Worldwide Phylogeography of the Citrus Brown Spot Pathogen, *Alternaria alternata*

T. L. Peever, A. Ibañez, K. Akimitsu, and L. W. Timmer

First author: Department of Plant Pathology, Washington State University, Pullman 99163; second and fourth authors: University of Florida, Citrus Research and Education Center, 700 Experiment Station Rd., Lake Alfred 33850; and third author: Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795, Japan.
Accepted for publication 4 March 2002.

ABSTRACT

Peever, T. L., Ibañez, A., Akimitsu, K., and Timmer, L. W. 2002. Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. *Phytopathology* 92:794-802.

Sixty-five isolates of *Alternaria alternata* were sampled from brown spot lesions on tangerines and mandarins (*Citrus reticulata*) and tangerine × grapefruit (*C. reticulata* × *C. paradisi*) hybrids in the United States, Colombia, Australia, Turkey, South Africa, and Israel to investigate the worldwide phylogeography of the fungus. Genetic variation was scored at 15 putative random amplified polymorphic DNA (RAPD) loci and 465 bp of an *endo*-polygalacturonase (*endo-PG*) gene was sequenced for each isolate. Cluster analysis of RAPD genotypes revealed significant differentiation between United State and Colombia isolates and Turkey, South Africa, Israel, and Australia isolates. Sequencing of *endo-PG* revealed 21 variable sites when the outgroup *A. gaisen* (AK-toxin-producing pathogen of Japanese pear) was included and 13 variable sites among the sampled isolates. Nucleotide substitutions at 10 of 13 variable sites represented silent mutations when *endo-PG* was translated in frame. Eight distinct *endo-PG* haplotypes were found among the sampled isolates and estimation of a phylogeny with *endo-PG* sequence data revealed

three clades, each with strong bootstrap support. The most basal clade (clade 1) was inferred based on its similarity to the outgroup *A. gaisen* and consisted exclusively of pathogenic isolates from the United States and Colombia. Clade 2 consisted of pathogenic and nonpathogenic isolates from the United States, Australia, South Africa, and Israel and clade 3 contained pathogenic and nonpathogenic isolates from Australia, South Africa, Israel, and Turkey. Quantitative estimates of virulence (disease incidence) were obtained for isolates from the United States, Colombia, South Africa, Israel, and Turkey by spray inoculating detached citrus leaves and counting the number of lesions 24 h after inoculation. Large differences in virulence were detected among isolates within each location and isolates from the United States were significantly more virulent than isolates from other locations. Several isolates from Colombia, South Africa, Israel, and Turkey had low virulence and 8% of all isolates were nonpathogenic. All but one of the nonpathogenic isolates were found in clade 2 of the *endo-PG* phylogeny, which also included the most highly virulent isolates sampled.

Additional keywords: *Alternaria citri*, *Citrus*, phylogenetic, polymerase chain reaction.

Two distinct pathotypes of the citrus brown spot pathogen, *Alternaria alternata* (Fr.:Fr.) Keissl., have been described based on their host specificity and toxin production (17). The first is known as the “rough lemon pathotype,” which is specific for rough lemon (*Citrus jambhiri* Lush), and the second is known as the “tangerine pathotype” which is specific for tangerines (*C. reticulata* Blanco) and tangerine × grapefruit (*C. reticulata* × *C. paradisi* Macfad.) hybrids. The rough lemon and tangerine pathotypes produce the host-specific toxins ACRL-toxin and ACT-toxin, respectively (17), which are thought to be pathogenicity factors in brown spot disease on both hosts (27). A closely related *Alternaria* sp., *A. gaisen* Nagano, causes black spot disease of Japanese pear (*Pyrus pyrifolia* (Burm. f.) Nakai) and produces the host-specific toxin, AK-toxin (23). AK-toxin is structurally related to ACT-toxin, produced by the tangerine pathotype (27), and at least two of the genes controlling ACT-toxin biosynthesis are homologous to *AKT* genes (22). Targeted mutation of several AK-toxin genes generated mutants which failed to produce toxin and were nonpathogenic (41,42), demonstrating the importance of toxin production in pathogenesis. Similar mutants of the tangerine pathotype also lose the ability to produce toxin and are nonpathogenic (K. Akimitsu, unpublished data).

The population structure and host specificity of the brown spot pathogen in Florida were recently determined using random amplified polymorphic DNA (RAPD) markers and pathogenicity assay (30,31). Isolates sampled from rough lemon and Minneola tangelo (*C. reticulata* × *C. paradisi*) were found to be host specific and highly genetically differentiated between hosts (30). In addition, all four populations examined were characterized by extremely high intrapopulation genetic diversity, with at least two genetically distinct RAPD groups or clusters occurring in each population. Significant differences in virulence were also detected between isolates from each RAPD cluster (30). In a related study, brown spot isolates were sampled from the tangerine × grapefruit hybrids Minneola and Sunburst in five locations in central Florida to assess the population structure on these hosts (31). Moderate levels of genetic differentiation (Nei's $G_{ST} = 0.12$) were found among isolates from both hosts which was hypothesized to be due to genetic drift and restricted gene flow among locations. Phylogenetic analysis of multiple protein-coding genes has recently been applied to morphologically similar fungi sampled from diverse geographic locations (9,18,26,49). This approach, known as phylogeography (1), allows an exploration of the relationship between gene genealogies or phylogeny and geography. Phylogeographic studies of medically and agriculturally important fungi have revealed substantial phylogenetic structure in populations of fungi which were previously considered homogeneous across their sampled range (9,13,18,26).

The taxonomic status of the citrus brown spot pathogen is currently unclear. The causal agent of citrus brown spot was origi-

Corresponding author: T. L. Peever; E-mail address: tpeever@wsu.edu

Publication no. P-2002-0422-02R
© 2002 The American Phytopathological Society

nally attributed to *A. citri* Ellis & N. Pierce in N. Pierce (14) based on its morphological similarity to the causal agent of citrus black rot, a postharvest disease (3). The pathogen has also been called *A. alternata* due to its morphological similarity to saprophytic *A. alternata* (24,38). The classification of the citrus brown spot fungus as either *A. citri* (14,48) or *A. alternata* (24,38) has been opposed by Simmons (33,34) who delineates small-spored species of *Alternaria* based on conidium catenulation and conidium morphology. Thus, 10 new morphological species were recently described based on brown spot isolates sampled from Minneola tangelo and rough lemon in different parts of the world (36). However, no quantitative data have been published in support of the morphological species concepts. Molecular data, including nuclear and mitochondrial restriction fragment length polymorphisms (RFLP) and nuclear rDNA internal transcribed spacer (ITS) sequence data have failed to differentiate the citrus brown spot isolates from other toxin-producing *Alternaria* spp. and from saprophytic *A. alternata* (19–21). Similarly, sequence data from more variable genomic regions, such as the mitochondrial large and small ribosomal subunits and the β -tubulin gene, have failed to differentiate the 10 described morphospecies associated with citrus (40).

Brown spot of citrus was first described on Emperor mandarin in Australia in 1903 (6); however, it was not until 1964 that Koch's postulates were fulfilled and the causal agent definitively identified as an *Alternaria* sp. (14). Brown spot disease was first observed in Florida in 1974 (48); Israel, South Africa, and Colombia in the 1980s (5,32,38); and Turkey and Spain in the 1990s (4,46). Florida currently is the only citrus production area in the United States where brown spot has been identified. The chronological pattern of disease spread suggests that Australia may have been the source population for subsequent introductions into other citrus-growing areas of the world. An alternative hypothesis is that the brown spot pathogen has been introduced into one or more of these regions from another source population. This putative source population may be southeast Asia, which is the center of origin of *Citrus* spp. (47) and may also be the center of origin of the pathogen. The objectives of this research were to (i) determine the worldwide phylogeography of the brown spot pathogen using molecular markers and sequence data, (ii) determine if quantitative differences in virulence exist among isolates from different citrus-growing areas of the world, and (iii) test the hypothesis that the brown spot pathogen was introduced into other citrus-growing regions of the world from Australia.

MATERIALS AND METHODS

Sampling. Sixty-five isolates of *A. alternata* were obtained from brown spot-infected leaves and fruit of tangerines and mandarins (*C. reticulata*) and tangerine hybrids (*C. reticulata* $\times$ *C. paradisi*) (Table 1). Isolates from Israel, Turkey, Colombia, and South Africa were obtained from Minneola tangelo in several geographically separated groves within each country. Isolates from Australia were sampled from different tangerine and tangelo cultivars in the Narara arboretum (Narara, New South Wales), and the U.S. sample consisted of a random subsample of isolates from a small area (2,500 m²) in a single Minneola grove in Florida obtained for a previous study (30). Single-conidial isolations were made from each original mass-hyphal isolate and stored as previously described (30). Isolates EGS 90-0512 and EGS 37-1321 of *A. gaisen* (35,37), the AKT-toxin-producing fungus which causes black spot of Japanese pear, were obtained from E. G. Simmons (Crawfordsville, IN) to use as outgroups in phylogenetic analyses. Both isolates were originally sampled from Japanese pear in Tottori prefecture, Japan (37).

DNA extraction. DNA was extracted from the sampled isolates as previously described (30). Briefly, lyophilized, powdered mycelium ($\approx$ 50 mg) was extracted with a lysis buffer containing 50 mM EDTA, 100 mM Tris buffer, pH 8, and 3% sodium do-

decyl sulfate for 30 to 45 min at 65°C. Mycelium was pelleted by centrifugation and the supernatant precipitated with 8M potassium acetate at -20°C for 15 min. Following centrifugation, the supernatant was subjected to two rounds of phenol-chloroform-isoamyl alcohol (25:24:1) extraction and one chloroform-isoamyl alcohol (24:1) extraction. The final supernatant was precipitated with two volumes of 100% EtOH and 0.5 M NaCl. Pellets were resuspended in water, precipitated again in 14% polyethylene glycol and 1 M NaCl, and resuspended in 50 μl of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNA was digested with RNase A at 20 $\mu\text{g}/\text{ml}$ (Sigma-Aldrich, St. Louis) at 37°C for 3 h. DNA concentrations were estimated visually in ethidium bromide-stained agarose gels by comparing band intensity with known quantities of phage lambda DNA (Life Technologies, Grand Island, NY).

RAPD-polymerase chain reaction. Fifteen-microliter polymerase chain reactions (PCR) with four random decamer nucleotide primers were performed in 96-well microtiter plates as previously described (30). Reaction mixtures contained 25 to 50 ng of DNA, 1.5 μl of 10 $\times$ reaction buffer (Sigma-Aldrich, Life Technologies, or Promega, Madison, WI), 200 μM dNTPs (Idaho Technologies, Idaho Falls, ID), 0.8 μM primer (Operon Technologies, Alameda, CA), and 0.6 units of *Taq* polymerase (Sigma-Aldrich, Promega, or Life Technologies). Mixtures were overlain with 40 μl of sterile mineral oil and placed in a thermocycler (MJ Research, Waltham, MA). Cycling conditions included an initial 2 min melt at 93°C followed by 44 cycles of 92°C for 1 min (melt), 37°C for 1 min (annealing), and 72°C for 2 min (extension). The final cycle was 92°C for 1 min, 37°C for 1 min, and 72°C for 8 min. PCR products were separated in 1.8% agarose gels (Life Technologies) with *HindIII*/*EcoRI*-digested lambda DNA (Promega) as size standards and visualized with a digital imaging system (UltraViolet Products, Upland, CA). RAPD markers were scored as previously described (30). Reproducibility of the RAPD markers was tested by performing PCR reactions with different concentrations (2 to 200 ng) of DNA template, with at least three independent DNA extractions from the same isolate and with different sources of *Taq* polymerase (Sigma-Aldrich, Promega, Life Technologies). Identical RAPD products were obtained with all treatments.

RAPD analysis. RAPD-PCR products were scored as binary data for each isolate as previously described (30). Isolates Colombia-4, Australia-7, and Australia-9 were not analyzed for RAPD-PCR variation. An unweighted pair group method with arithmetic means (UPGMA) phenogram was estimated among 62 isolates based on a distance matrix (Jaccard coefficient) using the FREETREE software package (29). Each isolate was treated as the operational taxonomic unit. Statistical support for phenogram branches was obtained using 1,000 bootstrapped samples of the data set generated in FREETREE and the consensus tree was visualized in TREEVIEW (28).

Endo-polygalacturonase amplification. Primers PG3 (5'-TACCATGGTTCTTTCCGA-3') and PG2 (5'-RCARTCRCTCYTGR-TT-3') were designed to amplify 489 bp of an *endo*-polygalacturonase (*endo-PG*) gene characterized from the rough lemon pathotype of *A. alternata* (11,12). The predicted 489-bp fragment was amplified from all isolates, including *A. gaisen* isolates 90-0512 and 37-1321. PCR reactions were carried out in thin-walled 0.2-ml PCR strip tubes (OPS, Petaluma, CA) in a total reaction volume of 25 μl . Reaction mixes contained 2.5 μl of 10 $\times$ PCR buffer (Promega), 2.5 mM MgCl₂ (Promega), 200 μM dNTPs (Idaho Technologies), 400 nM each PG3 and PG2 primers (Operon), 10 ng of DNA, and 1 unit of *Taq* polymerase (Promega). Cycling conditions consisted of 2 min of initial melt at 95°C, followed by 35 cycles of: 1 min of melt at 95°C, 1 min of annealing at 50°C, and 1 min of extension at 72°C. These cycles were followed by a final 5-min elongation cycle at 72°C. All PCR reactions were carried out in an Omn-E thermal cycler (Hybaid, Ashford, Middlesex, UK). Amplicons were purified through QiaQuick

TABLE 1. Isolates of *Alternaria alternata* sampled from citrus in different countries

Country, location	Isolate	Original code ^a	Species ^b	Host	Tissue	Collector	Date collected	Pathogenicity ^c
United States								
Polk City, Florida	Florida-1	SH-1 (46-136)	<i>A. citriarabusti</i>	Minneola tangelo ^d	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-4	SH-4 (46-139)	<i>A. tangelonis</i>	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-5	SH-5	nd	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-7	SH-7	nd	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-8	SH-8 (46-140)	<i>A. citriarabusti</i>	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-9	SH-9	nd	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-13	SH-13	nd	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-22	SH-22	nd	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-32	SH-32 (46-147)	<i>A. tangelonis</i>	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-35	SH-35	nd	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-37	SH-37 (46-149)	<i>A. tangelonis</i>	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-38	SH-38 (46-150)	<i>A. citriarabusti</i>	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Polk City, Florida	Florida-41	SH-41	nd	Minneola tangelo	Leaf	T. L. Peever	08/96	+
Colombia								
Pereira	Colombia-1	45-014	<i>A. tangelonis</i>	Minneola tangelo	Fruit	B. Castro	09/96	+
Pereira	Colombia-2	45-015	<i>A. tangelonis</i>	Minneola tangelo	Flower bud	B. Castro	09/96	+
Pereira	Colombia-3	45-016	<i>A. tangelonis</i>	Minneola tangelo	Leaf	B. Castro	09/96	+
Pereira	Colombia-4	45-017	<i>A. colombiana</i>	Minneola tangelo	Fruit	B. Castro	09/96	+
Pereira	Colombia-5	9-CEN-S	nd	Minneola tangelo	Leaf	B. Castro	09/96	+
Pereira	Colombia-6	10-CEN-s	nd	Minneola tangelo	Stem	B. Castro	05/96	+
Pereira	Colombia-7	11-CEN-S	nd	Minneola tangelo	Fruit	B. Castro	05/96	+
South Africa								
Tzaneen	South Africa-1	45-056	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	S. H. Swart	06/97	+
Tzaneen	South Africa-2	45-057	<i>A. turkisafrina</i>	Minneola tangelo	Fruit	S. H. Swart	02/97	+
Tzaneen	South Africa-3	45-058	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	S. H. Swart	05/97	+
Tzaneen	South Africa-4	45-059	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	S. H. Swart	04/97	+
Tzaneen	South Africa-5	45-060	nd	Minneola tangelo	Fruit	S. H. Swart	03/97	+
Tzaneen	South Africa-6	45-061	nd	Minneola tangelo	Fruit	S. H. Swart	01/97	+
Tzaneen	South Africa-7	45-062	nd	Minneola tangelo	Leaf	S. H. Swart	03/94	–
Turkey								
Abdioglu	Turkey-1	44-159	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Sihmurat	Turkey-2	44-160	<i>A. perangusta</i>	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Kazikli	Turkey-3	44-161	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Solakli	Turkey-4	44-162	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Solakli	Turkey-5	44-163	<i>A. turkisafrina</i>	Dancy tangerine ^e	Leaf	Y. Canihos	05/96	+
Yunusoglu	Turkey-6	44-164	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Kayarli	Turkey-7	44-165	nd	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Kuzucuoglu	Turkey-8	44-166	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Yassidam	Turkey-9	44-167	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Yolgecen	Turkey-10	44-168	nd	Minneola tangelo	Leaf	Y. Canihos	05/96	+
Australia								
Narara Arboretum	Australia-1	Australia-1	nd	Ponkan mandarin ^e	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-2	Australia-2	nd	Ponkan mandarin	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-3	Australia-3	nd	Ladu mandarin ^e	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-4	Australia-4	nd	Tangerine ^e	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-5	Australia-5	nd	Thornton tangelo ^d	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-6	Australia-6	nd	Tangerine tree 1 ^e	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-7	Australia-7	nd	Tangerine tree 2 ^e	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-8	Australia-8	nd	Emperor mandarin ^e	Fruit	P. Broadbent	04/98	–
Narara Arboretum	Australia-9	Australia-9	nd	Emperor mandarin	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-10	Australia-10	nd	Emperor mandarin	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-11	Australia-11	nd	Emperor mandarin	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-12	Australia-12	nd	Emperor mandarin	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-13	Australia-13	nd	Emperor mandarin	Fruit	P. Broadbent	04/98	nt
Narara Arboretum	Australia-14	Australia-14	nd	Emperor mandarin	Fruit	P. Broadbent	04/98	+
Narara Arboretum	Australia-15	Australia-15	nd	Emperor mandarin	Fruit	P. Broadbent	04/98	+
Israel								
Yedidya	Israel-1	45-001	nd	Minneola tangelo	Leaf	Z. Solel	07/96	+
Yedidya	Israel-2	45-002	nd	Minneola tangelo	Leaf	Z. Solel	07/96	+
Hadassim	Israel-3	45-003	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Z. Solel	07/96	+
Hadassim	Israel-4	45-004	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Z. Solel	07/96	+
Hadassim	Israel-5	45-005	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Z. Solel	07/96	+
Hadassim	Israel-6	45-006	<i>A. turkisafrina</i>	Minneola tangelo	Leaf	Z. Solel	07/96	+
Mayan Zvi	Israel-7	45-007	<i>A. dumosa</i>	Minneola tangelo	Leaf	Z. Solel	07/96	–
Mayan Zvi	Israel-8	45-008	nd	Minneola tangelo	Leaf	Z. Solel	07/96	–
Bet Lyd	Israel-9	45-009	nd	Minneola tangelo	Leaf	Z. Solel	07/96	+
Bet Lyd	Israel-10	45-010	nd	Minneola tangelo	Leaf	Z. Solel	07/96	–
Bet Lyd	Israel-11	45-011	<i>A. interrupta</i>	Minneola tangelo	Leaf	Z. Solel	07/96	+
Bet Lyd	Israel-12	45-012	nd	Minneola tangelo	Leaf	Z. Solel	07/96	+
Bet Lyd	Israel-13	45-013	nd	Minneola tangelo	Leaf	Z. Solel	07/96	+

^a Codes beginning with SH were assigned by T. Peever and L. W. Timmer (30); codes beginning with 44, 45, or 46 were assigned by E. G. Simmons (35); codes Australia-1 through Australia-15 were assigned by P. Broadbent. All isolates maintained in the culture collections of L. W. Timmer (University of Florida, CREC, Lake Alfred) and T. L. Peever (Department of Plant Pathology, Washington State University, Pullman) and are available upon request from the senior author.

^b Isolates identified to species by E. G. Simmons (35). Species not determined (nd).

^c Pathogenicity of each isolate was tested by spray inoculating detached Minneola leaves as described in the text; + = visible lesions after 24 h, – = no lesions after 48 h, nt = not tested.

^d *C. reticulata* Blanco cv. Dancy × *C. paradisi* Macf. cv. Duncan.

^e *C. reticulata* Blanco.

Columns (Qiagen, Valencia, CA) following the manufacturer's directions and DNA concentrations estimated visually in agarose gels as described above.

Endo-PG sequencing. Column-purified PCR amplicons were direct sequenced on both strands using cycle sequencing with the PG3 or PG2 primers described above (Operon). Each sequence reaction contained 40 to 90 ng of DNA, 320 nM primer, 4 µl of BigDye Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA), and sterile distilled water in 10-µl total volumes. Cycle sequence reactions were performed in an Omn-E thermal cycler and cycling conditions consisted of 25 cycles of 15 s at 96°C, 15 s at 50°C, and 4 min at 60°C. Products were purified using Centriflex Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD) and sequenced on a PE Biosystems Model 377 Automated DNA Sequencer (Applied Biosystems, Norwalk, CT). All sequencing was performed in the Laboratory for Biotechnology and Bioanalysis, School of Molecular Biosciences, Washington State University.

Phylogenetic analysis of *endo-PG*. Sequences were trimmed to 465 bp to eliminate ambiguous sites and aligned using Clustal-X 1.8 (44). This partial *endo-PG* sequence corresponds to bases 978-1442 of the coding region of *A. alternata endo-PG* (accession no. AB047682) and contains no introns. Sequences were translated and deduced amino acids were aligned with Jellyfish software using *A. alternata endo-PG* (accession no. BAB32923) as a guide (12). Partial *endo-PG* sequences were translated into deduced amino acid sequences of 154 residues corresponding to residues 62 to 215 of *A. alternata endo-PG* (accession no. BAB32923). Amino acid alignment of the variable nucleotide sequences allowed determination of number of nucleotide substitutions resulting in amino acid substitutions in the data set. A rooted *endo-PG* phylogeny was estimated using maximum likelihood with the DNAML program in PHYLIP 3.6 (PHYLIP [Phylogeny Inference Package], version 3.6a2; University of Washington, Seattle) (8). DNAML analysis was performed using default settings except for a transition/transversion ratio of 3.62 estimated from the data set, random input order of taxa (three jumbling steps), and a more thorough tree search. Statistical support for clades was estimated using 1,000 bootstrapped samples of the data set in the program SEQBOOT. *A. gaisen* isolates 90-0512 and 37-1321 were used to root the phylogeny. A majority-rule consensus tree was produced by the program CONSENSE and branch lengths were added to the tree using DNAML with the consensus tree as the input user tree. The consensus *endo-PG* phylogram was visualized in TREEVIEW (28).

Pathogenicity. Each isolate was tested for pathogenicity using a spray-inoculation assay with detached Minneola leaves as previously described (30). Conidia were produced as previously described (30), washed using centrifugation, and adjusted to 1×10^5 conidia per ml with a hemacytometer. Detached leaves (four leaves per isolate) were placed in 2-ml microcentrifuge tubes and sprayed to runoff with a thin layer chromatography sprayer (Crown Tools, Woodstock, IL). Inoculated leaves were incubated for 48 h at 25°C in moist chambers. Isolates were considered pathogenic if they produced at least one disease lesion on one of the four replicate leaves within 48 h of inoculation. Each isolate was tested in at least two independent experiments.

Virulence. Pathogenic isolates from Colombia, Florida, Israel, South Africa, and Turkey were tested for differences in virulence (quantitative differences in pathogenicity). Australian isolates were not tested. Five isolates from the Florida sample were selected based on differences in their RAPD genotypes (30,31) in order to maximize genetic variation among the Florida isolates. These isolates represented the entire range of RAPD variation sampled to date in Florida (29,30). Virulence assays were carried out as described for the pathogenicity assays above, except that conidial concentrations were reduced to 1×10^4 conidia per ml and disease incidence was estimated by counting the number of

lesions per leaf 24 h after inoculation. Due to the large number of isolates tested, assays were performed as four randomized complete block experiments with day of test as the blocking factor. Each block was inoculated on a separate day with the same five Florida isolates included in each block. Initial analysis of the Florida isolates revealed no significant block effect ($P = 0.18$) and subsequent analyses were collapsed into a completely randomized design with origin of isolate as the random treatment factor. Analysis of variance (ANOVA) was performed using the ONE-WAY procedure of Minitab statistical software (version 12; Minitab Inc., State College, PA). Comparison of country means was performed in Minitab using Hsu's multiple comparison with the

Fig. 1. Phenogram estimated among *Alternaria alternata* isolates sampled from brown spot lesions on mandarins, tangerines, and tangerine hybrids in different countries. Phenogram was estimated by clustering a distance matrix (Jaccard coefficient) of binary random amplified polymorphic DNA data using the unweighted pair group method with arithmetic means in the FREETREE program. Numbers at the major branches indicate the percentage occurrence of the cluster to the right of the branch in 1,000 bootstrapped data sets. Only branches occurring in 50% or more of the bootstrapped phenograms are labeled. Scale represents 10% differentiation.

best (MCB) (10) procedure with an experiment-wise (family) error rate of $P = 0.05$.

RESULTS

RAPD allele frequencies among locations. UPGMA cluster analysis of RAPD data revealed several clusters with low to moderate (56 to 80%) bootstrap support among the isolates (Fig. 1). The Florida–Colombia cluster had 57% bootstrap support, while another Florida cluster had 69% bootstrap support. No other major clusters of isolates (five or more isolates) from Australia, Israel, Turkey, or South Africa had bootstrap values greater than 50%. Other moderately supported clusters consisted of pairs of isolates from Florida, Australia, Israel, and South Africa (Fig. 1). All isolates from Colombia had the same RAPD genotype and this genotype was sampled twice in Florida (isolates Florida-7 and -35) (Fig. 1). Sixteen isolates from Turkey–South Africa–Israel shared another RAPD genotype (isolates Turkey-1, -2, -3, -4, -5, -8, -9; South Africa-1, -3, -4, -5; Israel-3, -4, -5, -12, -13) and this cluster had only 26% bootstrap support (Fig. 1).

Phylogenetic analysis of *endo-PG*. Sequencing and alignment of 465 bp of *endo-PG* from the 65 brown spot isolates revealed 21 phylogenetically informative sites when the outgroup taxon *A. gaisen* was included in the analysis and 13 informative sites among the sampled isolates (Table 2). A total of nine *endo-PG* haplotypes were identified among the isolates, including the outgroup taxon. *A. gaisen* isolates 90-0512 and 37-1321 shared an identical *endo-PG* haplotype. Nucleotide substitutions at 10 of the 13 variable sites were silent when the partial *endo-PG* sequences were translated in frame (Table 2) (12). Estimation of the *endo-PG* phylogeny revealed three clades with high (>80%) bootstrap values (Fig. 2). The most basal clade (clade 1) contained isolates from Florida and Colombia, while clade 3 contained isolates exclusively from Australia, Israel, South Africa, and Turkey. Clade 2 contained isolates from all locations except Turkey (Fig. 3). Colombian isolates were found exclusively within one clade (clade 1) whereas isolates from Turkey were found exclusively in clade 3. Israel isolates were the most diverse, with five distinct haplotypes distributed in two of the three clades. Additional phylogenetic analyses using maximum parsimony and distance methods also were implemented in the DNAPARS and DNADIST programs, respectively, of PHYLIP (8). Both analyses with 1,000 bootstrapped data sets produced phylograms with identical topology to that estimated with DNAML with similar support for three clades (data not shown).

Pathogenicity. Of the sampled isolates, 92% (59 of 64) were pathogenic to detached Minneola leaves (Table 1). The frequency of nonpathogens (8%) was slightly higher than that reported previously (3%) among a sample of 30 isolates from Minneola tangelo in Florida (30). The highest frequency of nonpathogens was found

in the Israel sample, where 3 of 13 (23%) of isolates were non-pathogenic.

Virulence. Significant differences in virulence were detected among the worldwide sample of isolates (Table 3). Florida isolates were more virulent than isolates from other locations (Fig. 3) and this was confirmed using Hsu’s MCB test with Florida isolates set as the “best” (data not shown). Upon removal of the Florida isolates from the analysis and repetition of the ANOVA, no significant differences in virulence were detected among isolates from Israel, Colombia, Turkey, and South Africa (Table 3). Clade 2 (Fig. 2) contained all of the nonpathogenic isolates except one (Israel-7) as well as some of the most virulent isolates (Florida-1, Florida-5, Israel-1, and Israel-2) (Figs. 2 and 3).

DISCUSSION

Phylogeographic analysis of the citrus brown spot pathogen revealed the existence of three strongly supported clades among 65 isolates sampled from seven of the countries where the disease has been reported. The *endo-PG* phylogeny was highly congruent with the phenogram estimated from RAPD data. Both analyses revealed substantial genetic differentiation between isolates from Florida and Colombia and isolates from Australia, Turkey, South Africa, and Israel. Rooting the *endo-PG* phylogeny by *A. gaisen* revealed polarity in the phylogeny and that the Florida–Colombia clade (clade 1) was ancestral to clades sampled from other parts of the world. The extent of sequence divergence among the sampled isolates relative to the short history of citrus cultivation in these countries suggests that at least three independent introductions of the pathogen have been made from a source population other than those sampled in this study.

Brown spot of citrus was first described in Australia in 1903 (6) and subsequently was identified in other citrus-growing areas of the world (4,5,38,46,48). The chronology of appearance of the disease suggests that the brown spot pathogen may have been introduced from Australia into other countries. The data presented here strongly reject this hypothesis. A much more likely explanation for the pattern of genetic variation observed among countries sampled in this study is that three independent introductions of the pathogen have been made into two or more of these countries, followed by migration of the pathogen among countries. The Minneola tangelo trees sampled in Israel, South Africa, and Turkey for this study are clonal descendents of a single progeny tree from a cross made in 1931 in Florida (39). Clonally propagated trees were moved from Florida to these countries and it is possible that clade 2 strains of the pathogen were moved from Florida to Australia, Israel, and South Africa with the host when Minneola groves were established in these countries. However, the complete absence of clade 2 strains in Turkey indicate that this was not true for Minneola transported to Turkey. Clade 3 strains were found in

TABLE 2. Distribution of nucleotide polymorphism in partial *endo*-polygalacturonase (*endo-PG*) sequences of *Alternaria alternata* from citrus

Isolate ^a	Nucleotide site												
	46	106	109	121	133	134	181	184	232	272	287	370	385
CON ^b	C	G	G	T	C	C	C	T	C	A	A	C	C
Florida-1	...	...	...	...	...	...	...	...	...	...	...	...	...
Florida-4	...	...	...	...	T	G	...	C	...	T	...	...	T
Israel-1	...	...	...	...	...	...	T	...	...	...	...	...	...
Israel-8	...	A	...	...	...	...	...	...	T	...	...	T	...
Israel-13	T	...	A	C	...	...	...	...	...	...	G	...	...
Turkey-1	...	...	A	C	...	...	...	...	...	...	G	...	...
Australia-1	...	...	A	C	...	...	...	...	...	...	...	...	...
Australia-9	T	...	A	C	...	...	...	...	...	...	...	...	...
Amino acid sub. ^c	...	...	...	...	...	Q/E	...	...	...	T/S	T/A	...	...

^a Isolate with representative *endo-PG* haplotype.
^b Consensus nucleotide at each variable site.
^c Amino acid replacement substitutions (consensus amino acid/substituted amino acid).

Israel, South Africa, Turkey, and Australia and it is unlikely that this lineage of the pathogen was moved from Florida to these locations. A more exhaustive sampling and sequence analysis of Florida isolates is required to rule out this possibility. It is most likely that clade 3 isolates were introduced into Australia, Turkey, South Africa, and Israel from a location other than Florida. The most basal clade (clade 1) of the *endo-PG* phylogeny, inferred by rooting the phylogeny by *A. gaisen*, consisted exclusively of pathogenic isolates from Florida and Colombia. This clade is basal to the three other clades; therefore, it appears that clade 1 isolates (or isolates most similar to them) represent the most recent common ancestor of isolates in clades 2 and 3.

Most of the historic introductions of citrus planting stock into citrus-growing areas of the world have gone undocumented and unregulated, but it seems likely that the major migratory route of the brown spot pathogen has been on infected citrus plants. The recovery of identical RAPD genotypes and *endo-PG* haplotypes from Florida and Colombia strongly supports the hypothesis of migration of the fungus from Florida to Colombia. Minneola plantings in Colombia were initiated using stock from Florida (L. W. Timmer, unpublished data) and it appears highly likely that the pathogen was introduced on infected plant material at the same time. Similarly, identical RAPD genotypes and *endo-PG* haplotypes were sampled from Australia, Turkey, South Africa, and Israel, suggesting that the pathogen has been moved extensively among these countries, probably on planting stock. The genus *Citrus* is native to the subtropical and tropical regions of Asia and the Malay archipelago and has been spread throughout the world by humans over a period of hundreds of years (47). Southeast Asia is the presumed center of origin of citrus; therefore, it is possible that the pathogen has evolved with the host in southeast Asia and has moved throughout the world with the host. If this hypothesis is correct, the genetic variation observed among brown spot isolates from citrus-growing regions of the world should represent a subset of the variation found at the center of origin. Unfortunately, we have no genetic information on the brown spot pathogen on *Citrus* spp. in southeast Asia. To our knowledge, the pathogen never has been isolated from Asia; however, the disease has been reported from Vietnam (P. Broadbent, personal communication). Collection and genetic characterization of brown spot isolates from southeast Asia would provide the necessary data to critically test this hypothesis.

Estimation of the UPGMA phenogram revealed significant differentiation among isolates from Florida. One Florida cluster could be differentiated from the other Florida cluster with 69% bootstrap support. Phylogenetic analysis of *endo-PG* similarly revealed two well-supported clades within the Florida sample. These isolates were collected in a very small area ($\approx 2,500$ m²) in one Minneola grove in central Florida in 1996 (30). This result clearly demonstrates that multiple phylogenetic lineages of this fungus can be distributed on a very fine scale. The two phylogenetic lineages identified in Florida in this study (clades 1 and 2) correspond to two RAPD clusters identified in a previous study of a larger sample from this same location (30). The *endo-PG* sequence data presented here confirms previous results, indicating that as much genetic variation exists within *A. alternata* populations in a single small area of a citrus grove as among populations throughout Florida (29,30). This data also indicates that RAPD markers are good predictors of phylogenetic lineages within populations of an asexually reproducing fungus such as the brown spot pathogen.

Phylogenetic analysis of protein-coding genes has been used recently to examine the evolutionary origins and phylogeographic structure of a number of plant- and human-pathogenic fungi (9,13, 18,26,49). All of these studies identified multiple phylogenetic lineages among morphologically similar fungi sampled from diverse geographic locations. The existence of well-supported clades among these fungi may, in some cases, be interpreted as distinct species in a

phylogenetic species context (40). The phylogenetic lineages identified in the above studies were correlated to the geographical origin of isolates in some cases (13,18,26) but not in others (9,49). In our study, a clear phylogeographic pattern was evident, with clade 3 isolates being limited to Turkey, Australia, Israel, and South Africa and clade 1 isolates limited to Florida and Colombia. Clade 2 isolates were the most geographically and pathogenically diverse, comprising isolates from all locations except Turkey, and included both nonpathogenic and highly virulent isolates. The present study has confirmed the utility of protein-coding genes for estimating phylogenies among closely related fungi. To our knowledge, this research represents the first use of an *endo-polygalacturonase* gene for phylogenetics. The *endo-PG* gene sequenced here was variable at the intraspecific level in *A. alternata* associated with citrus and may prove valuable for other fungi as well. Most of the variation (10 of 13 variable sites) observed among these isolates resulted in no amino acid substitutions when the sequences were translated in frame, indicating that the majority of the variation we detected was selectively neutral and that *endo-PG* sequence data is appropriate for phylogeographic analyses.

Eight new *Alternaria* spp. recently have been described from tangerines and tangerine hybrids based on morphological criteria (36). The description of these species was based on many of the isolates included in the present study; therefore, the *endo-PG* sequence data allows us an opportunity to test the congruence of species defined under both morphological and phylogenetic species concepts. If we use the presence of monophyletic groups and fixed differences in nucleotide substitutions in each group as criteria for phylogenetic species (2,25,43), our phylogenetic analysis of *endo-*

Fig. 2. *Endo-polygalacturonase* phylogeny estimated among *Alternaria alternata* isolates sampled from mandarins, tangerines, and tangerine hybrids in different countries. Phylogeny was estimated using maximum likelihood with the DNAML program in PHYLIP. *A. gaisen* isolate 90-0512 was used to root the phylogeny. Numbers at the major branches indicate the percentage of occurrence of the clade to the right of the branch in 1,000 bootstrapped data sets. Branch lengths are proportional to the inferred amount of evolutionary change and the scale represents 0.01 nucleotide substitutions per site. Clades (1, 2, and 3) inferred from bootstrap values greater than 80% are shown.

PG could be interpreted as delineating three species of *Alternaria* causing brown spot of citrus. The robustness of these clades needs to be tested with sequence data from other regions of the genome. Comparison of the morphological species with clades inferred from *endo-PG* sequence data revealed that clade 1 includes the morphological species *A. tangelonis* and *A. colombiana*, clade 2 contains the morphological species *A. citriarbusi*, and clade 3 contains the morphological species *A. dumosa*, *A. turkisafria*, *A. perangusta*, and *A. interrupta* (36). This is the opposite result of many phylogenetic studies of fungi where additional species have been revealed with phylogenetic analyses (43). We speculate that several of the morphospecies described among these brown spot isolates (36) are likely not tenable under a phylogenetic species concept and do not reflect the evolutionary history of these fungi. In order to test this hypothesis rigorously, additional variable genomic regions need to be sequenced from these isolates to define phylogenetic species based on the concept of genealogical concordance (43).

Statistically significant differences in virulence were detected among brown spot isolates sampled from different parts of the world. Isolates from Florida were more virulent than isolates from other locations. The Florida isolates were sampled from a single citrus grove in central Florida (30), whereas isolates from other parts of the world were sampled from multiple citrus groves within each country. This might explain the greater uniformity in virulence among the Florida; however, the Florida isolates were chosen deliberately to represent the maximum genetic variation occurring in Florida. In two previous studies of this Florida population (30,31), as much variation was detected within a single citrus grove as among several groves distributed across Florida. The virulence assay also identified large variation in virulence within each country, with isolates ranging from highly pathogenic to completely nonpathogenic. It is possible that the few nonpathogenic isolates recovered in this study were co-colonizing brown spot lesions or were colonizing leaves as endophytes and grew out preferentially during isolation procedures. *A. alternata* isolates with identical *endo-PG* haplotypes to the brown spot pathogens described here have recently been recovered from healthy citrus tissue (T. L. Peever and L. W. Timmer, *unpublished data*), which may indicate that *A. alternata* has a significant endophytic phase on citrus. A previous study of the same Florida isolates used in this research demonstrated significant differences in virulence between isolates in two RAPD clusters (clusters B and C) from a single central Florida location (30). These RAPD clusters correspond to *endo-PG* clades 1 and 2 identified in this study.

Correlation of the *endo-PG* sequence data and the results of the virulence assay revealed that the most virulent and least virulent (nonpathogenic) isolates occurred in clade 2. The existence of closely related nonpathogenic and highly virulent isolates in clade 2 may indicate that pathogenicity or virulence determinants are more unstable in this phylogenetic lineage. Repeated subculturing of Florida clade 1 and clade 2 isolates in the laboratory has indicated that clade 2 isolates are more likely to lose virulence than isolates in clade 1 (K. Akimitsu, *unpublished data*). Repeated re-isolation and culturing of single-conidial isolates of *A. gaisen* from two orchards in Japan revealed that a high percentage of isolates from one orchard lost pathogenicity after the first transfer, whereas isolates from another orchard maintained pathogenicity through many more transfers (45). Many of the nonpathogenic isolates were tested for AKT-toxin production and all nonpathogenic isolates were deficient in toxin production, leading the authors to conclude that the instability in pathogenicity observed was due to instability in AKT-toxin production. We speculate that the virulence of clade 2 isolates is less stable than virulence in other clades because of mutation of one or more sequences (*ACTT* sequences) controlling ACT-toxin production. This hypothesis could be tested experimentally by repeated subculturing of isolates from different clades, testing them for pathogenicity and toxin production, and determining the genetic basis for loss of toxin production.

Pathogenicity in this host-pathogen interaction is controlled by the production of host-specific toxins (15,16), but we have no knowledge of how quantitative differences in pathogenicity (i.e., virulence) may be controlled. It is possible that differences in

TABLE 3. Virulence of worldwide isolates of *Alternaria alternata* from Minneola

Comparison, source	df	MS	F	P
All countries ^a				
Location	4	1,570	4.69	0.004
Error	33	335	...	...
Total	37	...	...	...
Without Florida ^b				
Location	3	385	1.06	0.381
Error	29	362	...	...
Total	32	...	...	...

^a Sampled countries = United States, Colombia, South Africa, Israel, and Turkey.
^b Sampled countries = Colombia, South Africa, Israel, and Turkey.

Fig. 3. Virulence of *Alternaria alternata* isolates sampled from citrus in different locations. Heights of bars represent mean disease incidence (lesions per leaf) 24 h after inoculation. Numbers of isolates tested from each country were: Florida ($n = 5$), Colombia ($n = 7$), South Africa ($n = 6$), Israel ($n = 10$), and Turkey ($n = 10$). Four replicate leaves were inoculated for each isolate.

virulence between Florida isolates and those from other countries may be due to the production of differing amounts of ACT-toxin or to different relative amounts of structurally related toxin molecules which may differ in toxicity. The determination of toxin production and sequencing *ACTT* genes in brown spot isolates with statistically significant differences in virulence may allow us to address this hypothesis. Many of the same multilocus RAPD and *endo-PG* genotypes were sampled from Florida and Colombia, yet they differed significantly in virulence. Further research focusing on ACT-toxin production in these genetically similar isolates may provide a more detailed understanding of the mechanisms underlying quantitative differences in virulence. The present study has documented the existence of three phylogenetic lineages of the brown spot pathogen, each of which includes isolates differing in virulence and this variation needs to be considered in breeding programs for resistance to brown spot. The genetics of resistance to brown spot in citrus has not been intensively studied but preliminary results have indicated that susceptibility to the disease is controlled by a single, dominant genetic locus which appears to have originated in the tangerine cv. Dancy (7). To date, resistance screening has involved a single Florida isolate of *A. alternata*, but the results of this study indicate that additional isolates of the pathogen should be used which reflect the genetic and virulence diversity extant in this pathogen.

ACKNOWLEDGMENTS

PPNS 0328, Department of Plant Pathology, College of Agriculture and Home Economics Research Center, Project 0300, Washington State University, Pullman 99164. Research was supported in part by Florida Citrus Production Advisory Council Project 013-16P to L. W. Timmer. We thank L. Carris for reviewing the manuscript; P. Broadbent, Y. Canihos, S. H. Swart, Z. Solel, and B. Castro for providing isolates; L. Olsen for assistance with the DNA extractions and PCR reactions; and M. Rubio for maintaining plants in the greenhouse.

LITERATURE CITED

1. Avise, J. C. 2000. *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, MA.
2. Avise, J. C., and Ball, R. M., Jr. 1990. Principles of genealogical concordance in species concepts and biological taxonomy. *Oxf. Surv. Evol. Biol.* 7:45-67.
3. Bliss, D. E., and Fawcett, H. S. 1944. The morphology and taxonomy of *Alternaria citri*. *Mycologia* 36:469-502.
4. Canihos, Y., Erkilic, A., and Timmer, L. W. 1997. First report of *Alternaria* brown spot of Minneola tangelo in Turkey. *Plant Dis.* 81:1214.
5. Castro, B. L., Leguizamon, J., and Lopez, A. 1995. Determination of the causal agent of leaf spot in citrus plants in Colombian coffee growing region. *Fitopatol. Colomb.* 19:31-35.
6. Cobb, N. A. 1903. Letters on the diseases of plants—*Alternaria* of the citrus tribe. *Agric. Gaz. N. S. W.* 14:955-986.
7. Dalkiliç, Z. 1999. Identification and mapping of genes for *Alternaria* and *Phytophthora* disease resistance in citrus hybrids. Ph.D. thesis. University of Florida, Gainesville.
8. Felsenstein, J. 1993. *PHYMLIP* (Phylogeny Inference Package), 3.6a2 ed. University of Washington, Seattle.
9. Geiser, D. M., Pitt, J. I., and Taylor, J. W. 1998. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proc. Natl. Acad. Sci. USA* 95:388-393.
10. Hsu, J. C. 1984. Constrained two-sided simultaneous confidence intervals for multiple comparisons with the best. *Ann. Stat.* 12:1136-1144.
11. Isshiki, A., Akimitsu, K., Nishio, K., Tsukamoto, M., and Yamamoto, H. 1997. Purification and characterization of an endopolygalacturonase from the rough lemon pathotype of *Alternaria alternata*, the cause of citrus brown spot disease. *Physiol. Mol. Plant Pathol.* 51:155-167.
12. Isshiki, A., Akimitsu, K., Yamamoto, M., and Yamamoto, H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Mol. Plant-Microbe Interact.* 14:749-757.
13. Kasuga, T., Taylor, J. W., and White, T. J. 1999. Phylogenetic relationships of varieties and geographical groups of the human pathogenic fungus *Histoplasma capsulatum* darling. *J. Clin. Microbiol.* 37:653-663.
14. Kiely, T. B. 1964. Brown spot of Emperor mandarin. *Agric. Gaz. N. S. W.* 75:854-856.
15. Kohmoto, K., Akimitsu, K., and Otani, H. 1991. Correlation of resistance and susceptibility of citrus to *Alternaria alternata* with sensitivity to host-specific toxins. *Phytopathology* 81:719-722.
16. Kohmoto, K., Itoh, Y., Shimomura, N., Kondoh, Y., Otani, H., Kodama, M., Nishimura, S., and Nakatsuka, S. 1993. Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. *Phytopathology* 83:495-502.
17. Kohmoto, K., Scheffer, R. P., and Whiteside, J. O. 1979. Host-selective toxins from *Alternaria citri*. *Phytopathology* 69:667-671.
18. Koufopanou, V., Burt, A., and Taylor, J. W. 1997. Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitis*. *Proc. Natl. Acad. Sci. USA* 94:5478-5482.
19. Kusaba, M., and Tsuge, T. 1994. Nuclear ribosomal DNA variation and pathogenic specialization in *Alternaria* fungi known to produce host-specific toxins. *Appl. Environ. Microbiol.* 60:3055-3062.
20. Kusaba, M., and Tsuge, T. 1995. Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. *Curr. Genet.* 28:491-498.
21. Kusaba, M., and Tsuge, T. 1997. Mitochondrial DNA variation in host-specific toxin-producing pathogens in the genus *Alternaria*. *Ann. Phytopathol. Soc. Jpn.* 63:463-469.
22. Masunaka, A., Tanaka, A., Tsuge, T., Peever, T. L., Timmer, L. W., Yamamoto, M., Yamamoto, H., and Akimitsu, K. 2000. Distribution and characterization of *AKT* homologs in the tangerine pathotype of *Alternaria alternata*. *Phytopathology* 90:762-768.
23. Nakashima, T., Ueno, T., and Fukami, H. 1982. Structure elucidation of AK-toxins, host-specific phytotoxic metabolites produced by *Alternaria kikuchiana*. *Tetrahedron Lett.* 23:4469-4472.
24. Nishimura, S., and Kohmoto, K. 1983. Host-specific toxins and chemical structures from *Alternaria* species. *Annu. Rev. Phytopathol.* 21:87-116.
25. Nixon, K. C., and Wheeler, Q. D. 1990. An amplification of the phylogenetic species concept. *Cladistics* 6:211-223.
26. O'Donnell, K., Kistler, H. C., Tacke, B. K., and Casper, H. H. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *PNAS (USA)* 97:7905-7910.
27. Otani, H., Kohmoto, K., and Kodama, M. 1995. *Alternaria* toxins and their effects on host plants. *Can. J. Bot.* 73(suppl. 1):S453-S458.
28. Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12:357-358.
29. Pavlicek, A., Hrda, S., and Flegel, J. 1999. FreeTree—Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. *Folia Biol. (Praha)* 45:97-99.
30. Peever, T. L., Canihos, Y., Olsen, L., Ibanez, A., and Liu, Y.-C. 1999. Population genetic structure and host specificity of *Alternaria* spp. causing brown spot of Minneola tangelo and rough lemon in Florida. *Phytopathology* 89:851-860.
31. Peever, T. L., Olsen, L., Ibanez, A., and Timmer, L. W. 2000. Genetic differentiation and host specificity among populations of *Alternaria* spp. causing brown spot of grapefruit and tangerine × grapefruit hybrids in Florida. *Phytopathology* 90:407-414.
32. Schutte, G. C., and Beefon, K. V. 1994. The use of triazoles to control *Alternaria* brown spot of Minneola tangelo in the winter rainfall region of South Africa. *Citrus J.* 3:19-20.
33. Simmons, E. G. 1990. *Alternaria* themes and variations (27-53). *Mycotaxon* 37:79-119.
34. Simmons, E. G. 1992. *Alternaria* taxonomy: Current status, viewpoint, challenge. Pages 1-35 in: *Alternaria: Biology, Plant Diseases and Metabolites*. J. Chelkowski and A. Visconti, eds. Elsevier, Amsterdam.
35. Simmons, E. G. 1993. *Alternaria* themes and variations (63-72). *Mycotaxon* 48:91-107.
36. Simmons, E. G. 1999. *Alternaria* themes and variations (226-235): Classification of citrus pathogens. *Mycotaxon* 70:263-323.
37. Simmons, E. G., and Roberts, R. G. 1993. *Alternaria* themes and variations (73). *Mycotaxon* 48:109-140.
38. Solel, Z. 1991. *Alternaria* brown spot on Minneola tangelos in Israel. *Plant Pathol.* 40:145-147.
39. Staunt, J. 1990. *Citrus Varieties of the World*. Sinclair Int. Ltd., Norwich.
40. Su, G., Peever, T. L., and Timmer, L. W. 2001. Molecular systematics of citrus-associated *Alternaria* spp. (Abstr.) *Phytopathology* 91(suppl.):S190.
41. Tanaka, A., Shiotani, H., Yamamoto, M., and Tsuge, T. 1999. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. *Mol. Plant-Microbe Interact.* 12:691-702.
42. Tanaka, A., and Tsuge, T. 2000. Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity

- in the Japanese pear pathotype of *Alternaria alternata*. Mol. Plant-Microbe Interact. 13:975-986.
43. Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S., and Fisher, M. C. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31:21-32.
 44. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876-4882.
 45. Tsuge, T., Hayashi, N., and Nishimura, S. 1986. Metabolic regulation of host-specific toxin production in *Alternaria alternata* pathogens (3). Instability of pathogenicity in field isolates of *A. alternata* pear pathotype. Ann. Phytopathol. Soc. Jpn. 52:488-491.
 46. Vicent, A., Armengol, J., Sales, R., and Garcia-Jimenez, J. 2000. First report of *Alternaria* brown spot of citrus in Spain. Plant Dis. 84:1044.
 47. Webber, H. J., and Batchelor, L. D. 1949. The Citrus Industry, vol. 1. University of California Press, Berkeley.
 48. Whiteside, J. O. 1976. A newly recorded *Alternaria*-induced brown spot disease on Dancy tangerines in Florida. Plant Dis. Rep. 60:326-329.
 49. Xu, J., Vilgalys, R., and Mitchell, T. G. 2000. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. Mol. Ecol. 9:1471-1481.