ f. sp. _lactucae_, race 1 and 2 were associated with two distinct VC groups (VCG-1 and VCG-2 respectively), and race 3 was associated with four VCGs (VCG-3-1, VCG-3-2, VCG-3-3, and VCG-3-4) (Nashimura 2003). These studies yielded seemingly different results because of the use of different isolates. There is a need therefore to identify type isolates within the _lactucae_ population that can be used to identify VCGs in the future. Phylogenetic analysis using the partial sequence of the intergenic spacer region of the rDNA revealed three phylogenetic groups among 29 isolates examined (Fujinaga et al. 2003).
From that study, it was concluded that the three races are genetically distinct and had strong correlations with VCGs.

Although previous studies provide useful information regarding the genetic structure of this fungus, there is still a need for further investigations to elucidate the genetic diversity within the population of this fungus. Furthermore, understanding the distribution of genotypes and how this distribution is changing over time will allow evaluation of whether current phytosanitary measures are effective. If distinct genotypes remain geographically restricted, the current sanitary measures are likely to be having a positive impact. However, if genotypes unique to one region begin to appear elsewhere, then current measures may be inadequate. The evolutionary potential of the pathogen is also an important consideration in disease management and this can also be estimated from genetic diversity data.

Many methods have been used to detect genetic diversity within pathogen populations. Among these are restriction fragment length polymorphisms (RFLPs) and randomly amplified polymorphic DNAs (RAPDs) (Borokova and McClean 1993). Other types of molecular markers frequently used in studies of genetic variation are microsatellites. Microsatellite is a term collectively used for simple sequence repeats (SSR), short tandem repeats (STR), simple sequence length polymorphism (SSLP), or variable number tandem repeats (VNTR), and is a class of repetitive DNA sequences present in all organisms. Such repeats occur primarily due to slipped-strand mispairing and subsequent errors during DNA replication, repair, or recombination (Levinson and Gutman 1987).
The three popular types of markers containing microsatellite sequences that are presently used are: SSR generated by amplifying in a PCR reaction with the use of primers complementary to flanking regions, ISSR based on the amplification of regions between inversely oriented closely spaced microsatellites, and selective amplification of microsatellite loci, which utilizes amplified fragment length polymorphism (AFLP) (Rakoczy-Trajanowska and Bolibok 2004). Genetic variation within populations of some fungi have been also been assessed with minisatellites-primed PCR, and the primer of choice has been the M13 core sequence. Minisatellites are also tandem repetitive sequences that are a common feature of eukaryotic genomes. The repeats are hypervariable regions and thus are highly informative and sometimes exhibit allelic forms. The minisatellite M13 has been commonly used as a probe to generate fingerprint patterns in filamentous fungi (Meyer et al. 1991; Karlsson 1993; Stenlid et al. 1994). The technique thus offers an ideal method for studying intraspecific variation.

Mutations in these loci have been attributed to deletions or insertions of one or a few repeat units, and the mutation rates generally increase with an increase in the length of repeat tracks (Weidl et al. 1997; Dettman and Taylor 2004). This association is believed to exist because during replication, longer repeats are more prone to slipped-strand mispairing than shorter repeats and large numbers of repeats provide more opportunities for misalignment during the reannealing of the nascent strand (Dettman and Taylor 2004). Microsatellite loci show extensive length polymorphism and are highly dispersed in coding and noncoding regions in the eukaryotic genome and hence are used to estimate genetic variation in natural populations and evolutionary relationships.
Microsatellites can also originate in coding regions of the genome forming repetitive patterns in protein sequences, and the mechanisms involved in the generation of these repeats may be responsible for the rapid evolution of some proteins (Huntley and Golding 2000). They are known to serve as coding or regulatory elements and are conserved in relation to coding sequences. Repeat number variation of microsatellite tracts in coding regions is related to gene expression and function. Polyglutamine and polyproline tracts have been found in at least 67 transcription factors, including the human TATA-binding protein. Changes in repeat number can cause quantitative variation in protein function and gene activity and affect organismal physiology and development. The mutation process at a microsatellite locus can be considered to be a balance between the generation of replication of errors by slip-strand mispairing and the correction of some of these errors by exo-nucleolytic proofreading and mismatch repair (David et al. 1999; Katti et al. 2001). As regulatory elements, they are typically found upstream of promoter regions. An example includes the poly (GT) tracts found upstream of rRNA transcription unit in mouse, rat, and man. Such conservation of sequences across species is often an indication of critical biological function. In promoter regions microsatellites serve as enhancer elements in expression constructs and their deletion in promoter regions has been known to decrease promoter activity. They have also been shown to bind specific proteins as revealed by gel mobility shift experiments.

The key feature for microsatellite use in population genetics is their hypermutability with mutation rates estimated at $10^{-2}$-$10^{-6}$/generation (Ellegren 2000). Multilocus fingerprinting methods have been developed using oligonucleotides based on
microsatellites as primers in PCR amplifications (ISSRs) (Tsumura et al. 1996). This technique enables amplification of genomic DNA, provides information about many loci simultaneously, and has been successfully used to identify variability within fungal species (Thanos et al. 1996; Zeze et al. 1997). ISSR analysis is a simpler procedure than AFLP analysis because there is no need for an adapter ligation step. In addition, ISSRs appear to be more stable than RAPDs because they have longer primer sequences and use a higher annealing temperature during PCR (McCall et al. 2004).

The objective of the current study was to estimate genetic variability within *F. oxysporum* f. sp. *lactucae* populations in Arizona and California using ISSR techniques and to compare this variability to isolates from Italy, Japan and Taiwan. This will provide information relevant to controlling the disease through breeding of resistant cultivars, and development of improved phytosanitary strategies.

**MATERIALS AND METHODS**

**Fungal isolates**

Isolates from Arizona and California were collected over a period of four years from diseased lettuce roots and infested field soils and were used as representative of the population of isolates currently present in both states. Nine additional isolates including one race 1 and two race 2 isolates from Japan, three race 1 isolates from Italy, and three race 1 isolates from Taiwan were also included. These samples were collected to reflect the geographical distribution worldwide (Table D.1).
**DNA extraction and amplification**

Liquid cultures of all isolates were prepared by flooding agar plates (PDA) containing 5-day-old cultures of test fungi with 10ml of sterile deionized H2O and dislodging mycelia and conidia with a pipette tip. A 2 ml suspension was added to 100 ml of sterile liquid growth medium (20 g of D-glucose, 1.2 g of DL-asparagine, 1.2 g of K2HPO4, 0.5 g of yeast extract, 0.5 g of MgSO4.7H2O, and 0.1 g of NaCl per liter) in a 250-ml Erlenmeyer flask. The flasks were incubated on a rotary shaker at 120 rpm for 5 days at 22°C. Mycelia were harvested by filtration through Miracloth (Calbiochem, EMD Biosciences Inc., San Diego, CA), lyophilized, and stored desiccated at 4°C.

Extraction of fungal total genomic DNA was done using the BIO 101 Fast DNA kit (Qbiogene, Irvine, CA) according to the manufacturer’s instructions. Total DNA was resuspended in 50 ml TE buffer and stored at -20 °C. DNA concentrations were adjusted to 10 ng/µl with a fluorometer (DNA Fluorometer, TK0100, Hoefer Scientific Instruments, San Francisco). The stock DNA was diluted to 10 ng/µl and stored in TE buffer at 4 °C to standardize concentrations for working solutions.

**ISSR- and minisatellite-primed PCR**

A total of 10 primers previously reported in literature (Czembor and Arsiniuk 1999; Frederik and Otmar 2002; Leroy et al. 2001; Meyer et al. 1997), eight ISSRs and two minisatellites, were tested for the production of robust reproducible bands. PCR-amplifications were performed with 0.5 ng of genomic DNA in 25 µl reactions, 0.6 mM of each primer, 0.2 mM of each dNTP (Amersham Pharmacia), 2.5 mM of MgCl2, and 0.04 U of Amplitaq DNA polymerase in 1X Amplitaq buffer II (Applied Biosystems).
PCR reactions were carried out in a Peltier thermocycler (Model PTC-100, MJ Research, Inc.) programmed for the following parameters: 94° C for 1 min, 40 cycles at 94° C for 1 min, 55° C for 1 min 30 secs, and 72° C for 2 min. PCR-amplified DNA fragments were fractionated in 1% agarose gels in 0.5% tris-borate-EDTA (TBE) buffer and visualized by uv illumination after staining in ethidium bromide. Gels were photographed on a uv-transilluminator using a digital imaging system (UVP BioImaging Systems, UVP Inc., Upland, CA).

Data analysis

Bands were scored as biallelic loci (present or absent) with the aid of eight reference isolates (HL2, 2003-09, 2004-01, 2004-02, 2004-03, 2004-04, 2005-03, and 2005-05) included in each gel to ensure that all bands ascribed to a locus were of the same mobility. ISSR analyses were performed three times for each primer to confirm reproducibility of amplification products. Weak bands that were not reproducible in the PCR assays were not included in the analyses.

Data were transformed into a matrix to calculate genetic distances among isolates. Cluster analysis was performed by the UPGMA with Jaccard’s similarity coefficient (Sneath and Sokal 1973) and the goodness of fit of the phenogram for the similarity matrix was measured by cophenetic correlation (r) analysis using the NTSYSpc2.0 program (Exeter Software, Setauket, NY). Bootstrap values (1000 replicates) for each branch were calculated using Phylogenetic Analysis Using Parsimony package (PAUP, ver. 4.0b10; Sinauer Associates). As this study was continuous over several years,
isolates received each year were analyzed together with reference isolates that represented already identified haplotypes.

Population structure and differentiation were estimated using total genetic variation partitioned among geographic origin. Gene diversity within the total population \( H_t \) and within subpopulations \( H_s \) (Nei 1987). Genetic structure was analyzed using Nei’s coefficient of population differentiation which is an indication of the level of population subdivision, calculated using the formula \( G_{st} = \frac{H_r - H_s}{H_t} \) (Nei 1987). \( G_{st} \) values ranged from zero to one, with low values indicating that little variation is apportioned among populations while high values denote a large amount of variation among populations. An estimate of gene flow \( (N_m) \) was calculated using the coefficients \( G_{st} \) or \( G_{cs} \) where \( N_m = 0.5(1 - G_{st})/G_{st} \), to determine the level of migration between subpopulations. Nei’s measures of genetic distance and genetic identity between populations over all loci were computed to determine the level of genetic similarity between the subpopulations. Linkage disequilibria were calculated using each pair of loci within each of the three populations according to Lewontin (1960), using the equation \( D' = D/D_{max} \), where \( D' \) is the normalized linkage disequilibrium coefficient, \( D \) is the product of the coupling allele frequencies minus that of the repulsion allele frequencies, and \( D_{max} \) is the theoretical maximum for the observed allele frequencies. All these coefficients were computed using the program PopGen 1.31 (Yeh et al. 1999).
RESULTS

DNA amplification

From a total of 10 primers, seven were selected that provided clear, informative amplification products. The seven primers included two minisatellites, T3B and the core-sequence of the phage M13 specific primer, and five ISSR primers presented in Table D.2. The amplification patterns were reproducible within and between different PCR runs and duplicate DNA preparations of the same isolates. The number of amplification products varied from 1 to more than 15, with size range from 200 bp to 3000 bp (Fig. D.5).

Data analysis

For the Arizona isolates, a total of 74 unambiguous bands were generated with seven primers of which 52 were polymorphic (70.27%). The number of polymorphic bands ranged from six to 15 per primer. The reference isolates were clearly and consistently differentiated by the primers. The isolates from Arizona were grouped into three distinct clusters, I-A, I-B, and II, that corresponded to eight genetically distinct haplotypes (Fig. D.1). Three of these isolates formed a distinct cluster and were clearly separated from the other isolates by a bootstrap support value of 100%. The UPGMA tree, based upon the Jaccard’s similarity coefficients calculated from the combined data sets, is presented in Fig.D.1 (r = 0.997). The similarity between subgroups I-A and I-B was 80%, and between these two groups and haplotype group II was 58 %. Haplotype I-05 was widespread as it was recovered from 24 fields. Analysis over several years, revealed an increase from one haplotype in 2001 to eight haplotypes in 2005 (Table D.3).
The different haplotypes are mapped to the different fields in which they were recovered in Fig. D.6.

For the California isolates a total of 74 unambiguous bands were generated with the seven primers of which 58 were polymorphic (78.38%). Twenty-three genetically distinct haplotypes were identified that clustered into three subgroups. Subgroup 1-A comprised three isolates that belonged to haplotype I-05 together with five other haplotypes, while, subgroup I-C was made up of 14 haplotypes (Fig. D.2). The three subgroups shared a similarity index of 84.6%. Six isolates nonpathogenic to lettuce included in the analysis for comparison were scattered among the clusters. The dendrogram based upon Jaccard’s similarity coefficient calculated from combined data sets is presented in Fig. D.2 (r = 0.969).

Analysis of the cluster pattern of the combined taxa and data set revealed no correlation with either geographic origin or pathogenic race. Two isolates from Italy (FOL7 and FOL17) and one isolate from Japan (S-1), all associated with race 1, clustered within the major subgroup I-A (haplotype I-05) (Fig. D.3). Two race 2 isolates from Japan grouped together with a similarity index of 98%. Three isolates from Taiwan were interspersed among the different haplotypes showing no particular clustering pattern. One of these isolates grouped together with isolate FOL 10 from Italy, while the other associated with JCP165 from California. The UPGMA tree obtained from the combined data is presented in Fig.D.3 (r = 0.9783).

Gene diversities in the total population and within subpopulations were similar (0.1713 and 0.1557 respectively). The extent of differentiation was low (G_{st} = 0.0908)
and the level of gene flow was relatively high ($N_m = 5.0041$). Not all of the haplotypes encountered within the Arizona sample population were found within the Californian sample population and vice versa despite the amount of gene flow between the populations (Fig. D.4). Twenty-two haplotypes within the Californian sample were not represented in the Arizona sample. The genetic distance between the Arizona population and California population was 0.9740 and between Arizona and the other three together was 0.9745. The value was similar for California and the other countries. The genetic identity was relatively low between Arizona and California (0.0264) (Table D.5).

**DISCUSSION**

The most cost effective management of *Fusarium* wilt diseases is obtained through the use of resistant cultivars. However, the development of such cultivars is seriously impeded by pathogenic variability occurring within pathogen populations. *Fusarium oxysporum* f. sp. *lactucae* race 1 is widespread and has been reported in California, Arizona, Italy, Taiwan and Japan, while race 2 and 3 are found only in Japan. In this study, considerable genetic variation was revealed among isolates of race 1 recovered from lettuce, with haplotypes separating into three distinctly defined clusters within the Arizona isolate population. Given the high genetic similarity between subgroup I-A and I-B (91%) and their consistent appearance over a period of five years, the isolates within these groups may have evolved from the same ancestral strains. On the other hand, the low similarity index between group II and these two subgroups (58%) may suggest more than one introduction of the pathogen into Arizona.
Analysis over several years revealed an increase from two haplotypes in 2001 to eight haplotypes in 2005 in Arizona. Most of the isolates of 2001 fell within the main cluster (group I-A) in which the major haplotype was 1-05. This haplotype also encompassed the original strains isolated in 1990 from California. The second haplotype was represented by isolate JCP281 and was very distinct from haplotype I-05. In 2003, new haplotypes were identified that were closely related to the original genotypes. In 2004, more haplotypes were detected, some of which clustered with the main haplotype (I-05) and one of which clustered with JCP281. Therefore, the genetic diversity of this fungal population changed significantly in 2004.

The genetic distinctiveness of group II isolates suggests they may be the first recorded representatives of a third form of *lactucae* race 1 and possibly a new VCG. To date, all isolates tested have belonged to two VCGs within the race 1 population of isolates (Pasquali et al. 2005). Previously, high similarity levels have been used to distinguish VCGs from each other in the *F. oxysporum* complex. Assigbetse et al. (1994) distinguished six VCGs in *F. oxysporum* f. sp. *cubense* at an 80% similarity level using RFLP data. Thus genetic dissimilarities could be used to predict new VCGs prior to formal complementation tests (Rosewich et al. 1999).

As observed, the distribution of lactucae haplotypes in AZ fits three patterns: a single haplotype of high frequency (1-05), a single haplotype shared by two fields (I-01), and the rest appearing in single fields. This distribution suggests more than one independent introductions of the pathogen into AZ. Although the results are skewed by the low sample representation per field and per year, they establish a baseline distribution.
and a more representative sampling of isolates in the future may give a better representation of the haplotypes within AZ. Those isolates that were used as reference in this study may in future serve as representative isolates for further genotyping.

The California population was more diverse than the Arizona population, with a total of 23 haplotypes represented. The majority of these haplotypes were found within the two major clusters (subgroups I-A and IC). The data reported here may also provide an indication of where the f. sp. *lactucae* was first established in the United States. Although it is well known that the original site of diagnosis is not necessarily the site of origin, in this case, the fact that the disease was first diagnosed in California and that California contains a high level of diversity could very well argue for it being the site of origin in the US. Although the two clusters are considered as subgroups, the bootstrap support value was less than 50% indicating a high genetic similarity between the two (91%). In other studies of fungal diversity, there has generally been a strong correlation between center of origin and diversity (McDonald and Linde 2002). Following this reasoning therefore, CA is most likely the site of origin of the pathogen in the US. A single haplotype, 1-05, dominates in both states and was found throughout the five years of sample collection. The overrepresentation of this single haplotype suggests an extensive clonal reproduction of the f. sp. *lactucae*.

Race 2 isolates maintained a distinct fingerprint pattern from all the race 1 isolates in this study thus confirming their distinct origin. Phylogenetic evidence has shown that *F. oxysporum* f. sp. *lactucae* is of polyphyletic origin (Fujinaga et al. 2005; Mbofung et al. 2006 in press). This is not surprising as similar data revealing polyphyly have been
obtained from studies on *F. oxysporum* f. spp. *cubense*, *ciceri*, *lycopersici* and *melonis* (Jimenez-Gasco et al. 2004; Baayen et al. 2000; O’Donnell et al. 1998). In contrast, other formae speciales such as *albedinis*, *canariensis*, and *conglutinans* have been shown to be monophyletic (Baayen et al. 2000; Kistler 2001).

Gene diversity analysis of the populations showed similar values for both populations and the coefficient of genetic differentiation was quite low suggesting a similar population structure and a highly homogenous population. The amount of gene flow between Arizona and California was relatively high (5.0041). This suggests some form of movement of genotypes between the two states, although, the sampling was not robust enough to confirm this phenomenon since only single isolates were obtained per field for most of the fields sampled. The fact that the level of genetic differentiation was low further supports the fact that gene flow is occurring among the subpopulations in the different geographic locations. *Fusarium oxysporum* is believed to have strictly asexual reproduction and its spread is thought to be through a limited number of clonal lineages on a large geographic scale (Gordon and Martyn 1997). While a single haplotype may have dominated in 2001, the frequency of haplotypes changed over 5 years suggesting that the dominant haplotype can shift with time. These results are similar to those obtained in studies on *F. oxysporum* f. sp. *vasinfectum* in Australia (Wang et al. 2006).

Sampling bias in this study does not allow classification of the haplotypes into the different categories of high, moderate, and low frequencies. For example, a large number of haplotypes seem to be specific to certain fields, but this may not reflect the reality.
However, the mere presence of one dominant haplotype and several other singleton haplotypes suggests that there may have been movement across regions at different times.

The results of this study form a baseline distribution of the genotypes within the *F. oxysporum* f. sp. *lactucae* population sampled. Continued monitoring of the geographic distribution of these haplotypes and those that are yet to be identified will allow evaluation of the efficacy of current management practices. If in the course of this continued monitoring, region-specific haplotypes are identified then, management will require breeding of region-specific cultivars. On the other hand, if surveys indicate that haplotypes are continuing to move among states or regions, then, management would require more stringent cultural methods in addition to the breeding for cultivars resistant to more diverse genotypes.
REFERENCES


Table D.1. Isolates used in this study and their geographic origin.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Race&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Date&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Isolation substrate</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP1323</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1324</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1326</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1331</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1301</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1307</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1308</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1362</td>
<td>1</td>
<td>2001</td>
<td>soil</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1366</td>
<td>1</td>
<td>2001</td>
<td>soil</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1379</td>
<td>1</td>
<td>2001</td>
<td>soil</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1381</td>
<td>1</td>
<td>2001</td>
<td>soil</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1374</td>
<td>1</td>
<td>2001</td>
<td>soil</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>BMP1387</td>
<td>1</td>
<td>2001</td>
<td>soil</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>2001-01</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2001-03</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2001-06</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2001-07</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2001-08</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2001-10</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>JCP007</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>2002-01</td>
<td>1</td>
<td>2002</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2002-05</td>
<td>1</td>
<td>2002</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2002-09</td>
<td>1</td>
<td>2002</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2003-01</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2003-03</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2003-04</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>2003-05</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2003-06</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Bard, CA</td>
</tr>
<tr>
<td>2003-07</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Gila, AZ</td>
</tr>
<tr>
<td>2003-08</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Gila, AZ</td>
</tr>
<tr>
<td>2003-09</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Gila, AZ</td>
</tr>
<tr>
<td>2004-01</td>
<td>1</td>
<td>2004</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2004-02</td>
<td>1</td>
<td>2004</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2004-03</td>
<td>1</td>
<td>2004</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2004-04</td>
<td>1</td>
<td>2004</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2004-05</td>
<td>1</td>
<td>2004</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>2005-01</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2005-02</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2005-03</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
</tbody>
</table>

<sup>a</sup>NP nonpathogenic, <sup>b</sup>Year of collection of some of the isolates is unknown for those from Taiwan and Japan.
Table D.1 - *Continued.*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Race&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Date&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Isolation substrate</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005-04</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>JCP281</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>2005-05</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>Yuma, AZ</td>
</tr>
<tr>
<td>HL1</td>
<td>1</td>
<td>1990</td>
<td>lettuce</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>HL2</td>
<td>1</td>
<td>1990</td>
<td>lettuce</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP086</td>
<td>1</td>
<td>2002</td>
<td>lettuce</td>
<td>Watsonville, CA</td>
</tr>
<tr>
<td>JCP141</td>
<td>1</td>
<td>2002</td>
<td>soil</td>
<td>California</td>
</tr>
<tr>
<td>JCP158</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP161</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP165</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP173</td>
<td>1</td>
<td>2003</td>
<td>soil</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP181</td>
<td>1</td>
<td>2003</td>
<td>soil</td>
<td>Salinas, CA</td>
</tr>
<tr>
<td>JCP196</td>
<td>1</td>
<td>2003</td>
<td>soil</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP213</td>
<td>1</td>
<td>2003</td>
<td>soil</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP224</td>
<td>1</td>
<td>2003</td>
<td>soil</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP232</td>
<td>1</td>
<td>2003</td>
<td>soil</td>
<td>Huron, CA</td>
</tr>
<tr>
<td>JCP284</td>
<td>1</td>
<td>2003</td>
<td>-</td>
<td>California</td>
</tr>
<tr>
<td>JCP286</td>
<td>1</td>
<td>2003</td>
<td>-</td>
<td>Watsonville, CA</td>
</tr>
<tr>
<td>JCP288</td>
<td>1</td>
<td>2003</td>
<td>-</td>
<td>Watsonville, CA</td>
</tr>
<tr>
<td>JCP293</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Fresno, CA</td>
</tr>
<tr>
<td>JCP292</td>
<td>1</td>
<td>2003</td>
<td>lettuce</td>
<td>Fresno, CA</td>
</tr>
<tr>
<td>JCP324</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>California</td>
</tr>
<tr>
<td>JCP336</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>California</td>
</tr>
<tr>
<td>JCP319</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>California</td>
</tr>
<tr>
<td>JCP351</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>California</td>
</tr>
<tr>
<td>JCP353</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>California</td>
</tr>
<tr>
<td>JCP355</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>California</td>
</tr>
<tr>
<td>JCP356</td>
<td>1</td>
<td>2005</td>
<td>lettuce</td>
<td>California</td>
</tr>
<tr>
<td>FOL7</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Italy</td>
</tr>
<tr>
<td>FOL10</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Italy</td>
</tr>
<tr>
<td>FOL17</td>
<td>1</td>
<td>2001</td>
<td>lettuce</td>
<td>Italy</td>
</tr>
<tr>
<td>T11-13</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Taiwan</td>
</tr>
<tr>
<td>T24-22</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Taiwan</td>
</tr>
<tr>
<td>T32-14</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Taiwan</td>
</tr>
<tr>
<td>S-1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>Japan</td>
</tr>
<tr>
<td>F9501</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Japan</td>
</tr>
<tr>
<td>FK09701</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Japan</td>
</tr>
</tbody>
</table>

<sup>a</sup> NP nonpathogenic, <sup>b</sup> Year of collection of sm of the isolates is unknown for those from Taiwan and Japan.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Race&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Date&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Isolation substrate</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005-6CA</td>
<td>NP</td>
<td>2005</td>
<td>Cauliflower</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>JCP20</td>
<td>NP</td>
<td>2002</td>
<td>lettuce</td>
<td>Wellton, AZ</td>
</tr>
<tr>
<td>JCP126</td>
<td>NP</td>
<td>2002</td>
<td>soil</td>
<td>Salinas, CA</td>
</tr>
<tr>
<td>JCP289</td>
<td>NP</td>
<td>2003</td>
<td>-</td>
<td>Watsonville, CA</td>
</tr>
<tr>
<td>JCP020</td>
<td>NP</td>
<td>2002</td>
<td>soil</td>
<td>Watsonville, CA</td>
</tr>
<tr>
<td>JCP044</td>
<td>NP</td>
<td>2002</td>
<td>soil</td>
<td>Watsonville, CA</td>
</tr>
</tbody>
</table>

<sup>a</sup> NP nonpathogenic, <sup>b</sup> Year of collection of some of the isolates is unknown for those from Taiwan and Japan.
Table D.2. Minisatellite and ISSR primers used in this study. The two primers M13 and T3B are minisatellite primers and the rest are microsatellite primers.

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Sequence 5′→ 3′</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ACA)$_5$</td>
<td>ACA ACA ACA ACA ACA</td>
<td>45°C</td>
</tr>
<tr>
<td>(CAA)$_5$</td>
<td>CAA CAA CAA CAA CAA</td>
<td>45°C</td>
</tr>
<tr>
<td>(GACA)$_4$</td>
<td>GAC AGA CAG ACA GAC A</td>
<td>54°C</td>
</tr>
<tr>
<td>(GTG)$_5$</td>
<td>GTG GTG GTG GTG GTG GTG</td>
<td>60°C</td>
</tr>
<tr>
<td>M13</td>
<td>GAG GGT GGC GGT TCT</td>
<td>59°C</td>
</tr>
<tr>
<td>T3B</td>
<td>AGG TCG CGG GTT CGA ATC C</td>
<td>64.5°C</td>
</tr>
<tr>
<td>TGTC</td>
<td>TGT CTG TCT GTC TGT C</td>
<td>54°C</td>
</tr>
</tbody>
</table>
Table D.3. Haplotypes of *F. oxysporum* f.sp. *lactucae* recovered in Arizona over a five year sampling period.

<table>
<thead>
<tr>
<th>Year</th>
<th># of genotypes</th>
<th># of fields</th>
<th>(GTG)$_5$</th>
<th>T3B</th>
<th>M13</th>
<th>(GACA)$_4$</th>
<th>(ACA)$_5$</th>
<th>(CAA)$_5$</th>
<th>TGTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>2</td>
<td>24</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>B</td>
<td>C</td>
<td>C</td>
<td>E</td>
<td>C</td>
<td>B</td>
</tr>
<tr>
<td>2002</td>
<td>1</td>
<td>03</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>2003</td>
<td>2</td>
<td>08</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>5</td>
<td>05</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>B</td>
<td>C</td>
<td>C</td>
<td>E</td>
<td>C</td>
<td>B</td>
</tr>
<tr>
<td>2005</td>
<td>3</td>
<td>06</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>
Table D.4. Genetic diversity analysis of populations of *Fusarium oxysporum* f. sp. *lactucae* from Arizona, California, Japan, Italy and Taiwan. The isolates from Japan, Italy and Taiwan are analyzed as a single population.

<table>
<thead>
<tr>
<th>Population (not corrected)</th>
<th>Number of haplotypes</th>
<th>Number of polymorphic loci</th>
<th>$H^t$</th>
<th>$H^s$</th>
<th>$G_{st}$</th>
<th>$N_m$</th>
<th>$h^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arizona</td>
<td>8</td>
<td>52 (70.27%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1003</td>
</tr>
<tr>
<td>California</td>
<td>23</td>
<td>58 (78.38%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1707</td>
</tr>
<tr>
<td>Others</td>
<td>7</td>
<td>44 (59.46%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1962</td>
</tr>
<tr>
<td>All regions</td>
<td>39</td>
<td>72 (97.30%)</td>
<td>0.1713</td>
<td>0.1557</td>
<td>0.0908</td>
<td>5.0041</td>
<td>-</td>
</tr>
</tbody>
</table>

*Gene diversity in the total population as described by Nei (1987).  
Gene diversity within subpopulations as described by Nei (1987).  
Coefficient of genetic differentiation $G_{st} = (H_t - H_s)/H_t$ as described by Nei (1987).  
The amount of gene flow between subpopulations $N_m = 0.5 \times (1 - G_{st})/G_{st}$ as described by Slatkin (1987).  
Gene diversity as described by Nei (1973).
Table D. 5. Genetic identity represented in the upper diagonal, genetic distance represented in the lower diagonal and the number of pairwise loci with significant linkage disequilibrium estimated using the population analysis program, PopGen ver. 1.31 for the three groups of isolates. All the pairwise tests of linkage disequilibria were significant indicating all the loci were in linkage disequilibrium (LD).

<table>
<thead>
<tr>
<th>Population identity</th>
<th>Arizona</th>
<th>California</th>
<th>Others</th>
<th>*Linkage Disequilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arizona</td>
<td>-</td>
<td>0.9740</td>
<td>0.9745</td>
<td>3308</td>
</tr>
<tr>
<td>California</td>
<td>0.0564</td>
<td>-</td>
<td>0.9711</td>
<td>1896</td>
</tr>
<tr>
<td>Others</td>
<td>0.0259</td>
<td>0.0294</td>
<td>-</td>
<td>1420</td>
</tr>
</tbody>
</table>

* $P < 0.05$
Figure D.1. Dendrogram derived from fingerprint analysis of 33 *F. oxysporum* f. sp. *lactucae* isolates using ISSR primers (ACA)$_5$, CAA$_5$, (GACA)$_4$, (GTG)$_5$, and TGTC, and minisatellite primers M13 and T3B. One *F. oxysporum* isolate (NP) nonpathogenic to lettuce was included for comparison. Numbers on branches represent bootstrap values greater than 50% based on 1000 replicates. The bottom scale is the percentage of similarity by Jaccard’s similarity coefficient.
Figure D.2. Dendrogram derived from fingerprint analysis of 32 *F. oxysporum* f. sp. *lactucae* isolates using ISSR primers (GACA)$_4$, (GTG)$_5$, and the minisatellite primers M13 and T3B. Six *F. oxysporum* isolates (NP) nonpathogenic to lettuce were included for comparison. Numbers on branches represent bootstrap values greater than 50% based on 1000 replicates. The bottom scale is the percentage of similarity by Jaccard’s similarity coefficient. The original *F. oxysporum* f. sp. *lactucae* isolates HL2 and HL1 belong to haplotype I-05.
Figure D.3. Dendrogram of combine data of all the *F. oxysporum* isolates using ISSR primers GACA$_4$, GTG$_5$, and the minisatellite primers M13 and T3B. Six *F. oxysporum* isolates (NP) nonpathogenic to lettuce were included for comparison. Isolates of *F. oxysporum* f. sp. race 2 are presented in red. Numbers on branches represent bootstrap values greater than 50% based on 1000 replicates. The bottom scale is the percentage of similarity by Jaccard’s similarity coefficient.
Fig.D. 4. Diagrammatic representation of the distribution of the 30 haplotypes of *Fusarium oxysporum* f. sp. *lactucae* among four lettuce-growing regions. Haplotype shared by regions is represented in the bars connecting the circles. The different haplotypes found in each region is represented within the circles.
Fig. D.5. Amplification patterns obtained with ISSR primer (GTG)₅ using reference isolates (1-8) and some 23 *F. oxysporum* f. sp. *lactucae* isolates from California. Isolate 9 is a nonpathogenic *F. oxysporum* from cauliflower included for comparison.
Fig.D.6. Arizona winter lettuce production fields along the Gila valley. Map of the distribution of the eight haplotypes within the sampled population of *F. o. f. sp. lactucae.*
Figure D.7. Pattern of distribution of Fusarium wilt symptoms in a lettuce field with lettuce heads almost at maturity. Photograph: (Photo source: Dr. Mike Matheron, Department of Plant Sciences, University of Arizona, 2002).